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TATA Box-Binding Protein (TBP) Is a Constituent of the Polymerase I-Specific Transcription Initiation Factor TIF-IB (SL1) Bound to the rRNA Promoter and Shows Differential Sensitivity to TBP-Directed Reagents in Polymerase I, II, and III Transcription Factors

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The role of the *Acanthamoeba castellanii* TATA-binding protein (TBP) in transcription was examined. Specific antibodies against the nonconserved N-terminal domain of TBP were used to verify the presence of TBP in the fundamental transcription initiation factor for RNA polymerase I, TIF-IB, and to demonstrate that TBP is part of the committed initiation complex on the rRNA promoter. The same antibodies inhibit transcription in all three polymerase systems, but they do so differentially. Oligonucleotide competitors were used to evaluate the accessibility of the TATA-binding site in TIF-IB, TFIID, and TFIIIB. The results suggest that insertion of TBP into the polymerase II and III factors is more similar than insertion into the polymerase I factor.

While the transcription systems of eukaryotic RNA polymerases I, II, and III obviously share some characteristics, initiation mechanisms for these transcription systems have been largely studied separately. The polymerases themselves have five subunits in common, seven in the case of the "odd pols," RNA polymerases I and III (45). Nevertheless, the general transcription factors involved with each polymerase have been examined in isolation, perhaps masking important generalizations about their functions. This approach began to change when it was discovered that some of the genes for small nuclear RNAs (snRNAs) are transcribed by polymerase III whereas most are transcribed by polymerase II (27, 32, 35, 47). All, however, have regulatory sequences which more closely resemble polymerase II than polymerase III promoters: they are entirely upstream of the transcribed gene, have TATA boxes at approximately −30, and use a common upstream activator protein, Oct 1, which also affects polymerase II protein gene promoters (20, 22, 28, 31, 51, 52). Most surprisingly, simple changes in spacing between the promoter elements could change the RNA polymerase which expressed the snRNA (18, 52). These observations prompted Margottin et al. (26) to test the yeast RNA polymerase II transcription factor which binds to the TATA sequence element (now known as TFIIDτ or TBP) for function in U6 snRNA transcription. TBP was found to be required for yeast U6 transcription by polymerase III and later by the cognate HeLa U6 RNA transcription system (23, 46). Subsequently, TFIIA (53) and a gene product related to TFIIB (4, 5, 17, 25) were found to participate in polymerase III transcription.

The full impact of this factor overlap was perhaps not realized because polymerase I still appeared to use dedicated factors. However, a flurry of studies (6, 7, 44, 56) revealed that TBP was required for transcription of all genes. TBP is now known to be a subunit of TFIID (41), TFIIIB (17, 24, 48, 55), and human TIF-IB (SL1) (6). TBP is associated with additional subunits (TAFs) to make up the functional factors (reviewed in reference 41). The TAFs appear to be different for each polymerase system (reviewed in reference 42), although overlap of TAFs has not been rigorously ruled out. All the TBP-containing factors are pivotal for their respective polymerases. Indeed, TFIIIB and TIF-IB are fundamental transcription factors; i.e., they appear to be responsible for the repetitive recruitment of RNA polymerase during successive rounds of initiation (16; reviewed in references 36 and 37).

The manner by which the TBP-containing factor is recruited to the promoter differs from gene to gene. For RNA polymerase III genes with type I (5S RNA) or type II (tRNA, VAI, Alu, EBER, 7SL, 4.5S) internal control regions, additional general transcription factors are required to assemble TFIIIB onto the promoter (reviewed in references 10–12). Initiation complex formation on 5S and tRNA genes is an organized process in which the factors bind in an obligatory order, each relying on protein-DNA and protein-protein interactions with a previously bound factor(s) to join the complex. On some genes the DNA interaction site for TFIIIB is sequence specific, whereas on others specific sequence recognition is not involved. Binding is not dependent on a canonical TATA box in most cases (12; however, see reference 50 for an exception). In all cases, TFIIIB is positioned over its site by the assembly factor(s). Even if there is a specific recognition sequence, the fundamental

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initiation factor cannot bind without the aid of the assembly factor(s).

In contrast, TFIID can bind many polymerase II gene promoters by itself, apparently via the DNA-binding domain of TBP (33). Nevertheless, on "TATA-less" protein-gene promoters (40; reviewed in reference 41) and snRNA genes (8), TFIID is tethered by other DNA-binding proteins distinct from the general transcription factors used on TATA-containing protein-gene promoters. We use the terms "tethering" and "assembly" factors interchangeably, although we do not imply synonymous mechanisms.

In the RNA polymerase I system, the fundamental initiation factor has characteristics similar to either TFIID or TFIIIB, depending on the species. Binding to the rRNA promoter of human, *Xenopus*, and *Acanthamoeba* TIF-IB closely follows the TFIIIB paradigm (3, 21, 29, 59). Binding is sequence specific and requires UBF as an assembly factor. In contrast, mouse (43) and rat (reviewed in reference 37) TIF-IB may not require UBF for function, although transcription is stimulated by the factor UBF. TIF-IB may therefore mimic TFIID in exhibiting a differential requirement for tethering factors on different promoters.

We answered three questions concerning the role of TBP in eukaryotic transcription. First, TBP is required for all three transcription systems in *Acanthamoeba castellanii*. Furthermore, specific antibodies were used to confirm that TBP is indeed a subunit of the fundamental rRNA transcription initiation factor, TIF-IB. Second, we demonstrated for the first time that TBP is a component of the committed transcription complex for RNA polymerase I. Third, we investigated in detail the puzzling report that TATA box oligonucleotides inhibit transcription of polymerase III genes regardless of whether the promoter of the gene has a TATA box element (56). We compared the sensitivity of all three polymerase systems to inhibition by TATA box-containing oligonucleotides. When we used a range of oligonucleotide concentrations in place of a single-point assay as in the earlier study (56), we found a less pronounced differential effect of the various oligonucleotides for a given polymerase system. Interesting differences among the three polymerase systems were observed, however, suggesting a variety of architectures in which TBP is assembled in the three systems.

MATERIALS AND METHODS

Template and oligonucleotide DNAs. A 448-bp DNA fragment isolated from plasmid pEBH10 (1) after cleavage with *Eco*RI and *Nde*I was used as the template for RNA polymerase I transcription. This fragment contained 120 bp of DNA upstream of the transcription start site, 80 bp of the pre-rRNA coding region, and 248 bp of vector (pUC8) DNA. Polymerase I transcription reactions with this DNA template produce a 309-nucleotide runoff transcript. The plasmid pBS+/5S.3 described previously (15) was used as the template for RNA polymerase III transcription of 5S RNA. A plasmid containing a single copy of the initiator $tRNA_1^{Met}$ gene from *Arabidopsis thaliana* (generously provided by Mike Zwick and W. Folk, University of Missouri-Columbia) was used as template for the tRNA transcription reactions. Plasmid pDTBP-97, described previously (58) was cleaved with *Bam*HI and used as the template for RNA polymerase II transcription.

Complementary oligonucleotides were synthesized by Macromolecular Resources, Colorado State University, Fort Collins. Equal molar amounts of the oligonucleotides were mixed and annealed by standard methods. The TATAAAA oligonucleotide contains the TATA box region of the adenovirus major late promoter from −45 to −15: TCCTGAAGGGGGGCTATAAAAGGGGGTGGGG. The TAGAGAA oligonucleotide is the same as the preceding oligonucleotide except for two base changes in the TATA box (underlined): TCCTGAAGGGGGGCTAGAGAAGGG GGTGGGG. The nonspecific oligonucleotide 1S contains the first 21-bp repeat from human T-cell leukemia virus type I: GATCTAAGGCTCTGACGTCTCCCCA (34). The TIF-IB control oligonucleotide contains the promoter region of the *A. castellanii* rRNA gene from −55 to −10 with five point mutations (underlined) previously shown (19) to increase transcription and committed complex formation (data not shown): CGACTGGAGCACTTTTCTTGCACCTAAAC TGGTTGCACCGTCCGAA.

Preparation of nuclear extracts. Nuclear extracts from vegetative *A. castellanii* cells were prepared as described previously (15, 60) with the following modifications. A 40-liter culture of cells was grown to a density of 2×10^6 to 4×10^6 cells per ml and harvested in a JCF-Z continuous-flow rotor (Beckman) at 6,000 rpm and 4°C. After the cells were washed with 15 mM KCl in HEM (10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES]-KOH [pH 7.9], 0.1 mM EDTA, 2 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM phenylmethanesulfonyl fluoride, 1 mM benzamidine), they were homogenized in two packed-cell volumes of the same buffer with 25 passes in a Potter-Elvehjem type homogenizer and centrifuged as described previously (15, 60). The crude nuclear pellet was suspended in two packed-cell volumes of HEM, and the nuclei were lysed at 0.5 M $(NH_4)_2SO_4$ (pH 7.9). The extract was centrifuged at 150,000 × *g* in a Beckman Ti70.1 rotor for 80 min at 4°C. The supernatant was collected, and solid ammonium sulfate was added (0.1735 g/ml) with gentle stirring on ice. The precipitated proteins were collected by centrifugation at 100,000 × *g* for 30 min at 4°C. After dialysis as previously described (60), the nuclear extract was cleared by centrifugation in the Beckman JA-20 rotor at 12,100 × *g* for 10 min at 4°C. The extract was divided into aliquots and stored at −70°C.

Protein fractionation. Nuclear extracts prepared from vegetative *Acanthamoeba* cells were sequentially fractionated on DEAE-Fast Flow (Pharmacia) and Bio-Rex 70 (Bio-Rad) columns and then subjected to two passages over a DNA affinity column. The elution profile of TIF-IB was determined by in vitro runoff transcription assays (14). TIF-IB from the second DNA affinity column was pooled and subjected to glycerol gradient sedimentation and fractionation. After this step in the fractionation scheme, TIF-IB is essentially homogeneous (unpublished data). Partially purified TIF-IB, used as noted in the experiments described below, is from the first DNA affinity pool (see Fig. 4A and 6). RNA polymerase I and *Acanthamoeba* UBF (aUBF) were partially purified from whole-cell extracts as detailed previously (14, 59). RNA polymerase I used in the transcription assays was from a heparin-Sepharose (Pharmacia) pool. The aUBF used in the gel mobility shift assays was from the peak fraction eluted from a MonoQ column (Pharmacia).

Preparation of anti-TBP immunogloblins. *Acanthamoeba* TBP was cloned, expressed in *Escherichia coli*, and purified by an extension of our previously published procedure (57). This TBP was purified by sodium dodecyl sulfate (SDS)-gel electrophoresis and used to produce polyclonal antibodies in rabbits. The immunoglobulins were purified from preimmune and immune serum by passage over a column contain-

ing immobilized protein G (no. 20398; Pierce) as specified by the manufacturer.

A Western immunoblot analysis (see below for conditions) of TBP expressed from full-length and deleted constructs of the cDNA clone was used to show that these immunoglobulins recognize epitopes in the N-terminal nonconserved domain (57).

Oligonucleotide and anti-TBP antibody inhibition of RNA polymerase I and III transcription. The indicated amount of each oligonucleotide was mixed with 3 μl of nuclear extract or 1 μl of partially purified TIF-IB in a final volume of 14 μl and a KCl concentration of 100 mM. The samples were incubated at 25°C for 30 min. Nucleotides, template DNA, $MgCl_2$, KCl, dithiothreitol, and buffer were then added to yield final reaction conditions that are optimal for transcription by each polymerase (14, 15). For the RNA polymerase I reactions, 2 μl of partially purified polymerase was added to start transcription. The final volume for all of the reactions was 30 μl. The RNA polymerase I and III transcription reactions were incubated at 25°C for an additional 30 and 75 min, respectively. The reactions were terminated and the transcripts were processed as detailed previously (15). The transcription products were electrophoresed through 6% polyacrylamide gels containing 8 M urea, and the RNA was visualized and quantified with a Phosphorimager gel scanner (Molecular Dynamics, Sunnyvale, Calif.) and Image Quant 3.22 software. All of the transcriptions were repeated multiple times.

In one-half the final transcription reaction volume, the appropriate amount of preimmune and/or anti-TBP immunoglobulin G (IgG) was mixed with partially purified TIF-IB or nuclear extract, water, and phosphate-buffered saline to keep the total salt concentration (KCl plus NaCl) between 100 and 150 mM. The antibody-protein samples were incubated at 25°C for 1 h, and then nucleotides, template DNA, $MgCl_2$, KCl, dithiothreitol, and buffer were added and the reactions were completed as noted above, except that the final reaction volume was 50 μl for the polymerase I transcriptions.

Oligonucleotide and anti-TBP antibody inhibition of RNA polymerase II transcription. The indicated amount of oligonucleotide or preimmune and/or anti-TBP IgG was mixed with 4 μl of nuclear extract and 6 μl of 3.3× transcription buffer (1× transcription buffer is 25 mM HEPES [pH 7.5], 10 mM $MgCl_2$, 70 mM potassium acetate, 0.2 mM EDTA, 2% glycerol, and 0.5 mM dithiothreitol) in a final volume of 20 μl. The reactions were incubated at 25°C for 30 min, and then transcription was started by adding 9 μl of 3.3× transcription buffer, 2.5 μl of 8 mM nucleoside triphosphates, 1 U of human placental RNase inhibitor (GIBCO BRL), and 100 ng of template DNA in a final volume of 50 μl. The transcription reactions were incubated at 30°C for 60 min. Transcription was then terminated, and the products were analyzed by primer extension as described previously (58). The primer extension products were quantified with a Betagen detector after autoradiography. All of the transcriptions were repeated multiple times.

Immunoblotting. Aliquots (200 μl) of fractions 13 through 19 from the TIF-IB glycerol gradient were precipitated with chloroform-methanol (54). The samples were resuspended in 1× SDS loading buffer and electrophoresed through an SDS–10% polyacrylamide gel by standard methods. Protein was transferred from the gel to a nitrocellulose membrane (Schleicher & Schuell) by a Genie electrophoretic blotter as specified by the manufacturer (Idea Scientific). The membrane was blocked with 5% bovine serum albumin in Tris-buffered saline (TBS-BSA) for 1 h at room temperature and then incubated with anti-TBP IgG (1:1,000 dilution) in TBS-BSA overnight at room temperature. The membrane was washed four times for 15 min with TBS containing 0.05% Tween 20 (TTBS) and then incubated with 15 ml of TBS-BSA containing 3 μl of ^{125}I-protein A (ICN Biochemicals, Inc.) for 2 h at room temperature. The membrane was washed four times for 15 min with TTBS, and cross-reactive protein bands were visualized and quantified by phosphorimage analysis as above.

Mobility shift assays. The *Eco*RI-*Hin*dIII fragment from pEBH10, which contains the rRNA promoter, was end labeled as described previously (1). This 240-bp DNA contains rRNA gene sequence from −120 to +80, plus 40 bp of multiple-cloning sequence from pUC8 (the insert was cloned into the *Hin*cII site). [^{32}P]DNA fragment (1 to 5 ng; 20,000 cpm) was incubated with the protein sample in 20 mM Tris-HCl (pH 8.0)–0.5 mM EDTA–10 mM $MgCl_2$–100 mM KCl–10% glycerol, with pBR322 DNA as the nonspecific competitor at 1.0 μg/μg of total protein. Committed complexes were allowed to form for 20 min at 25°C by incubation with aUBF and TIF-IB purified as described above. Then the indicated amounts of IgG were added and incubated for an additional 1 h at 25°C. In some reactions, aUBF and TIF-IB were omitted. The reactions were stopped by putting the tubes on ice, and the samples were loaded immediately on a cold gel. A low-cross-linking gel (5% acrylamide [5% T, 1.2% C], 5% glycerol, 20 mM Tris-HCl [pH 8.0], 1 mM EDTA) was prerun with a reservoir buffer of 20 mM Tris-HCl (pH 8.0)–1 mM EDTA–4.6 mM β-mercaptoethanol for 2 h at 4°C, with one change of buffer after 1 h. After prerunning, the buffer was changed again and the wells were rinsed with fresh reservoir buffer. The protein-DNA complex samples were then loaded on the gel and electrophoresed at a constant 18 mA (approximately 250 V) at 4°C for 2.25 h. The gel was dried and visualized on a phosphorimager as above.

RESULTS

Because TBP contributes to the activities of factors involved in transcription by all three polymerases, we asked whether its role in initiation was the same in each system. We were also intrigued by the apparent differences in the modes by which the individual factors are recruited to promoters. The common theme is that all are DNA-binding proteins. Therefore, we first tested the differential effect of oligonucleotides whose interaction with TBP is predictable.

TATA box oligonucleotide preferentially inhibits transcription by RNA polymerases II and III but not transcription by polymerase I. We titrated in vitro transcription extracts (crude nuclear extracts) with increasing amounts of three different blunt-ended double-stranded oligonucleotides: one contained a wild-type TATA box in the adenovirus major late promoter context (TATAAAA), and the second had the identical sequence except that the third and fifth bases of the TATA box were changed (TAGAGAA) (13). Alteration of these residues has been shown to strongly reduce but not totally eliminate TBP binding (13). The third oligonucleotide is a random sequence with approximately the same G+C content. Each of these oligonucleotides had a differential effect on transcription by the three polymerase systems. The primary data are shown in Fig. 1, and quantitative comparisons are presented in Fig. 2. At very large amounts of oligonucleotide, the polymerase II and III systems were inhibited, whereas polymerase I appeared to be less sensitive (see below). Inhibition by the two nonspecific oligonu-

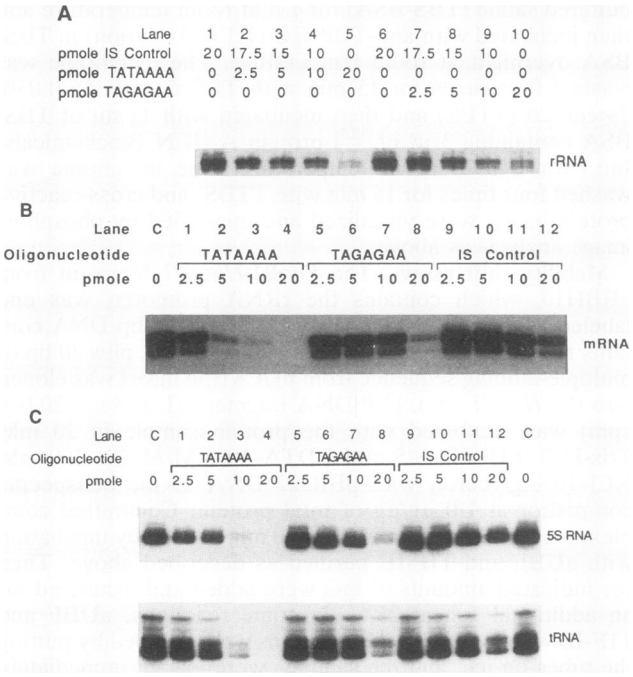

FIG. 1. Effect of oligonucleotide competitors on RNA polymerase I, II, and III transcription. The indicated amount of each oligonucleotide was preincubated with crude nuclear extract for 30 min at 25°C prior to the addition of DNA template and transcription initiation. Transcription conditions and processing of transcription products were as detailed in Materials and Methods. Lane C in panels B and C contains the products of a control reaction in which the nuclear extract was incubated in the absence of oligonucleotide. (A) RNA polymerase I in vitro transcription runoff products. (B) RNA polymerase II in vitro transcription products analyzed by primer extension. (C) RNA polymerase III in vitro transcription products from *Acanthamoeba* 5S RNA or *Arabidopsis* $tRNA_1^{Met}$ genes, obtained in separate experiments.

cleotides is lower than inhibition by the wild-type TATA oligonucleotide (Fig. 2B to D). The polymerase II and III systems show a differential effect of the TATAAAA and TAGAGAA oligonucleotides, with the wild-type oligonucleotide inhibiting most strongly. The polymerase II system, as expected, is inhibited strongly by the oligonucleotide containing the wild-type TATA box. The wild-type TATA box is required for transcription of this template (58). In the polymerase III system, transcription is similarly inhibited, showing that the TATA-binding domain of TBP is available and interaction with a TATA box oligonucleotide partially blocks a required step in transcription. However, no TATA element is found in these two polymerase III templates (60). Therefore, the inhibitory effect of the oligonucleotide may be on a step of transcription other than promoter DNA binding of TFIIIB (see Discussion).

Despite this differential effects of the wild-type, mutant, and random oligonucleotides at intermediate concentrations, all three exhibit a degree of inhibition at all concentrations. Therefore, comparisons at a fixed oligonucleotide concentration can inappropriately exaggerate or mask the differences (see Discussion).

Complex effects of the oligonucleotides in the crude transcription system were evident in the polymerase I transcription study. The addition of even small amounts of oligonucleotide to the crude nuclear extract strongly stimulated rRNA transcription (data not shown), in contrast to the polymerase II and III systems. We carried out the titration experiments with highly purified RNA polymerase I, aUBF, and TIF-IB (data not shown). The stimulatory effect of added oligonucleotides was eliminated in the purified system, suggesting that the oligonucleotides bind nonspecific inhibitors present in the crude system. Therefore, in the crude system, titrations at constant total oligonucleotide concentration (20 pmol) were carried out by varying the ratio of random 1S to wild-type or mutant TATA oligonucleotides (Fig. 1A). With this method the results were identical in the crude and purified systems. In these experiments, in contrast to the other systems, we saw no difference between the wild-type and mutant oligonucleotides. These results suggest that the sequence-specific TATA box-binding site of TBP in TIF-IB (see below) is neither used nor available in the rRNA transcription system. In contrast, this domain appears to be accessible in the polymerase II and III factors (see Discussion).

Antibodies against TBP inhibit all three transcription systems. An alternative explanation for the lack of differential inhibition of the polymerase I system is that TBP does not form part of the required transcription complex. Despite clear evidence that TBP is a component of free TIF-IB (6), there is no direct evidence that TBP is present in the committed complex on the rRNA promoter. To test this notion and to examine the requirement for TBP in all three of these systems, we used an immunological approach. Polyclonal antibodies were produced in rabbits against cloned (57), expressed, and gel-purified *Acanthamoeba* TBP. The IgG fraction was purified from immune and preimmune sera, and the purified IgGs were tested for their ability to inhibit the three in vitro transcription systems. In each case, transcription was strongly inhibited by addition of the anti-TBP antibodies (Fig. 3). Two additional points concerning this experiment are worth noting. First, we found that incubation of the transcription system components with preimmune IgGs resulted in a loss in activity when compared with incubation with an equivalent amount of BSA. The presence of protein stabilizes the transcriptional components during the incubation (data not shown), and different proteins have a differential stabilizing effect. Therefore, in the experiments whose results are shown in Fig. 3, a constant amount of IgG was maintained by varying the ratio of immune and preimmune IgG fractions. We also note that whereas the polymerase II and III systems can be completely inhibited, the polymerase I system reproducibly (three experiments) retains about 20% of its activity (Fig. 3D). This suggests that the antibody interferes slightly less efficiently in the polymerase I system (see Discussion).

From these results, it is clear that TBP is a required component in all three *Acanthamoeba* transcription systems. However, these experiments do not prove that TBP is in the complex of factors bound to the promoter.

TBP is a component of *Acanthamoeba* TIF-IB and of the committed complex on the rRNA promoter. The IgG fraction was used to test TIF-IB purified to near homogeneity for the presence of TBP. Two approaches were used. A Western blot of fractions across a peak of TIF-IB from a glycerol gradient was probed with the antibody and compared with the transcriptional activity of each fraction. There is a compelling correspondence between the amount of TIF-IB-dependent transcription activity and TBP detected in the Western blot (Fig. 4). Our purest preparations of TIF-IB contain polypeptides of 145, 99, 95, 91, and 32 kDa (TBP). Its native molecular mass has been estimated to be 289 kDa

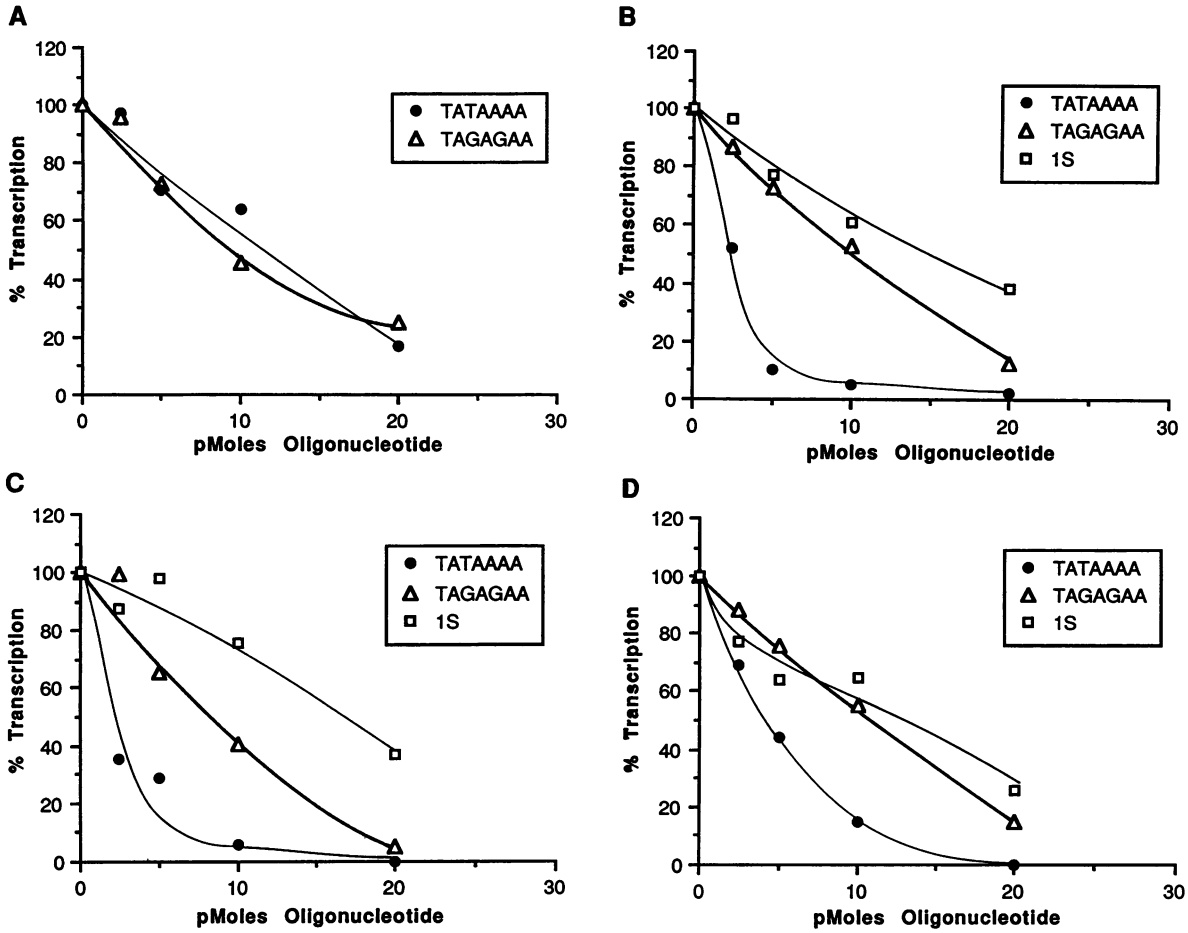

FIG. 2. Quantitative comparisons of oligonucleotide inhibition of RNA polymerase I, II, and III transcription. In each panel the percent transcription relative to the control reaction is plotted versus the amount of oligonucleotide present during the preincubation. (A) Transcription level for RNA polymerase I plotted versus the amount of oligonucleotide added. (B) Transcription level for RNA polymerase II plotted versus the amount of oligonucleotide added. (C) 5S RNA transcription level for RNA polymerase III plotted versus the amount of oligonucleotide added. (D) *Arabidopsis* $tRNA_1^{Met}$ transcription level for RNA polymerase III plotted versus the amount of oligonucleotide added.

(14), which is inconsistent with all of the polypeptides being subunits of TIF-IB. Further experiments are in progress to determine the subunit architecture of TIF-IB.

We also tested for the presence of TBP in the committed complex on the rRNA promoter. rRNA promoter DNA is retarded in its migration in native polyacrylamide gel electrophoresis by the binding of TIF-IB in the presence of aUBF to form the committed complex (38). This complex is expected to be additionally retarded (supershifted) by the binding of immunoglobulin against TBP to the committed complex. Neither immune (Fig. 5A, lane 1) nor preimmune (lane 2) IgG causes retardation of the free promoter DNA. Preincubation of TIF-IB and aUBF with DNA before addition of preimmune IgG resulted in the formation of the characteristic retarded band attributable to the committed complex (lane 3, DNA-TIF). When immune IgG was substituted for preimmune IgG, a supershifted band was obtained (lane 4). At higher concentrations of immune IgG, higher-order supershifted bands were obtained as additional epitopes were recognized by IgGs in the polyclonal preparation (Fig. 5B). This is the first direct demonstration that TBP is present in the committed complex on an rRNA promoter.

DISCUSSION

We show here that all three transcription systems in the small free-living amoeba *A. castellanii* have TBP as a required component. This extends the phylogenetic range in which this is true beyond human (reviewed in reference 42) and yeast (7, 44) cells. Second, we verified that TBP is a component of *A. castellanii* rRNA transcription factor TIF-IB. Previously, this had been published only for the cognate human factor (SL-1) (6) and reported for several other closely related vertebrate homologs (37). Third, we demonstrated that TBP is present in the committed complex on the rRNA promoter. This had not been formally tested previously. Finally, a series of oligonucleotide and antibody inhibition studies suggest that TBP is assembled in distinct ways in the three transcription systems, as has been suggested previously (42, 44).

One can consider several mechanisms by which the oligonucleotides or the antibodies might inhibit transcription. (i) They could bind and sequester the entire TBP-containing factor, making it unavailable for promoter binding. (ii) They could interact with the factor-promoter complex, eliciting an inactivating conformational change or sterically hindering

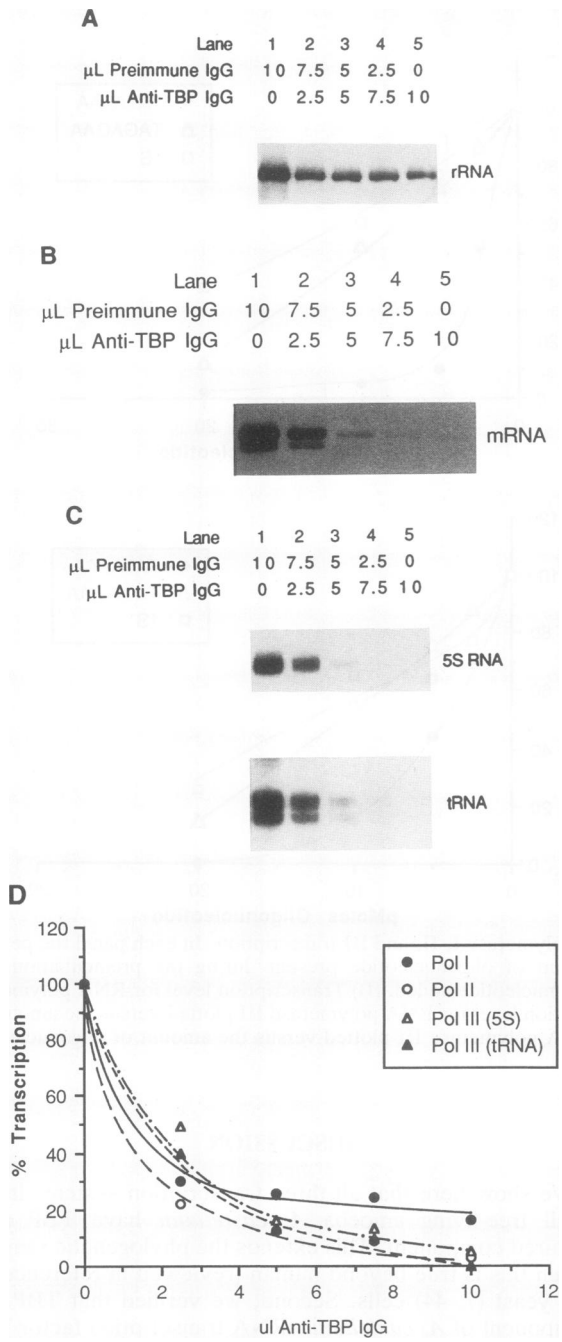

FIG. 3. Anti-TBP IgG inhibition of polymerase I, II, and III transcription. Nuclear extract or partially purified TIF-IB was preincubated with preimmune IgG and/or anti-TBP IgG prior to addition of template and transcription initiation as described in Materials and Methods. The total amount of IgG added was held constant by varying the ratio of preimmune IgG to anti-TBP IgG in the reactions. In panels A to C the amount of each specific IgG is shown above the transcription products. (A) RNA polymerase I in vitro transcription runoff products. (B) RNA polymerase II in vitro transcription products analyzed by primer extension. (C) RNA polymerase III in vitro transcription products from *Acanthamoeba* 5S RNA and *Arabidopsis* $tRNA_1^{Met}$ genes. (D) Quantitative comparison of anti-TBP IgG inhibition of RNA polymerase I, II, and III transcription. The percent transcription relative to the control reaction containing only preimmune IgG (100%) is plotted versus the amount of anti-TBP IgG present in the preincubation.

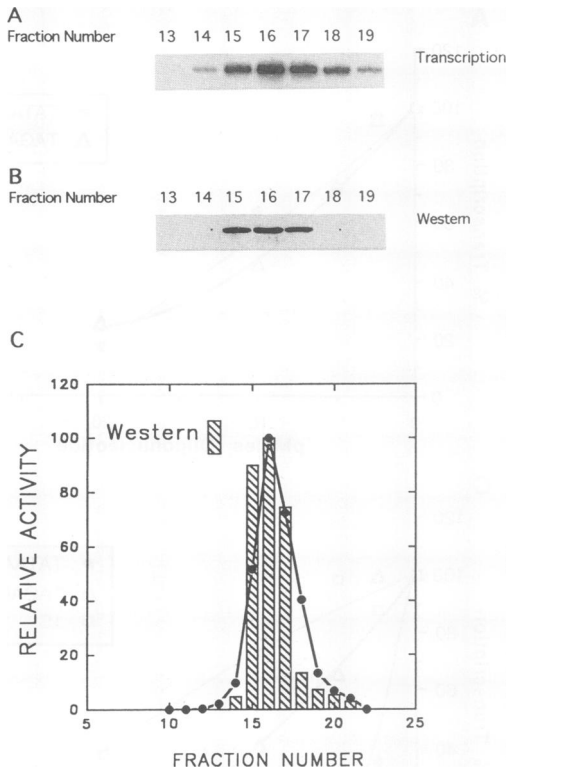

FIG. 4. Extensively purified TIF-IB and TBP cofractionate on a glycerol gradient. (A) Individual fractions from a glycerol gradient were assayed for TIF-IB activity in reconstituted RNA polymerase I transcription reactions. The runoff transcription products are shown below the corresponding fraction number. (B) Western analysis across the peak of TIF-IB from the glycerol gradient, using anti-TBP IgG and ^{125}I-protein A detection. (C) Quantitative comparison of the transcriptional activity and the amount of TBP detected in the Western analysis of the TIF-IB fractions from the glycerol gradient. Relative activities were determined by comparison with the peak fraction (fraction 16). Transcription levels are indicated by the solid circles and line graph. TBP levels are represented by the cross-hatched bar graph.

later interactions in the transcription process. (iii) Free TBP could be in a dynamic equilibrium with the native transcription factor (TBP plus TAFs), and the oligonucleotides or antibodies could sequester the free TBP subunit in solution. There exists evidence for a facile exchange of exogenous TBP into HeLa TFIIIB (55). However, since there is evidence against a significant free TBP pool (49), we will not consider this mechanism further in the context of our experiments. In addition, the same mechanism does not necessarily apply to all three transcription systems.

For polymerase II, the first mechanism seems most likely. Since the majority of polymerase II genes contain a functional TATA element, the TATA-binding site of TBP must be exposed in native TFIID. Furthermore, it has been experimentally demonstrated that the same oligonucleotides used in the present study differentially inhibit the binding of TFIID (13). In TFIID, the TAFs are tightly associated with the TBP subunit (reviewed in reference 41).

Similarly, U6 snRNA and *Neurospora* 5S RNA genes, both transcribed by RNA polymerase III, have required TATA boxes (see Introduction). This suggests that the TATA-binding site of TFIIIB is accessible in the native

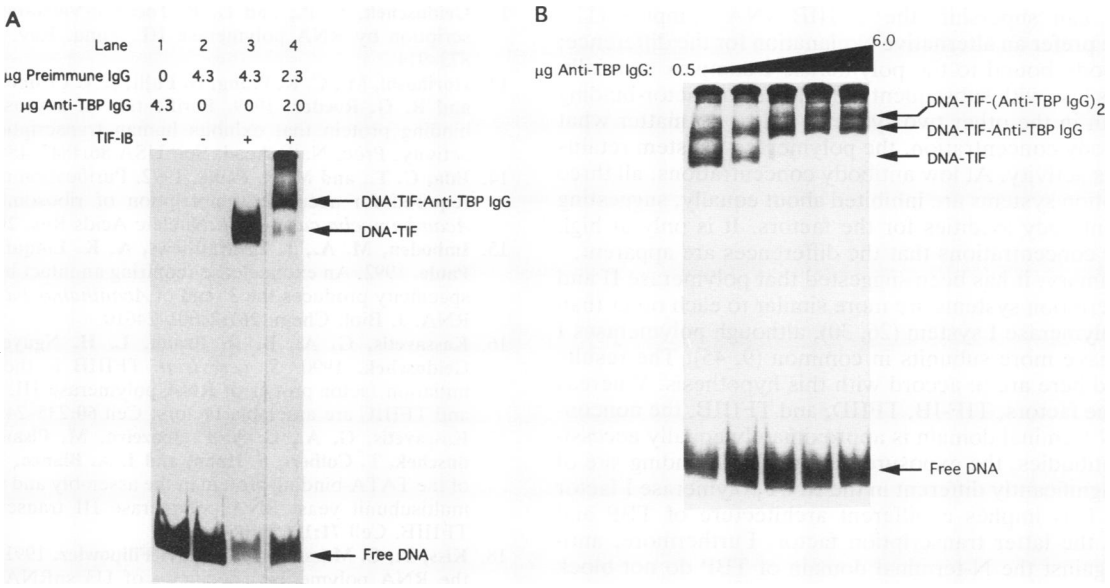

FIG. 5. TBP is a component of the RNA polymerase I committed initiation complex. (A) Labeled promoter DNA was incubated for 20 min at 25°C with or without TIF-IB as indicated to allow the initiation complex to form. Preimmune IgG and/or anti-TBP IgG was then added, and incubation was continued for an additional 1 h. Protein-DNA complexes were resolved from free promoter DNA by electrophoresis through a 5% (5% T, 1.2% C) polyacrylamide gel under native conditions. The locations of free DNA and interpretations of DNA-protein complexes are shown to the right of the panel. (B) Increasing amounts of anti-TBP IgG were added to preformed initiation complexes on labeled promoter DNA. The incubation conditions and electrophoretic analysis are the same as noted for panel A.

factor. However, on yeast 5S RNA and tRNA genes, TBP cannot be cross-linked to the DNA in the TFIII(B+C)-DNA complex, leading to the conclusion that it is not in proximity to the DNA in the initiation complex (17). The similarity of inhibition profiles by oligonucleotides of polymerases II and III are consistent with similar availability of the TATA-binding site of TBP in TFIID and TFIIIB. Thus, inhibition of polymerase III transcription could occur by direct interaction of the TATA-oligonucleotide with the DNA-binding site of TBP, causing an inactivating conformational change, sequestration of the factor, or steric hindrance of protein-protein interactions.

In contrast, results presented here for polymerase I are most consistent with the conclusion that the TATA-binding site of TBP in TIF-IB is not available for interaction with TATA sequences. First, we observed little differential effect of the TATA and mutant TATA oligonucleotides on rRNA transcription (Fig. 2A). Second, there is a perfect TATA box (TATAAA) surrounding +1 in the *Acanthamoeba* and some other eukaryotic (39) rRNA genes, but this site is not footprinted by TIF-IB (1, 2). The rRNA promoter-binding site for TIF-IB in *Acanthamoeba* cells does not resemble a consensus TATA element (19).

We raise a note of caution in the use of single concentrations of oligonucleotides in transcription inhibition experiments. Especially in crude systems, complex and opposite effects can be obtained on addition of oligonucleotides which bind both required factors and nonspecific inhibitors. Because of the latter effect, direct comparison with the activity in the absence of added oligonucleotide is critical.

In contrast to the differential accessibility of the TATA-binding site of TBP in TIF-IB, TFIID, and TFIIIB, the accessibility of the N-terminal domain of TBP to antibodies in all three factors appears similar (Fig. 3). Polyclonal antibodies against homologous TBP, but not preimmune immunoglobulins, inhibit transcription by *Acanthamoeba* RNA polymerases I, II, and III. Like TBP from all other eukaryotes, the sequence of TBP from *A. castellanii* can be divided into two segments (57). The C terminus is highly conserved and folds into the saddle-shaped DNA-binding domain which contains the TATA-binding site. The N-terminal 80 amino acids constitute the nonconserved domain, which varies considerably from organism to organism (33). Our antibodies recognize epitopes in this nonconserved N-terminal domain (data not shown). Inhibition of polymerase I, II, and III transcription by these immunoglobulins shows that the N-terminal domain is exposed in all three TBP-containing initiation factors. In the TIF-IB–rRNA gene complex, a significant portion of the N-terminal domain must be exposed because at least two distinct supershifted bands are obtained (Fig. 5B), corresponding to association of one and two immunoglobulins. Alternatively, this result could be interpreted as two TBP molecules in the committed rRNA gene promoter complex, either as a duplicated subunit of TIF-IB or as two TIF-IBs bound to the core promoter.

Even at Ig concentrations four times that needed to produce the double supershifted bands, the polymerase I system still retains 20% of its transcriptional activity. This contrasts with the other two systems, which are inhibited completely (Fig. 3). We conclude that antibodies bound to the N-terminal domain in the polymerase I factor interfere less extensively with important interactions in the transcription process than in the other two systems. The ability to obtain a supershifted complex with TIF-IB demonstrates that the anti-TBP antibody does not completely eliminate the ability of the factor to bind the rRNA promoter. In the polymerase II and III systems, binding to antibody or to promoter DNA might be mutually exclusive, resulting in complete dissociation of the complex at high antibody titers. In opposition to this notion, we note that in yeast cells, TBP

antibody can supershift the TFIIIB-DNA complex (17). Thus, we prefer an alternative explanation for the difference: the antibody bound to the polymerase I complex sterically interferes less with subsequent polymerase or factor-binding steps than in the other two systems. Thus, no matter what the antibody concentration, the polymerase I system retains 20% of its activity. At low antibody concentrations, all three transcription systems are inhibited about equally, suggesting similar antibody avidities for the factors. It is only at high antibody concentrations that the differences are apparent.

In summary, it has been suggested that polymerase II and III transcription systems are more similar to each other than to the polymerase I system (26, 30), although polymerases I and III have more subunits in common (9, 45). The results presented here are in accord with this hypothesis. Whereas in all three factors, TIF-IB, TFIID, and TFIIIB, the nonconserved N-terminal domain is approximately equally accessible to antibodies, the exposure of the TATA-binding site of TBP is significantly different in the RNA polymerase I factor TIF-IB. This implies a different architecture of TBP and TAFs in the latter transcription factor. Furthermore, antibodies against the N-terminal domain of TBP do not block subsequent interactions, presumably protein-protein, as effectively in the polymerase I system as in the other two systems (Fig. 3). This implies a fundamental difference between these interactions in the polymerase I system.

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