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TATA-Box DNA Binding Activity and Subunit Composition of RNA Polymerase III Transcription Factor IIIB from *Xenopus laevis*

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The RNA polymerase III transcription initiation factor TFIIIB contains the TATA-box-binding protein (TBP) and polymerase III-specific TBP-associated factors (TAFs). Previous studies have shown that DNA oligonucleotides containing the consensus TATA-box sequence inhibit polymerase III transcription, implying that the DNA binding domain of TBP is exposed in TFIIIB. We have investigated the TATA-box DNA binding activity of *Xenopus* TFIIIB, using transcription inhibition assays and a gel mobility shift assay. Gel shift competition assays with mutant and nonspecific DNAs demonstrate the specificity of the TFIIIB-TATA box DNA complex. The apparent dissociation constant for this protein-DNA interaction is ~0.4 nM, similar to the affinity of yeast TBP for the same sequence. TFIIIB transcriptional activity and TATA-box binding activity cofractionate during a series of four ion-exchange chromatographic steps, and reconstituted transcription reactions demonstrate that the TATA-box DNA–protein complex contains TFIIIB TAF activity. Polypeptides with apparent molecular masses of 75 and 92 kDa are associated with TBP in this complex. These polypeptides were renatured after elution from sodium dodecyl sulfate-gels and tested individually and in combination for TFIIIB TAF activity. Recombinant TBP along with protein fractions containing the 75- and 92-kDa polypeptides were sufficient to reconstitute TFIIIB transcriptional activity and DNA binding activity, suggesting that *Xenopus* TFIIIB is composed of TBP along with these polypeptides.

Transcription of the genes encoding the tRNAs, 5S rRNA, and other small cellular and viral RNAs by RNA polymerase III (pol III) requires the ordered formation of a transcription initiation complex containing the factors TFIIIB and TFIIIC and, in the case of 5S RNA genes, the gene-specific factor TFIIIA (for extensive reviews and references, see references 7 and 38). Transcription complex assembly on tRNA and small virus-associated RNA genes is initiated by the direct binding of TFIIIC to the intragenic A-box and B-box promoter elements (7, 38). For the 5S RNA genes, TFIIIA binds to the internal control region of the gene and subsequently recruits TFIIIC to the TFIIIA-5S DNA binary complex. In contrast, the genes encoding U6 and 7SK RNAs, which are also transcribed by pol III (7, 38), are regulated by upstream TATA-box and proximal sequence elements, and these genes require TFIIIB, pol III, and a proximal sequence binding factor (termed PTF or SNAPc) for transcription in vitro (12, 28, 38, 43). TFIIIC is a multisubunit protein consisting of six polypeptides in *Saccharomyces cerevisiae* (1, 7) and at least this number of polypeptides in vertebrates (19, 30). The genes encoding the 220-kDa subunit that binds the B-box promoter element (20, 21) and a 110-kDa subunit (30) have been cloned from mammalian cells. For the tRNA genes, once TFIIIC (or TFIIIA and TFIIIC for the 5S genes) is bound to the template, TFIIIB binds upstream from the start site for transcription in a sequence-independent manner (15, 22, 25). In the yeast system, TFIIIB remains stably bound to class III genes after dissociation of TFIIIC (and TFIIIA) with salt or heparin and can serve to direct pol III to the template in the absence of these other factors (15).

Several studies have documented that TFIIIB is a target of regulation during cell growth, differentiation, and the cell cycle (8, 34, 39, 41). Thus, understanding the mechanism of TFIIIB binding and the polypeptide composition and regulatory modifications of TFIIIB is of central importance in elucidating class III gene regulation. The polypeptide composition of yeast TFIIIB is well established: it consists of the TATA-box-binding protein (TBP) and pol III-specific TBP-associated factors (TAFs) of 70 and 90 kDa (16). Each of these TAFs has been cloned in *S. cerevisiae* (4, 6, 17, 24), and the 70-kDa subunit (TFIIIB70; also known as Brf) is similar in sequence to the pol II transcription factor TFIIB, which interacts with TBP (in TFIID) and with pol II (reviewed in reference 18). TFIIIB70 has been shown to interact with the 135-kDa subunit of TFIIIC, with TBP, and with the 34-kDa subunit of pol III (2, 18, 37), consistent with the role of TFIIIC in recruitment of TFIIIB and the role of TFIIIB in pol III recruitment. In contrast, with the exception of TBP, the polypeptide composition of vertebrate TFIIIB is controversial. Polypeptides associated with TBP in a TFIIIB fraction from a phosphocellulose column have been identified by immunoprecipitation with antibody to TBP, but different polypeptide constituents have been observed in different laboratories (5, 23, 33). These differences may reflect the presence of multiple TBP-containing complexes in the starting phosphocellulose fraction, namely, TFIIIB and the putative pol II factor B-TFIID (26). A 90-kDa TFIIIB subunit (TFIIIB90) has recently been isolated by a combination of conventional chromatography and anti-TBP immunoprecipitation, and a cDNA for this subunit was cloned

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(36). TFIIIB90 is similar in sequence to TFIIB and to the IIB-related domain of yeast TFIIIB70; TFIIIB90 also contains high-mobility-group box domains which could participate in DNA binding.

On the basis of the presence of TBP in TFIIIB and the observation that DNA oligonucleotides containing the consensus TATA-box sequence will inhibit pol III transcription (27, 40), presumably by binding TBP, we have examined the TATA-box DNA binding activity of TFIIIB from *Xenopus laevis*. We find that proteins present in chromatographic fractions containing TFIIIB transcriptional activity will bind a TATA-box oligonucleotide in a sequence-specific and highly stable manner. Moreover, this binding activity cofractionates with TFIIIB under conditions that separate human TFIIIB from B-TFIID (26). The TATA-box DNA binding activity of TFIIIB has been used to identify the polypeptide subunits of *Xenopus* TFIIIB. Reconstituted transcription reactions suggest that *Xenopus* TFIIIB is composed of TBP and two polypeptides with apparent molecular masses of 75 and 92 kDa. The 92-kDa polypeptide likely corresponds to human TFIIIB90 (36).

MATERIALS AND METHODS

Oligonucleotides. Double-stranded oligonucleotides were prepared by annealing equal molar amounts of the two complementary strands. The wild-type TATA-box oligonucleotide was derived from the adenovirus major late promoter and had the top-strand sequence 5′-GATCGGGGGCTATAAAAGGGGGTG GG-3′. A second TATA-box oligonucleotide had the top-strand sequence 5′-TCCTGAAGGGGGGCTATAAAAGGGGGTGGGG-3′. The mutant TATA-box oligonucleotide had the top-strand sequence 5′-TCCTGAAGGGGGGTA GAGAAGGGGGTGGGG-3′ (the two base changes within the TATA box are underlined). The latter two oligonucleotides were the generous gift of M. Paule (Colorado State University, Fort Collins). A 23-bp oligonucleotide corresponding in sequence to the C-box promoter element of the *Xenopus* somatic-type 5S RNA gene (nucleotide positions +75 to +97 of the 120-bp gene) was used as a nonspecific oligonucleotide. Oligonucleotides were end labeled with [γ-^{32}P]ATP and polynucleotide kinase.

Egg extracts and protein fractions. A high-speed supernatant from unfertilized *Xenopus* egg extracts was prepared as described previously (10) and contained ~3 to 5 mg of protein per ml. The egg extract was dialyzed and subjected to chromatography on phosphocellulose P-11 (Whatman) as described previously (29). PC-B refers to the 0.1 to 0.35 M KCl fraction (~1.6 mg of protein per ml, containing TFIIIB and RNA pol III), and PC-C refers to the 0.35 to 0.65 M KCl fraction (~440 μg of protein per ml, containing TFIIIC and the majority of the RNA pol III activity). PC-C+D refers to a 0.35 to 1 M KCl fraction. Protein fractions were dialyzed against buffer A-100, containing 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.9), 100 mM KCl, 2 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 0.1 mM EDTA, and 20% (vol/vol) glycerol. One-half milliliter of the dialyzed PC-B fraction was applied to a 1-ml Mono Q FPLC (fast protein liquid chromatography) column (Pharmacia). The column was washed with 5 volumes of buffer A-100, and proteins were eluted with a 20-ml linear gradient of KCl from 100 to 600 mM in buffer A. Aliquots from the peak fractions of TFIIIB transcription activity from the Mono Q column were pooled and concentrated by precipitation with methanol. This TFIIIB protein fraction was then dissolved in sodium dodecyl sulfate (SDS) sample buffer and subjected to electrophoresis on an SDS–10% polyacrylamide gel. Prestained protein size markers (Rainbow markers; Amersham) were run on both sides of the TFIIIB sample, and appropriate gel slices were excised on the basis of the migration of the prestained standards. Proteins were eluted from SDS-polyacrylamide gel slices overnight at ambient temperature as described previously (9), with the modification that the samples (supplemented with 100 μg of bovine serum albumin per ml) in 6 M guanidine-HCl were diluted fivefold with buffer A-100 and then concentrated fivefold with a Centricon 30 concentrator. This procedure was repeated for a total of four dilution and concentration steps to remove guanidine and to renature the proteins. Because of the addition of bovine serum albumin, the concentration of the eluted proteins could not be determined. However, the final volume of concentrated protein (200 μl) was approximately the same as the starting volume of the Mono Q-purified TFIIIB fraction prior to SDS-gel electrophoresis. TFIIIB was also purified by sequential chromatography on phosphocellulose, DEAE-Sepharose (200 to 350 mM KCl step, in buffer A), heparin-agarose, and Mono Q FPLC. The DEAE-Sepharose TFIIIB fraction was diluted with an equal volume of buffer A without KCl and applied directly to heparin-agarose without a dialysis step. The heparin-agarose resin was washed with buffer A-100, and bound proteins were eluted with a linear gradient of KCl from 200 to 600 mM (in buffer A). Fractions containing TFIIIB activity were pooled and dialyzed against buffer A-100 prior to chromatography on Mono Q FPLC as described above.

Gel mobility shift assays. Binding reaction mixtures contained the amounts of ^{32}P-labeled double-stranded oligonucleotides and protein fractions indicated in the figure legends in a final volume of 20 μl. The buffer for the binding reactions consisted of 20 mM HEPES (pH 7.9), 50 to 60 mM KCl, 1 mM $MgCl_2$, 20 μM $ZnCl_2$, 5 mM dithiothreitol, 0.1 mM EDTA, and 12% (vol/vol) glycerol except for analysis of the Mono Q fractions, in which case the KCl concentrations ranged from 55 to 135 mM. Reaction mixtures generally contained 1 ng of labeled oligonucleotide and 200 ng of pBluescript DNA (Stratagene) and were incubated for a minimum of 20 min prior to electrophoresis on 6% polyacrylamide gels in 25 mM Tris–190 mM glycine (pH 8.5)–1 mM EDTA (3) at ambient temperature (22 to 23°C). Gels measuring 20 cm (height) by 16 cm (width) by 0.75 mm (depth) were subjected to preelectrophoresis at 150 V for 2 h prior to electrophoresis at 150 V for 2.5 to 3 h. Extent of binding was estimated either by laser densitometry of the gel autoradiograms or by Molecular Dynamics PhosphorImager analysis of the dried gels. For estimation of equilibrium binding constants, data were plotted as [bound DNA] versus [free DNA] and analyzed with the KaleidaGraph program (Synergy Software, Reading, Pa.) for the Apple Macintosh computer. For isolation of the TATA/PC-B protein complex, a reaction mixture consisting of 100 μl of the PC-B fraction, 20 ng of the ^{32}P-labeled TATA-box oligonucleotide, and 1 μg of unlabeled oligonucleotide in a total volume of 200 μl was subjected to electrophoresis in all lanes of a 1-mm-thick gel. The protein-DNA complex was excised from the gel (with the aid of an autoradiogram of the wet gel) and eluted into buffer A supplemented with 6 M guanidine-HCl. After incubation with gentle rocking for 16 h at 4°C, eluted proteins were applied to a spin column containing 100 μl of DEAE-Sepharose A25 equilibrated in buffer A-100 plus bovine serum albumin at 100 μg/ml. Guanidine was removed, and the bound proteins were renatured by washing the column five times with 100-μl aliquots of buffer A-100 (without bovine serum albumin); proteins were separated from the oligonucleotide by elution of the protein with buffer A containing 0.36 M KCl. Fractions of 100 μl were collected, and the peak of eluted protein was identified with the Bradford assay (Bio-Rad). The concentration of the eluted protein was too low to be accurately determined.

Transcription reactions. Reaction mixtures contained the *tyrD* tRNA gene of *X. laevis* (32) and volumes of protein fractions indicated in the figure legends. The reaction mixtures also contained 20 mM HEPES (pH 7.9), 65 mM KCl, 6 mM $MgCl_2$, 2 mM dithiothreitol, 0.1 mM EDTA, 12% (vol/vol) glycerol, 10 U of recombinant RNasin (Promega), 0.6 mM ATP, UTP, and CTP, 20 μM GTP, and 10 μCi of [α-^{32}P]GTP (DuPont NEN) in a final volume of 20 μl. Protein fractions and DNA template were incubated for 20 min prior to the addition of the labeled nucleotide. Reactions were carried out for 1 to 2 h at ambient temperature and were terminated with a stop buffer consisting of 20 mM Tris-HCl (pH 7.5), 1% SDS, 0.3 M sodium acetate, and 10 mM EDTA; in some experiments, equal amounts of radiolabeled 5S RNA or a labeled DNA fragment were added to each reaction to act as a recovery standard. RNAs were purified by extraction with RNAzol (Tel-Test Inc., Friendswood, Tex.) and precipitation with isopropanol and were analyzed by electrophoresis on denaturing 6% polyacrylamide gels (29:1 acrylamide to bisacrylamide) containing 8.3 M urea in 88 mM Tris-borate (pH 8.3)–2 mM EDTA. After electrophoresis, the gels were dried and subjected to autoradiography with Kodak Biomax film. For quantitation, dried gels were subjected to PhosphorImager analysis with a Molecular Dynamics instrument, and the relative amounts of radioactivity in the RNA transcripts were determined by using the Molecular Dynamics ImageQuant software.

Cloning of *Xenopus* TBP, protein expression, and TAF depletion. A cDNA expression clone for *Xenopus* TBP was generated by PCR amplification of the TBP coding sequence from total DNA isolated from a *Xenopus* oocyte cDNA library prepared in λZap II (Stratagene; a gift of A. Wolffe, National Institutes of Health). The following oligonucleotides were used for amplification: 5′-GTA CGGATCC**ATG**GATCAAAACAACAGCATACCCC-3′ and 5′-CCAGGAAT TC**CTATTA**CGTTGTTTTTCTGAAGCCTTTAAGG-3′. The coding regions of these oligonucleotides were derived from the *Xenopus* TBP sequence deposited in GenBank by Hashimoto et al. (11). The 5′ oligonucleotide contains an *Bam*HI restriction site (underlined) and initiating ATG codon (boldface), while the 3′ oligonucleotide contains an *Eco*RI site (underlined) and stop codons (boldface). After digestion with *Bam*HI and *Eco*RI, the PCR product was isolated after purification on a 1.6% agarose gel and cloned in pGEX-2T (Pharmacia). Sequence analysis revealed two differences between two clones that we isolated and the sequence in GenBank (11); these are at nucleotide positions 163 and 529, which correspond to changes at amino acid positions 55 (Gln to Glu) and 177 (Met to Ile). These sequence differences could reflect either allelic or animal variation, but the possibility that these differences are due to PCR artifacts cannot be excluded. Protein expression was obtained in *Escherichia coli* BL21(DE3) cells (Novagen) after induction for 4 h with 0.4 mM isopropylthiogalactopyranoside (IPTG). The glutathione *S*-transferase fusion protein was isolated by chromatography on glutathione-Sepharose as recommended by the supplier (Pharmacia). TAFs and other TBP-binding proteins were depleted from the PC-C fraction by incubation of 100 μl of PC-C with 100 μl of packed glutathione-Sepharose (in buffer A-100) containing 25 μg of bound GST-TBP for 30 min at 4°C. After centrifugation for 30 s in a microcentrifuge, the supernatant was removed and designated PC-CΔTAFs. The *Bam*HI-*Eco*RI fragment from the TBP cDNA was also cloned in the vector pET-21a (Novagen) for expression of a hexahistidine-tagged protein. Protein expression was in *E. coli*

BL21(DE3) cells, and His$_6$-TBP was purified by Ni^{2+}-nitrilotriacetic acid-agarose chromatography (Qiagen). This protein sample (1.2 to 1.5 mg of TBP per ml) was the generous gift of P. Labhart (Scripps Research Institute). This recombinant protein was active in DNA binding and supported pol III transcription in vitro (see below). Recombinant human TBP and monoclonal antibody to human TBP were purchased from Promega.

RESULTS

TATA-box DNA binding activity of proteins present in a phosphocellulose fraction from a *Xenopus* egg extract. Previous studies (27, 40) have documented that DNA oligonucleotides containing the RNA pol II TATA-box promoter element will inhibit transcription of a variety of genes that are transcribed by RNA pol III, presumably by binding to the TBP subunit of the RNA pol III transcription factor TFIIIB (see the introduction). These studies were performed with extracts and chromatographic fractions from human (40) and *Acanthamoeba* (27) cells. We tested the effect of a TATA-box oligonucleotide, derived from the sequence of the adenovirus major late promoter (27), on transcription of a *Xenopus* $tRNA^{Tyr}$ gene in a crude extract prepared from unfertilized *Xenopus* eggs. Figure 1A shows that this oligonucleotide is a potent inhibitor of class III gene transcription (lanes 3 to 6). Similar to results in the *Acanthamoeba* system (27), higher concentrations of a mutant TATA-box oligonucleotide (TAGAGAA) were required for inhibition (lanes 8 to 11). A control oligonucleotide, corresponding in sequence to the binding site for TFIIIA within a 5S RNA gene, was without effect on tRNA transcription (lanes 14 to 17). No inhibition was observed even at DNA concentrations 10-fold higher than those found to inhibit transcription with the TATA-box oligonucleotide (lanes 16 and 17). A quantitative comparison of the effects of the wild-type and mutant TATA-box oligonucleotides on tRNA transcription is shown in Fig. 1B. Approximately threefold-higher concentrations of mutant oligonucleotide are required for levels of inhibition similar to those obtained with the wild-type oligonucleotide.

On the basis of these results, we tested the TATA-box DNA binding activity of TFIIIB with a gel mobility shift assay using the radiolabeled TATA-box oligonucleotide (Fig. 2A). A single protein-DNA complex is observed with this DNA probe and either the unfractionated extract isolated from unfertilized *Xenopus* eggs (lanes 2 and 3) or the PC-B fraction (lanes 4 and 5) isolated from this extract. We will refer to this complex as the TATA/PC-B protein complex. The proteins that generate this complex either are absent from or are present in vastly reduced concentration in the PC-C fraction (lane 6). Similarly, the TATA/PC-B protein complex is not observed with a PC-C+D fraction (data not shown). To assess whether TBP is a component of this complex, we tested the effect of a monoclonal antibody to human TBP on the formation and mobility of the complex. This antibody blocks the formation of the complex if PC-B proteins and antibody are incubated prior to the addition of the DNA probe (Fig. 2B, lane 3); however, the antibody has no effect on the formation or mobility of the complex if added after incubation of protein and DNA (lane 2). Nonspecific antibodies (antiactin) and preimmune rabbit serum do not block the formation of the TATA/PC-B protein complex (data not shown). Further, preincubation of the antibody and recombinant human TBP prior to the addition of the PC-B fraction prevents the inhibitory effect of this antibody on the formation of the TATA/PC-B complex (data not shown). Thus, TBP is a component of this protein-DNA complex. These results also suggest that the epitope(s) recognized by the antibody is not exposed in the protein-DNA complex. Using the Tris-glycine-EDTA gel system (3), we find that recombinant TBP (rTBP) alone does not generate a stable protein-

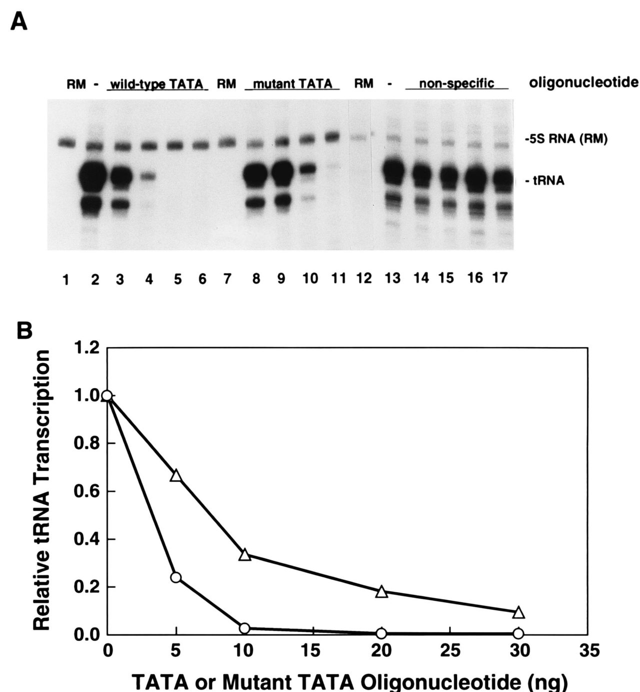

FIG. 1. Inhibition of tRNA transcription with a TATA-box DNA oligonucleotide. (A) Aliquots of 2.5 μl of an unfertilized egg extract were incubated with the amounts of oligonucleotide indicated below for 30 min at 22°C prior to the addition of a tRNA gene template and labeled and unlabeled nucleotides as described in Materials and Methods. Transcription reactions were terminated after 1 h, and equivalent aliquots of radiolabeled 5S RNA were added to each reaction to act as a recovery marker (RM; lanes 1, 7, and 12). The reactions shown in lanes 1 to 11 and 12 to 17 were from different experiments. Control reactions, without added oligonucleotide, are shown in lanes 2 and 13. Wild-type TATAAA oligonucleotide was added to the reactions of lanes 3 to 6, and mutant TAGAGA oligonucleotide was added to the reactions of lanes 8 to 11: 5 ng (0.24 pmol), lanes 3 and 8; 10 ng (0.49 pmol), lanes 4 and 9; 20 ng (0.98 pmol), lanes 5 and 10; 30 ng (1.5 pmol), lanes 6 and 11. A C-box 5S gene oligonucleotide was added to the reactions of lanes 14 to 17: 30 ng (2 pmol), lane 14; 100 ng (6.6 pmol), lane 15; 200 ng (13 pmol), lane 16; 300 ng (20 pmol), lane 17. Oligonucleotide sequences are given in Materials and Methods. (B) Quantitation of inhibition data. The relative tRNA transcription (corrected for the 5S RNA recovery marker) at each oligonucleotide concentration was determined by PhosphorImager analysis of the dried gel. Data are shown for the wild-type TATAAA oligonucleotide (circles) and for the mutant TAGAGA oligonucleotide (triangles).

DNA complex (assaying up to a 90-fold molar excess of rTBP [data not shown, but see Fig. 6C]); however, a stable TBP–TATA-box oligonucleotide complex is observed with both recombinant human and *Xenopus* TBP in a Mg-containing gel system (data not shown). In combination, these observations indicate that the TATA/PC-B complex contains TBP and associated polypeptides.

We next tested the specificity of this complex for the TATA-box DNA sequence by competition with either a wild-type (TATAAAA) or a mutant (TAGAGAA) oligonucleotide (Fig. 3A). Fifty percent reduction in binding was observed with a ~2.5-fold molar excess of unlabeled wild-type competitor DNA in the reaction mixture, while a ~16-fold molar excess of unlabeled mutant oligonucleotide was required to observe a similar 50% reduction in binding (Fig. 3B). This finding suggests that the proteins that generate the TATA/PC-B protein complex have a ~6-fold-higher affinity for the wild-type over the mutant sequence. This difference in affinity is also similar (within a factor of 2 to the relative inhibitory effects of these same wild-type and mutant TATA-box oligonucleotides on

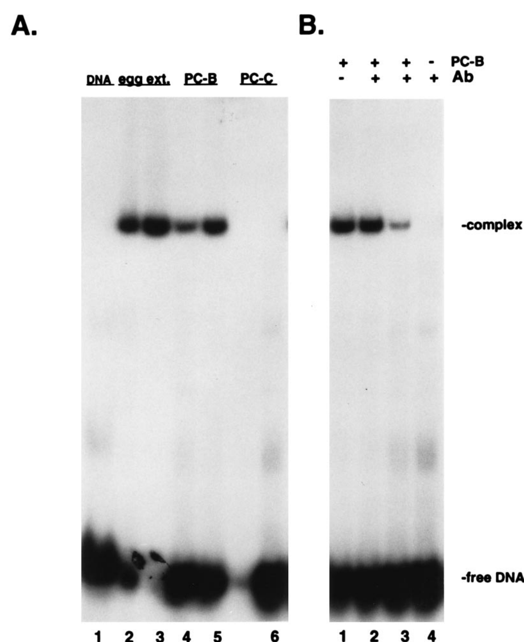

FIG. 2. Formation of a protein-DNA complex with a TATA-box oligonucleotide and proteins present in the PC-B fraction isolated from a *Xenopus* egg extract. (A) Each reaction mixture contained 1 ng of the ^{32}P-labeled double-stranded TATA-box oligonucleotide and other reaction components as described in Materials and Methods, plus the following additions: lane 1, DNA alone; lane 2, 0.5 μl of egg extract (ext.); lane 3, 1 μl of egg extract; lane 4, 0.5 μl of PC-B fraction; lane 5, 1 μl of PC-B fraction; lane 6, 1 μl of PC-C fraction. (B) Antibody to TBP blocks formation of the TATA/PC-B protein complex. Each reaction mixture contained 1 ng of the labeled TATA-box oligonucleotide and, where indicated at the top, 1 μl of the PC-B fraction and/or 1 μl of anti-human TBP monoclonal antibody (Ab; 0.18 mg/ml; Promega). In the reaction of lane 2, antibody was added 20 min after addition of PC-B, while in the reaction of lane 3, antibody and PC-B proteins were incubated for 20 min prior to the addition of DNA. Samples were subjected to electrophoresis after an additional 20-min incubation.

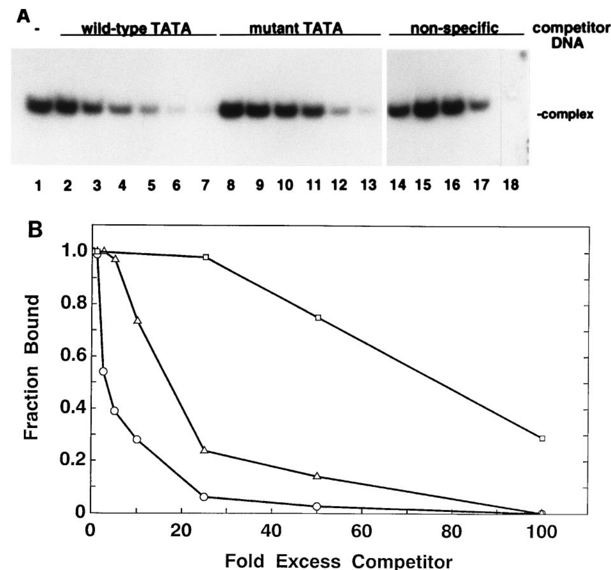

FIG. 3. Competition of the TATA/PC-B protein complex with specific, mutant TATA-box DNA and nonspecific DNAs. Each reaction mixture contained 0.6 ng (lanes 1 to 13) or 1 ng (lanes 14 to 17) of the labeled oligonucleotide, 1 μl of the PC-B fraction, and either no competitor (lanes 1 and 14) or the following competitor DNAs: lanes 2 to 7, 0.6, 1.5, 3, 6, 15, and 30 ng of the wild-type TATAAA oligonucleotide, respectively; lanes 8 to 13, 0.6, 1.5, 3, 6, 15, and 30 ng of a mutant TAGAGA oligonucleotide, respectively; lanes 15 to 17, 20, 50, 100, and 200 ng of pBluescript SK+ DNA (Stratagene). In panel A, the portion of the autoradiogram containing the protein-DNA complex is shown, and a graphical representation of the data is shown in panel B. Lanes 1 to 13 and 14 to 17 were from separate experiments. Additional data, not shown in panel A, are included in the graphical treatment shown in panel B. Circles indicate competition with the wild-type oligonucleotide, triangles indicate competition with the mutant TAGAGA oligonucleotide, and squares denote competition with plasmid DNA.

RNA pol III gene transcription (Fig. 1B and reference 27) and the relative effects of mutations at these positions on RNA pol II transcription (31). An ~80-fold mass excess of nonspecific plasmid DNA was required for 50% reduction in binding, suggesting a ~30-fold preference for the TATA sequence over nonspecific DNA.

The TATA-box binding activity in PC-B corresponds to TFIIIB. Since human PC-B fractions contain both TFIIIB and B-TFIID, two complexes that contain TBP (26), we subjected the PC-B fraction to chromatography on Mono Q FPLC to determine whether the TATA-box DNA binding activity and TFIIIB transcriptional activity reside in the same complex. Mono Q chromatography has been used previously in the purification of TFIIIB (5, 23, 27) and readily separates human TFIIIB from B-TFIID (26). Figure 4A shows the results of a TATA-box gel mobility shift assay with the input PC-B fraction and the fractions from the Mono Q column, and Fig. 4B shows the results of a tRNA gene transcription experiment with the input PC-B fraction and these same Mono Q fractions. In the transcription experiment, TFIIIC and RNA pol III activities were supplied by the PC-C fraction, which absolutely requires TFIIIB for activity (Fig. 5, lanes 1 and 2). Clearly, both the gel shift and TFIIIB transcriptional activities coelute from the resin in the same fractions, with the peak of both activities in fraction 16 (corresponding to elution at 300 to 400 mM KCl, in agreement with previous studies [5, 8, 23, 27]). This experiment suggests that the gel shift activity does not correspond to B-TFIID, since human B-TFIID has been reported to elute from Mono Q at a lower KCl concentration (150 mM) than TFIIIB (26); however, we have no data on the chromatographic properties of B-TFIID from *X. laevis*. Additionally, the TATA-box DNA–protein complex was found to cofractionate with TFIIIB during sequential chromatography on four ion-exchange resins (phosphocellulose, DEAE-Sepharose, heparin-agarose, and Mono Q FPLC [data not shown]).

To demonstrate further that the TATA/PC-B protein complex corresponds to TFIIIB, we eluted this complex from the nondenaturing gel and tested the transcriptional activity of the eluted proteins (separated from the oligonucleotide by chromatography on DEAE-Sepharose A25) in combination with TFIIIC and RNA pol III (provided by the PC-C fraction). In this experiment, the TATA/PC-B complex was isolated from a scaled-up reaction in which 1 μg of double-stranded TATA-box oligonucleotide was incubated with 200 μl of PC-B and subjected to electrophoresis in all lanes of a nondenaturing gel. The relative migration of the TATA/PC-B complex was the same for this reaction as for standard reaction conditions containing 1 ng of TATA oligonucleotide and a 200-fold mass excess of nonspecific competitor DNA (Fig. 2). In preliminary experiments, we found that the PC-C fraction supplemented with recombinant *Xenopus* TBP (rX-TBP) gave a low level of pol III gene transcription (Fig. 5, lane 3), suggesting that the PC-C fraction contains a low level of pol III TAF activity. To assay the gel-purified proteins, the PC-C fraction was depleted of TAF activity by TBP affinity chromatography using a glutathione S-transferase–TBP fusion protein bound to glutathione-Sepharose (as described in Materials and Methods). This TAF-

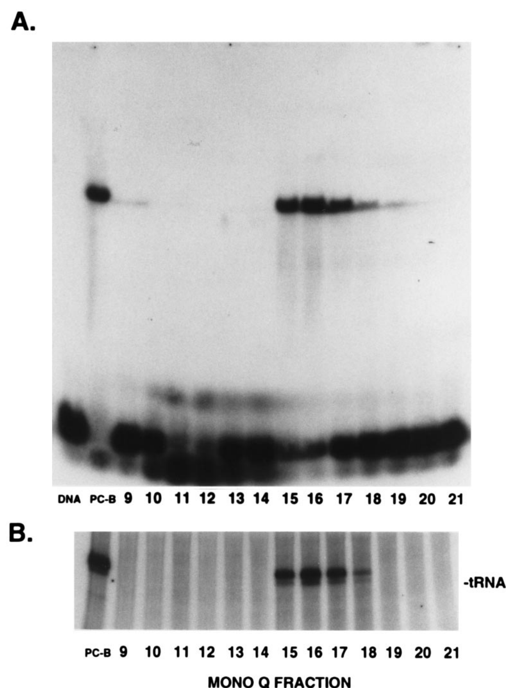

FIG. 4. Cofractionation of the TATA/PC-B protein complex with TFIIIB activity by chromatography on Mono Q FPLC. In panel A, 5-μl aliquots of each of the indicated Mono Q fractions were incubated with 1 ng of the ^{32}P-labeled TATA-box oligonucleotide (in 20-μl reactions), and formation of protein-DNA complexes was analyzed on a nondenaturing polyacrylamide gel. The lane marked DNA did not contain protein, and the reaction of the lane marked PC-B contained 1 μl of the PC-B fraction. In panel B, TFIIIB transcriptional activity was analyzed with 300 ng of the *tyrD* tRNA gene plasmid DNA, 8 μl of PC-C (containing TFIIIC and RNA pol III), and either 3 μl of PC-B or 3-μl aliquots of the indicated Mono Q fractions. Other reaction components and conditions were as described in Materials and Methods. The position of tRNA is shown at the right.

depleted PC-C (PC-CΔTAF) supplemented individually with either rX-TBP (lane 7) or the eluted proteins (lane 8) did not support tRNA gene transcription; however, in combination, the eluted proteins, rX-TBP, and PC-CΔTAF supported ~12% of the level of tRNA transcription as did PC-B plus PC-CΔTAF (lane 9). These data demonstrate that the TATA/PC-B protein complex contains the TFIIIB TAF activity. The requirement for exogenous TBP suggests that TBP either was not recovered from the nondenaturing gel or was inactivated

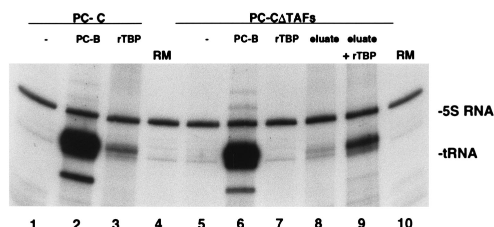

FIG. 5. The TATA/PC-B protein complex contains TFIIIB TAFs. Transcription reaction mixtures contained 320 ng of the *tyrD* tRNA gene plasmid DNA, 10 μl of PC-C (lanes 1 to 3), or 10 μl of PC-CΔTAF (lanes 5 to 9), other reaction components, and either no additions (lanes 1 and 5) or the following: lanes 2 and 6, 5 μl of PC-B; lanes 3, 7, and 9, 1 μl (120 ng) of rX-TBP; lanes 8 and 9, 5 μl of proteins eluted from the TATA/PC-B protein complex. After termination of the reactions, equivalent aliquots of a radiolabeled 5S RNA transcript were added to each of the reactions to serve as a recovery marker (RM). Lanes 4 and 10 contained only 5S RNA. The positions of the 5S and tRNAs are indicated.

by the elution and purification protocols that we used. Consistent with this observation, a silver-stained SDS-polyacrylamide gel of the eluted proteins failed to reveal a polypeptide at the expected position for *Xenopus* TBP (33 kDa [Fig. 6A, lane 1]). A 33-kDa polypeptide was observed when the TATA/PC-B complex was eluted from the nondenaturing gel with a buffer containing 1% SDS (not shown). Three major bands corresponding to polypeptides with apparent molecular masses of 65, 75, and 92 kDa were present in the eluted protein fraction. Other minor components were also present in the eluted protein fraction. The 65-kDa polypeptide was also observed when PC-B proteins were subjected to electrophoresis in the absence of the TATA-box oligonucleotide and a gel slice was taken from the nondenaturing gel at the position of the TATA/PC-B complex (data not shown); however, neither the 75- nor the 92-kDa polypeptide was observed in the absence of the TATA-box oligonucleotide. Densitometry of the silver-stained gel suggests that these two polypeptides are present in stoichiometric amounts (assuming equivalent silver staining of the two polypeptides on a molar basis), suggesting a 1:1 molar ratio of these polypeptides in TFIIIB.

Renaturation of TFIIIB TAF activity of Mono Q-purified TFIIIB subjected to SDS-gel electrophoresis. The peak fractions containing TFIIIB transcriptional activity and TATA-box DNA binding activity from the Mono Q column (Fig. 4) were pooled, and the proteins in these fractions were precipitated and subjected to SDS-gel electrophoresis. Prestained molecular weight markers were run along side the TFIIIB protein fraction, and slices from the gel were excised at the expected positions of the 65-, 75-, and 92-kDa polypeptides that were observed in the TATA-box gel shift complex. Figure 6A (lane 2) shows the silver stain of this Mono Q-purified TFIIIB. Polypeptides were eluted from the SDS-gel and renatured from 6 M guanidine as described by Hager and Burgess (9), with the modifications noted in Materials and Methods. Aliquots of the renatured proteins were tested both individually and in combination for the ability to support tRNA transcription with rX-TBP and the PC-CΔTAF fraction (as a source of TFIIIC and RNA pol III). As before, neither PC-CΔTAF alone nor PC-CΔTAF supplemented with rX-TBP supports accurate tRNA transcription (Fig. 6B, lanes 1 and 2); however, a nonspecific transcript is observed when the reaction is supplemented with rX-TBP (lane 2; band above the recovery marker). Addition of PC-B proteins to the PC-CΔTAF fraction results in active transcription of the $tRNA^{Tyr}$ gene (lane 3). None of the eluted and renatured proteins tested individually supported tRNA transcription (lanes 4 to 6); only the combination of the polypeptides from the 75- and 92-kDa gel slices or the mixture of polypeptides eluted from all three gel slices (lanes 9 and 10, respectively) supported tRNA transcription. These eluted proteins in combination with TBP and the PC-CΔTAF fraction supported ~10% of the level of tRNA transcription as did the PC-B fraction. Consistent with the artifactual comigration of the 65-kDa polypeptide with the TATA/PC-B complex, the protein fraction containing this polypeptide is not required for specific tRNA transcription. These data suggest that *Xenopus* TFIIIB is composed of TBP and two polypeptides with apparent molecular masses of 75 and 92 kDa.

We next tested whether the eluted and renatured polypeptides could reconstitute the TATA-box gel shift complex when supplemented with rX-TBP. The fractions containing the 65, 75-, and 92-kDa polypeptides were tested individually and in combination for gel shift activity (Fig. 6C). In the Tris-glycine-EDTA gel system, a 60-fold molar excess of rX-TBP does not yield a stable complex with the TATA-box oligonucleotide

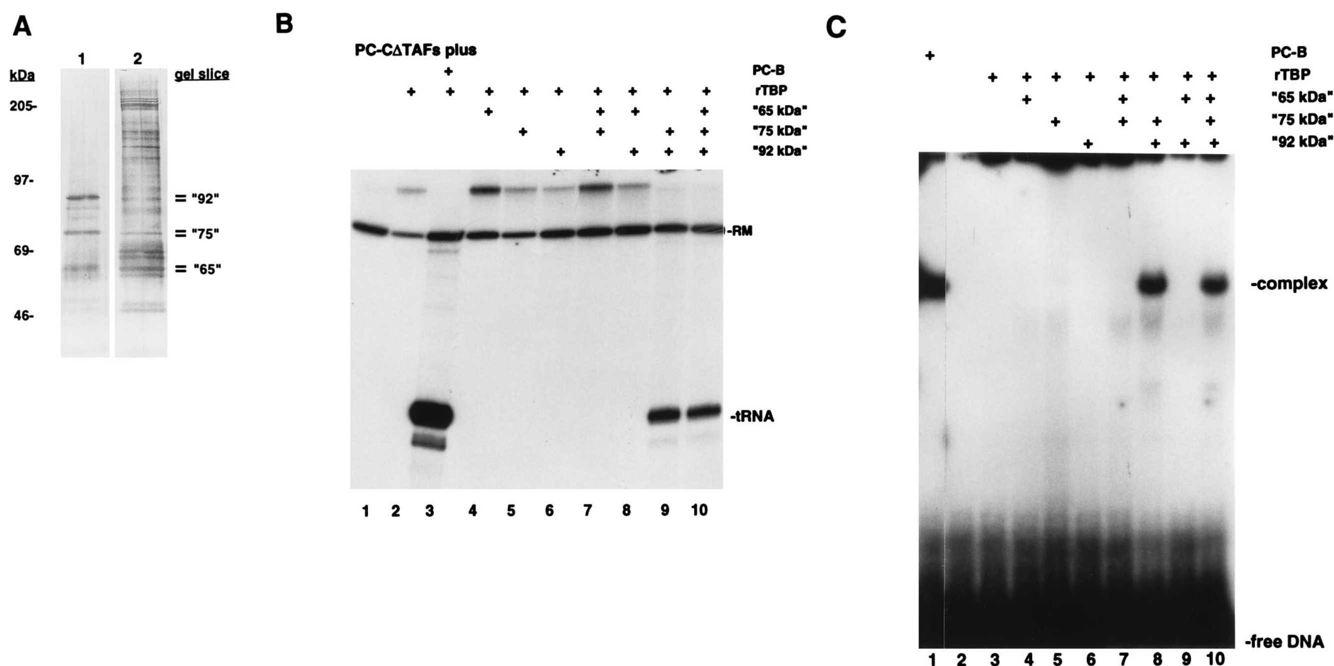

FIG. 6. Identification of TFIIIB TAFs. (A) SDS-gel electrophoresis and silver staining of polypeptides eluted from the TATA/PC-B gel shift complex (lane 1) and Mono Q-purified TFIIIB (lane 2). The positions of prestained protein standards are shown at the left, and the centers of the positions of the gel slices taken for elution and renaturation are indicated at the right. Each gel slice was approximately 0.5 cm from a 12 cm-long gel. (B) Reconstituted transcription reaction mixtures containing the $tRNA^{Tyr}$ gene (200 ng per reaction) and 7.5 μl of the PC-CΔTAF fraction were incubated for 30 min prior to the addition of rX-TBP (120 ng), 7.5 μl of PC-B, or 2.5-μl aliquots of the eluted and renatured protein fractions as indicated. Reaction mixtures were incubated for an additional 30 min prior to the addition of nucleotides. Reactions were supplemented where necessary with buffer to a final volume of 20 μl. The reactions were terminated after a 2-h incubation, and aliquots of a radiolabeled 474-bp DNA restriction fragment were added to each of the reactions to serve as a recovery marker (RM). The position of the tRNA transcript is indicated. (C) Reconstitution of gel shift activity. Each 20-μl binding reaction mixture contained 1 ng of the radiolabeled TATA-box oligonucleotide, 100 ng of vector DNA, and, where indicated, 120 ng of rX-TBP and 5-μl aliquots of the indicated eluted and renatured protein fractions or 1 μl of the PC-B fraction. Most of the region of the gel containing the free DNA probe is not shown.

(lane 3). None of the individual eluted protein fractions along with rX-TBP reconstituted TATA-box DNA binding activity; however, the mixture of all three protein fractions and TBP (lane 10) or just the 75- and 92-kDa fractions and TBP (lane 8) reconstituted DNA binding activity at approximately 5 to 10% of the activity of the PC-B fraction. As found for reconstitution of TFIIIB transcription activity, the 65-kDa protein fraction was not necessary for reconstitution of gel shift activity. The mixture of TBP and the 75-kDa protein fraction yielded a minor protein-DNA complex of higher mobility than the TATA/PC-B complex, suggesting that the 75-kDa polypeptide might form a low-affinity complex with TBP in the absence of the 92-kDa polypeptide.

Apparent dissociation constant and stability of the TATA/PC-B protein complex. The apparent dissociation constant for the formation of the TATA-TFIIIB protein complex was determined in the gel mobility shift assay by DNA titration with a constant amount of Mono Q-purified TFIIIB in each reaction (Fig. 7). Under the conditions of this experiment, maximum complex formation is observed within 10 min of incubation (data not shown). Protein-DNA complexes were incubated for a sufficient time prior to gel electrophoresis (30 min) to ensure that the reactions had reached equilibrium. For determination of an apparent dissociation constant, data were obtained over a range of DNA concentrations spanning 2 log units (0.075 to 6 nM). The relative amounts of bound and free DNA for each input DNA concentration were determined by PhosphorImager analysis of the gel, and Fig. 7 shows a graphical representation of this data, where [bound DNA] is plotted versus [free DNA] and the apparent dissociation constant was obtained by using a quadratic fit to a single-component binding equation. This analysis yields a dissociation constant of 0.34 ± 0.07 nM. Similar values for the apparent dissociation constant were obtained in binding experiments performed either with or without nonspecific competitor DNA (plasmid DNA). This equilibrium dissociation constant is similar to the kinetic dissociation constant (0.3 nM) measured for the interaction of recombinant yeast TBP with the same TATA-box sequence (at 60 mM KCl [14]). In agreement with previous studies with

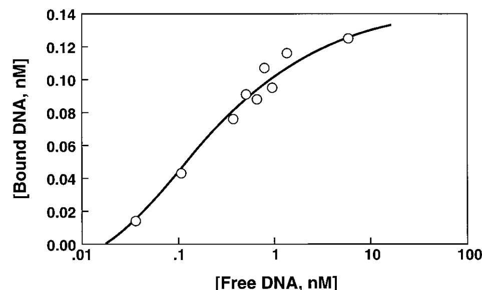

FIG. 7. Determination of the apparent dissociation constant for formation of the TATA-TFIIIB complex by DNA titration. Binding reaction mixtures were incubated for 30 min prior to analysis by gel electrophoresis and contained 0.25 μl of the Mono Q TFIIIB fraction and the following final concentrations of the ^{32}P-labeled double-stranded TATA-box oligonucleotide in a 20-μl volume: 0.075, 0.15, 0.45, 0.6, 0.75, 0.9, 1.05, 1.5, 3, and 6 nM. Quantitation of bound and free oligonucleotide in each reaction was by PhosphorImager analysis of the nondenaturing polyacrylamide gel. The concentration of bound DNA is plotted against the concentration of free DNA for each reaction.

yeast TFIIIB (15), once the TATA/PC-B protein complex has been formed, it is highly resistant to dissociation. Negligible dissociation of the complex is observed upon competition with a 100-fold excess of unlabeled oligonucleotide for up to 2 h prior to electrophoresis (data not shown). In contrast, the recombinant yeast TBP–TATA-box DNA complex dissociates in solution with a half-life of between 65 to 100 min (depending on the KCl concentration in the reaction [14]).

DISCUSSION

Previous studies have shown that TATA-box DNA oligonucleotides inhibit pol III transcription in vitro, using extracts and protein fractions from either human or *Acanthamoeba* cells (27, 40). This same TATA-box sequence is also inhibitory to transcription of a tRNA gene as assayed with an unfractionated extract from *Xenopus* eggs (Fig. 1). Further, we have shown that a TATA-box DNA–Sepharose resin will retain TFIIIB activity (8). Thus, it was reasonable to suspect that the TBP subunit of TFIIIB would be available to bind a TATA-box element and give rise to a stable protein-DNA complex that could be resolved by nondenaturing gel electrophoresis. A previous report from this laboratory demonstrated the cofractionation of TFIIIB transcriptional activity and TATA-box DNA binding activity on Mono Q FPLC (8); however, it was not determined in that study whether the gel mobility shift activity was due to TFIIIB, to free TBP, or to other TBP-containing complexes. The present report provides evidence that this protein-DNA complex corresponds to TFIIIB. It is conceivable that the TATA-box DNA binding activity of vertebrate TFIIIB that we describe does not reflect an activity utilized in vivo since TATA-containing pol III genes (such as the U6 small nuclear RNA gene) require other upstream binding factors for transcription (28, 43). However, a recent report has shown that *Drosophila* pol III can transcribe artificial DNA templates containing only TATA elements and the initiation sites derived from small nuclear RNA genes (35). Pol III must be recruited to these templates by a TBP-TFIIIB complex since these DNA constructs lacked all other pol III promoter elements.

Several lines of evidence indicate that the TATA/PC-B protein complex that we have identified by gel mobility shift assay contains TBP and that this complex corresponds to TFIIIB, rather than to free TBP or to other TBP-containing complexes. First, an antibody to TBP will block the formation of the complex if the antibody and PC-B proteins are incubated together prior to the addition of the TATA-box oligonucleotide (Fig. 2B), and prebinding of TBP to the antibody will reverse the inhibitory effect of the antibody. These results show that TBP is indeed part of this complex. Once the TATA/PC-B protein complex is formed, however, this antibody is without effect on either the abundance or the mobility of the complex. This finding suggests that the TBP epitope(s) recognized by this monoclonal antibody is masked either by the DNA, by conformational changes in TBP within the protein-DNA complex, or by other components of the complex. Second, the specificity of the complex for the TATA element was established by competition with wild-type (TATAAAA) and mutant (TAGAGAA) oligonucleotides (Fig. 3). The results of this experiment established a ~6-fold preference for the wild-type over the mutant sequence and a ~30-fold preference over nonspecific plasmid DNA. These relative affinities are similar, within a factor of 2 to the affinities of *Acanthamoeba* and *Xenopus* TBP for the same sequences as measured by inhibition of pol III transcription (Fig. 1B and reference 27). Third, the apparent dissociation constant for formation of the TATA/PC-B protein complex is similar to that observed for the interaction of recombinant yeast TBP with the same TATA element (14). The finding that rTBP alone will not generate a stable protein-DNA complex with the Tris-glycine-EDTA gel system (3) suggests that the TATA/PC-B complex contains polypeptides in addition to TBP. These polypeptides must confer complex stability in this gel system. Additionally, the TATA/PC-B complex, once formed, is far more stable in solution than the rTBP-TATA complex. The half-life of the rTBP complex has been reported to be 60 to 100 min (14), while we observe no dissociation of the TATA/PC-B complex over this time period (data not shown). This stability is similar to that reported for yeast TFIIIB bound to class III genes (15).

Several lines of evidence document that the TATA/PC-B complex corresponds to TFIIIB rather than to other TBP-containing complexes, such as the pol II factor TFIID or B-TFIID, or to the pol I factor SL1 or the U6 small nuclear RNA gene-specific factor PTF or SNAPc (reviewed in reference 13). First, the TATA/PC-B complex is most abundant in the PC-B fraction that contains TFIIIB and B-TFIID (Fig. 2A). TFIID and SL1 are most abundant in the PC-D fraction (0.6 to 1 M KCl), while SNAPc is most abundant in the PC-C (0.35 to 0.6 M KCl) fraction (13, 28). Human TFIIIB and B-TFIID can be separated by chromatography of PC-B proteins on Mono Q FPLC, where B-TFIID elutes from the resin at 0.15 M KCl while TFIIIB elutes at 0.3 to 0.4 M KCl (26, 36). We find that both the *Xenopus* TATA/PC-B gel shift activity and TFIIIB transcriptional activity coelute from the Mono Q resin between 0.3 and 0.4 M KCl (Fig. 4). The TATA-box DNA binding activity that we describe cofractionates with TFIIIB during chromatography on four ion-exchange resins. Further, TFIIIB TAF activity has been recovered from the TATA/PC-B complex eluted from the nondenaturing polyacrylamide gel (Fig. 5). TBP activity is not recovered from the complex with our isolation and renaturation protocol, and recombinant TBP was needed to observe TFIIIB transcriptional activity. No polypeptide of the expected molecular mass of *Xenopus* TBP (33 kDa) was observed in a silver-stained SDS-polyacrylamide gel of the eluted proteins; however, a polypeptide corresponding to 33 kDa was observed when proteins were eluted from the TATA/PC-B complex with a buffer containing SDS.

SDS-gel electrophoresis and silver staining of the polypeptides present in the TATA/PC-B gel shift complex revealed two major specific polypeptides with apparent molecular masses of 75 and 92 kDa (Fig. 6A). A 65-kDa polypeptide was also recovered from the nondenaturing gel at the position of the TATA/PC-B complex when PC-B proteins were subjected to electrophoresis either in the presence or in the absence of the TATA-box oligonucleotide, showing that this polypeptide is not a TFIIIB subunit. Densitometry of the silver-stained gel revealed that the 75- and 92-kDa polypeptides are present in approximately equal molar ratios (assuming equivalent silver staining of each polypeptide), suggesting that they are present in a 1:1 stoichiometry in the TATA/PC-B complex. It is evident that the latter two polypeptides are TFIIIB TAFs from the experiment in which Mono Q-purified TFIIIB was subjected to SDS-gel electrophoresis and polypeptides were eluted from gel slices corresponding to the expected migration positions for these polypeptides. These polypeptides were renatured by using a modification of the method of Hager and Burgess (9) and then tested for transcriptional activity in combination with rX-TBP, TFIIIC, and pol III (provided by a TAF-depleted PC-C fraction). The protein fractions containing the 75- and 92-kDa polypeptides were sufficient to support tRNA transcription in this reconstituted system (Fig. 6B) and were sufficient, along with rX-TBP, to reconstitute TATA-box DNA

binding activity in the Tris-glycine-EDTA gel system (Fig. 6C). We conclude that *Xenopus* TFIIIB contains, minimally, TBP and these 75- and 92-kDa polypeptides. Since previous studies have indicated that TFIIIB consists of polypeptides that are both tightly and loosely associated with TBP (33, 36), we do not know whether the TATA/PC-B complex contains the full complement of TFIIIB polypeptides or only the tightly bound TAF(s). Other TFIIIB components could have been provided by the PC-C fraction used in the transcription assays. Nonetheless, our experiments provide evidence that the TATA/PC-B complex corresponds to TBP and two tightly bound TFIIIB TAFs with apparent molecular masses of 75 and 92 kDa.

Previous studies have identified TBP-associated polypeptides in the PC-B fraction by immunoprecipitation with antibody to TBP (5, 23, 33). Polypeptides of 190, 87, and 60 kDa (5), 150, 82, and 54 kDa (23), and 172 kDa (33) were identified. The large polypeptide identified in each of these studies likely corresponds to a B-TFIID TAF since it can be separated from TFIIIB by methods that separate TFIIIB from B-TFIID (36). Similarly, we do not observe a high-molecular-weight TAF in the TATA/PC-B gel shift complex. The 92-kDa polypeptide that we observe in the TATA/PC-B complex most likely corresponds to human TFIIIB90, recently cloned by Wang and Roeder (36). These authors found that the immunopurified 92-kDa polypeptide or the recombinant TFIIIB90 polypeptide and TBP were sufficient to reconstitute TFIIIB activity when assayed with TFIIIC and RNA pol III (provided by a conventional PC-C fraction), while we find that both the 75- and 92-kDa polypeptides along with TBP are needed to reconstitute TFIIIB activity. One likely explanation for this difference is that we depleted our TFIIIC fraction of TBP-binding proteins prior to assaying for TFIIIB activity. If human TFIIIB contains multiple TAFs, the TFIIIC fraction used by Wang and Roeder could have provided the loosely associated TAFs (33). Indeed, Wang and Roeder noted that additional factors were needed for TFIIIB activity when the TBP-TFIIIB90 complex was assayed with highly purified TFIIIC and pol III (36). Alternatively, the polypeptide compositions of *Xenopus* and human TFIIIB could differ. A 67-kDa human TFIIIB TAF has also been described (5, 36). Cloning and expression of the *Xenopus* TFIIIB TAFs will be needed to unambiguously define the subunits of TFIIIB and will provide needed reagents for future studies on the regulation of TFIIIB activity during differentiation and the cell cycle (8, 34, 39, 41).

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