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Mechanism of TATA-Binding Protein Recruitment to a TATA-Less Class III Promoter

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Summary

The TATA-binding protein (TBP) is required for transcription by RNA polymerase III (pol III), even though many pol III templates, such as the adenovirus VA_1 gene, lack a consensus TATA box. We show that TBP alone does not form a stable, productive interaction with VA_1 DNA. However, it can be incorporated into an initiation complex if the other class III basal factors, TFIIIB and TFIIIC, are also present. TFIIIB can associate with the evolutionarily conserved C-terminal domain of TBP in the absence of DNA or TFIIIC, suggesting that TFIIIB exists in solution as a complex with TBP. The stable association of TBP with an essential component of the pol III transcription apparatus may account for the ability of TATA-less class III genes to recruit TBP.

Introduction

A requirement for the TATA-binding protein (TBP) was originally thought to be restricted to TATA box–dependent transcription by RNA polymerase II (pol II). However, it has recently been shown to be necessary also for transcription in vitro of TATA-containing pol III templates (Lobo et al., 1991; Margottin et al., 1991; Simmen et al., 1991), TATA-less pol II templates (Pugh and Tjian, 1991), TATA-less pol III templates (White et al., 1992a, 1992b), and pol I templates (Comai et al., 1992). The in vivo relevance of these biochemical studies has been confirmed using yeast genetics (Cormack and Struhl, 1992; Schultz et al., 1992). Therefore, TBP is required for transcription by all three nuclear RNA polymerases found in eukaryotes (reviewed by Green, 1992; Sharp, 1992; White and Jackson, 1992).

Whereas recombinant TBP exists as a monomer in solution, TBP in extracts from human or Drosophila cells occurs in high molecular weight complexes (Pugh and Tjian, 1990; Dynlacht et al., 1991; Pugh and Tjian, 1991; Tanese et al., 1991; Timmers and Sharp, 1991; Comai et al., 1992). This is due to its interaction with polypeptides referred to as TBP-associated factors (TAFs) (reviewed by Pugh and Tjian, 1992; Sharp, 1992). Several distinct TBP-containing complexes have been identified in HeLa extracts. One of these is TFIID, which functions in pol II transcription, contains at least 10 TAFs, and elutes from phosphocellulose in the high salt D fraction (PC-D; 0.6–1.0 M KCl) (Pugh and Tjian, 1990, 1991; Tanese et al., 1991; Timmers and Sharp, 1991). A second complex that contains TBP is the pol I basal factor SL1, which contains 3 TAFs and also elutes in the PC-D fraction (Comai et al., 1992). A distinct TBP-containing complex, referred to as B-TFIID, elutes from phosphocellulose in a 0.04–0.35 M KCl fraction and will support pol II transcription after further fractionation (Timmers and Sharp, 1991). It has been suggested that different TBP–TAF complexes are specific for different classes of RNA polymerase, and that distinct sets of TAFs in each complex confer class specificity (Comai et al., 1992; Sharp, 1992; White and Jackson, 1992). Although SL1 and TFIID represent TBP–TAF complexes for pols I and II, respectively, no such complex has yet been identified for the pol III system.

Two activities were originally identified as being required for transcription of tRNA and VA_1 genes by pol III; these elute in the phosphocellulose B (PC-B; 0.1–0.35 M KCl) and C (PC-C; 0.35–0.6 M KCl) fractions and were named TFIIIB and TFIIIC, respectively (Segall et al., 1980). Both of these factors have proved complex and difficult to study, and their molecular compositions remain controversial (reviewed by Gabrielsen and Sentenac, 1991). However, it is clear that TFIIIC is involved in promoter recognition and that TFIIIB is only assembled into a complex on a tRNA or VA_1 gene once TFIIIC is bound (Lassar et al., 1983; Fuhrman et al., 1984; Carey et al., 1986; Dean and Berk, 1988; Kassavetis et al., 1989, 1990; reviewed by Geiduschek and Tocchini-Valentini, 1988; Gabrielsen and Sentenac, 1991; Wolffe, 1991). These studies were all conducted prior to the discovery that TBP is also an essential component of the pol III transcription machinery (Cormack and Struhl, 1992; Schultz et al., 1992; White et al., 1992a, 1992b). The question therefore arises as to where TBP fits into the reaction pathway for transcription complex assembly on class III genes.

For TATA-containing class II genes, TBP binds directly to DNA, and this first step in promoter recognition serves to nucleate the assembly of a transcription complex (reviewed by Greenblatt, 1991; Roeder, 1991). This might also be the case for class III genes with TATA boxes, such as the U6 snRNA genes. However, the majority of vertebrate class III genes lack TATA sequences. In these cases, two alternative strategies can be envisaged for recruiting TBP. One is that TBP binds DNA directly, but at a sequence other than a TATA box. A variety of sequences bearing little or no resemblance to the consensus TATA element (TATAAA) can functionally replace the TATA box of a yeast pol II promoter and support wild-type levels of expression in vivo, as well as TBP-dependent transcription in vitro (Singer et al., 1990). It is therefore possible that some class III genes without consensus TATA boxes nevertheless have functional binding sites for TBP. The alternative strategy for recruiting TBP to a TATA-less promoter is by protein–protein interactions. For example, TBP alone does not recognize the human rRNA promoter; the upstream binding factor (UBF) initiates transcription complex formation in this case and TBP is then recruited as part of SL1 (Bell et al., 1988; Comai et al., 1992). An analogous mechanism might involve TBP recruitment in association with a pol III factor.

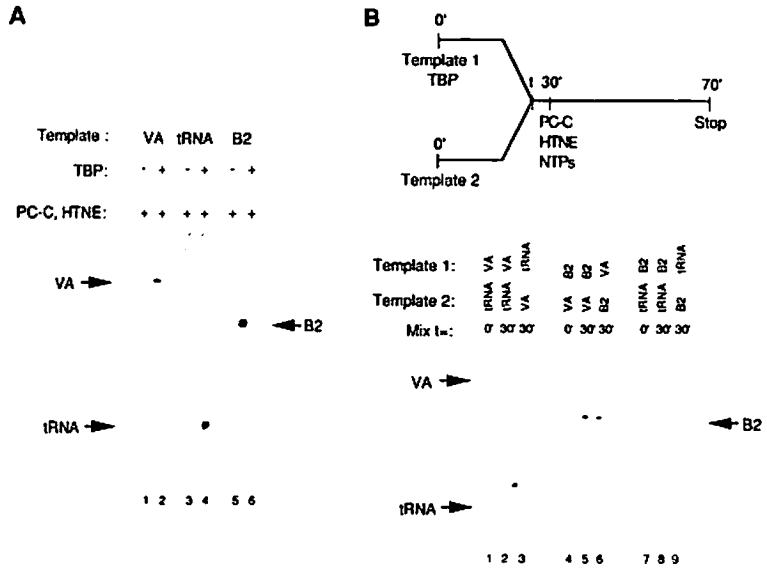

Figure 1. TBP Does Not Form a Stable Productive Complex on Naked VA_1 DNA

(A) Demonstration that TBP is limiting for transcription of VA_1, $tRNA^{Leu}$, and B2 genes in the presence of PC-C and HTNE. One microgram of pBRVA$_1$ (lanes 1 and 2), pLeu (lanes 3 and 4), or pTB14 (lanes 5 and 6) templates was preincubated with 2 μl of PC-C and 1.6 μl of HTNE for 15 min at 30°C in the presence (lanes 2, 4, and 6) or absence (lanes 1, 3, and 5) of 5 ng of recombinant TBP. Nucleotides were then added and transcription was allowed to proceed for 40 min at 30°C. Relatively low amounts of TBP were used in the experiments in Figures 1–3 in order to ensure that this factor did not saturate the first template; as a result, the levels of induction obtained were less than maximal.

(B) Incubation of VA_1, $tRNA^{Leu}$, or B2 genes with TBP in the absence of other factors does not result in a stable productive interaction. Template 1 (0.5 μg), as indicated, was preincubated with 5 ng of TBP under transcription reaction conditions for 30 min at 30°C. Template 2 (0.5 μg), as indicated, was added either at the beginning (lanes 1, 4, and 7) or at the end (lanes 2, 3, 5, 6, 8, and 9) of the preincubation. PC-C (2 μl), 1.6 μl of HTNE, and nucleotides were then added and transcription was allowed to proceed for 40 min at 30°C.

We have addressed the molecular mechanism whereby TBP is recruited to the adenovirus 2 VA_1 gene. This gene lacks a TATA sequence, but does contain the sequence GATAAA (+24 to +29) within the transcribed region. Since TBP has weak affinity for a similar sequence in the chicken β-globin promoter (Fong and Emerson, 1992), and since internal TBP-binding sites can be functional in class II genes (Carcamo et al., 1990), it seemed possible that this sequence serves as a recognition site for TBP. However, we were unable to detect direct binding of cloned TBP to the VA_1 gene. In contrast, VA_1 is able to sequester TBP stably into an active transcription complex if the other pol III factors are also present. We find that TBP interacts with a component of TFIIIB. Since this association can occur in solution in the absence of TFIIIC or a promoter, TFIIIB may exist as a complex with TBP. These findings demonstrate the existence of a TBP-associated pol III factor and establish a mechanism by which TATA-less class III genes are able to utilize TBP.

Results

TBP Alone Does Not Bind Directly to the VA_1 Gene

We tested whether cloned TBP could bind directly to VA_1 sequences. Preliminary DNAase I footprinting experiments provided no evidence for a direct interaction between TBP and VA_1 DNA (data not shown). However, TBP can be difficult to footprint and DNAase I footprinting may only detect relatively strong interactions. We therefore employed the more sensitive template commitment assay to look for binding. In such experiments, a stable interaction between a gene and a limiting factor can be detected by the inhibition of transcription of a subsequently added second gene that requires the same factor; this reflects the ability of the first gene to bind and sequester the limiting factor so that it is no longer available to interact with the second gene. Whereas interactions of low affinity can be displaced by DNAase I, such interactions can be detected using a template commitment assay if the competing second template also has a low affinity for the factor in question. This approach was used to determine whether the documented involvement of TBP in VA_1 transcription (White et al., 1992a, 1992b) involves direct binding to DNA.

Cloned, purified TBP was incubated with template for extended periods of time under transcription conditions. A second template was then added along with a PC-C fraction and HeLa extract that had been heat treated at 47°C for 15 min. This mild heat treatment inactivates TBP and TFIIIC, but not TFIIIB (Nakajima et al., 1988; Simmen et al., 1991; White et al., 1992a, 1992b). The PC-C fraction provides active TFIIIC, leaving reaction conditions in which TBP is rate limiting for pol III transcription (White et al., 1992a, 1992b). Figure 1A shows this to be the case for VA_1, $tRNA^{Leu}$, and B2 genes, since addition of TBP stimulates transcription of each of these in the presence of heat-treated nuclear extract (HTNE) and PC-C. The low levels of expression observed in the absence of added TBP reflect the presence of some active TBP in the PC-C fraction (White et al., 1992a). If template 1 is able to sequester TBP during the preincubation step, then it will be preferentially transcribed relative to the subsequently added template 2 under these conditions. Figure 1B shows that this is not the case and that the ratio of transcription of the VA_1, $tRNA^{Leu}$, and B2 genes is not affected by which gene is preincubated with TBP. The preincubation time in these experiments was 30 min, but the same result is obtained if it is extended to 1 hr (data not shown). Therefore TBP does not interact stably with these genes when they are presented as naked DNA templates.

TBP Is Stably Sequestered onto the VA_1 Promoter In the Presence of TFIIIB and TFIIIC

We tested whether TBP is recruited onto the VA_1 promoter in the presence of the other class III basal factors using

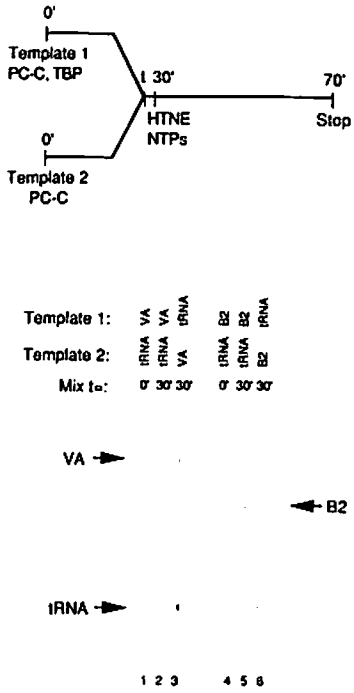

Figure 2. TFIIIC Is Insufficient to Allow TBP to Form a Stable Productive Complex with the VA$_I$ Gene

Template 1 (0.5 μg), as indicated, was preincubated with 5 ng of TBP and 2 μl of PC-C under transcription reaction conditions for 30 min at 30°C. Template 2 (0.5 μg), as indicated, was preincubated in parallel with 2 μl of PC-C under transcription reaction conditions for 30 min at 30°C. The reactions were mixed either at the start (lanes 1 and 4) or at the end (lanes 2, 3, 5, and 6) of the preincubation. HTNE (1.6 μl) and nucleotides were then added and transcription was allowed to proceed for 40 min at 30°C.

modifications of the template commitment protocol employed above. Transcription complex assembly on the VA$_I$ gene is known to involve DNA recognition by TFIIIC followed by TFIIIB binding to the TFIIIC–DNA complex (Lassar et al., 1983; Fuhrman et al., 1984; Carey et al., 1986; Dean and Berk, 1988). We therefore used the template commitment assay to ascertain whether TBP interacts productively with VA$_I$, tRNA^{Leu}, and B2 genes in the presence of TFIIIC. Template 1 was preincubated with TBP and PC-C, while template 2 was preincubated in parallel with PC-C alone. The reactions were then mixed, HTNE and nucleotides were added, and transcription was allowed to proceed. Figure 2 shows that no competitive advantage is conferred upon any of these genes by preincubation with TBP in the presence of TFIIIC. These results suggest that TBP does not interact stably with the complexes formed between TFIIIC and these genes.

We next tested whether TBP can be recruited to complexes involving both TFIIIB and TFIIIC bound to these promoters. Template 1 was preincubated with TBP, PC-C, and HTNE, while, in a parallel reaction, template 2 was preincubated with PC-C and HTNE in the absence of added TBP. The reactions were then mixed, nucleotides were added, and transcription was allowed to proceed. Figure 3A shows that the inclusion of PC-C and HTNE in the preincubation reaction enables VA$_I$ to sequester TBP stably so that transcription of subsequently added tRNA^{Leu} or B2 genes is reduced. Similarly, preincubation of tRNA^{Leu} or B2 genes with TBP, PC-C, and HTNE provides a competitive advantage relative to a subsequently added second template that was preincubated with just PC-C and HTNE. The ability of the tRNA^{Leu} gene to reduce transcription of a second template appears somewhat less than that of the VA$_I$ and B2 genes. The reason for this has not been investigated, but it may be that TFIIIB associates less stably with this template. Differences between class III genes in the stability of factor binding have previously been reported (Lassar et al., 1983; Carey et al., 1986; Dean and Berk, 1988). In no case is exclusion complete, since the PC-C fraction itself contains some TBP. However, the preferential transcription of VA$_I$ when preincubated with TBP and the other class III factors and substan-

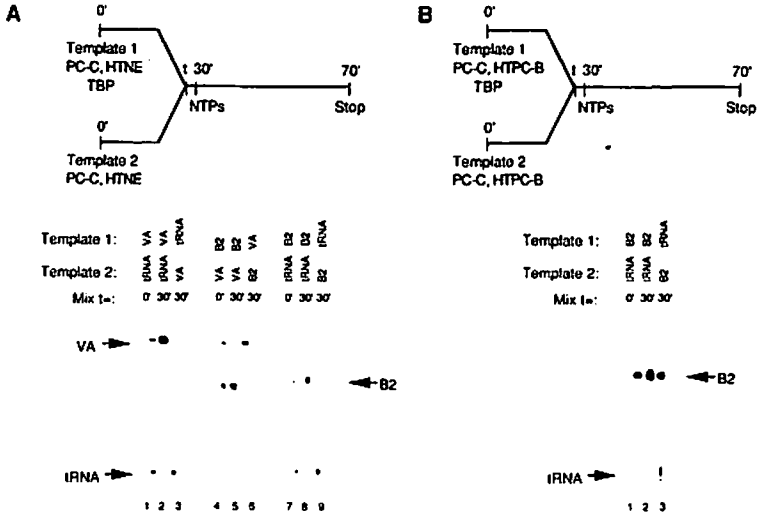

Figure 3. TBP Forms a Stable Complex on the VA$_I$ Gene in the Presence of TFIIIC and TFIIIB

(A) TBP can be sequestered in the presence of PC-C and HTNE. Template 1 (0.5 μg), as indicated, was preincubated with 5 ng of TBP, 2 μl of PC-C, and 1.6 μl of HTNE under transcription reaction conditions for 30 min at 30°C. Template 2 (0.5 μg), as indicated, was preincubated in parallel with 2 μl of PC-C and 1.6 μl of HTNE under transcription reaction conditions for 30 min at 30°C. The reactions were mixed either at the start (lanes 1, 4, and 7) or at the end (lanes 2, 3, 5, 6, 8, and 9) of the preincubation. Nucleotides were then added and transcription was allowed to proceed for 40 min at 30°C.

(B) TBP can be sequestered in the presence of PC-C and HTPC-B. Template 1 (0.5 μg), as indicated, was preincubated with 5 ng of TBP, 2 μl of PC-C, and 2 μl of HTPC-B under transcription reaction conditions for 30 min at 30°C. Template 2 (0.5 μg), as indicated, was preincubated in parallel with 2 μl of PC-C and 2 μl of HTPC-B under transcription reaction conditions for 30 min at 30°C. The reactions were mixed either at the start (lane 1) or at the end (lanes 2 and 3) of the preincubation. Nucleotides were then added and transcription was allowed to proceed for 40 min at 30°C.

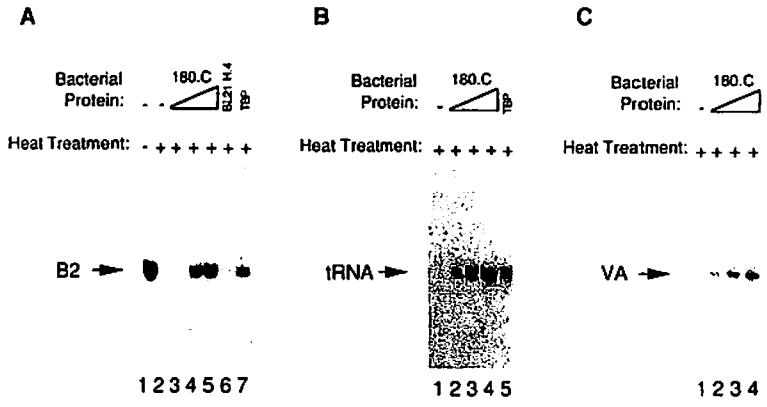

Figure 4. The C-Terminal 180 Amino Acid Residues of TBP Are Sufficient for Pol III Transcription

(A) The 180.C protein supports B2 transcription. pTB14 (1 μg) was preincubated for 15 min at 30°C with 1.6 μl of unheated extract (lane 1) or with 2 μl of PC-C and 1.6 μl of HTNE (lanes 2–7) in the presence of the following bacterially produced protein: lanes 1 and 2, none; lane 3, 5 ng of 180.C; lane 4, 15 ng of 180.C; lane 5, 20 ng of 180.C; lane 6, 200 ng of the equivalent fraction from the same strain of bacteria lacking an expression vector; lane 7, 10 ng of full-length TBP. Nucleotides were then added and transcription was allowed to proceed for 40 min at 30°C.

(B) The 180.C protein supports tRNA transcription. pLeu (1 μg) was preincubated for 15 min at 30°C with 2 μl of PC-C and 1.6 μl of HTNE in the presence of the following bacterial protein: lane 1, none; lane 2, 10 ng of 180.C; lane 3, 15 ng of 180.C; lane 4, 20 ng of 180.C; lane 5, 8 ng of full-length TBP. Nucleotides were then added and transcription was allowed to proceed for 40 min at 30°C.

(C) The 180.C protein supports VA_1 gene transcription. pBRVA_1 (1 μg) was preincubated for 15 min at 30°C with 2 μl of PC-C and 1.6 μl of HTNE in the presence of the following bacterial protein: lane 1, none; lane 2, 5 ng of 180.C; lane 3, 10 ng of 180.C; lane 4, 15 ng of 180.C. Nucleotides were then added and transcription was allowed to proceed for 40 min at 30°C.

tial exclusion of the second template demonstrate that TBP can be stably incorporated into a transcription complex formed on the TATA-less VA_1 promoter. This is also the case for the B2 and, to a lesser extent, $tRNA^{Leu}$ genes. This effect is obtained when TFIIIC and TFIIIB are both included in the preincubation step, but not with TFIIIC alone. In the previous experiments HTNE was used to provide active TFIIIB. A similar response is obtained if a heat-treated PC-B fraction (HTPC-B) is instead used as a source of partially purified TFIIIB (Figure 3B). Therefore, TBP recruitment to TATA-less class III promoters requires the presence of additional proteins, including a source of TFIIIB.

The Conserved C-Terminal Domain of TBP Is Sufficient to Mediate Pol III Transcription

In each case examined to date, proteins that contact TBP directly have been found to associate with its conserved C-terminal domain (Lee et al., 1991; Lieberman and Berk, 1991; Buratowski and Zhou, 1992; Hagemeier et al., 1992). Since we have implicated protein–protein interactions in recruiting TBP to TATA-less class III genes, we asked whether the C-terminal region of TBP is sufficient to mediate transcription of these genes. Figure 4A shows that this is indeed the case. The C-terminal 180 amino acid residues of TBP (180.C) expressed in bacteria are sufficient to activate transcription of a B2 gene in a TBP-dependent reaction containing PC-C and HTNE. Proteins from the same bacterial strain without an expression vector do not have this effect. Transcription levels obtained with unheated extract and with full-length TBP are shown for comparison. The 180.C protein also activates transcription of the $tRNA^{Leu}$ and VA_1 genes in this assay (Figures 4B and 4C). Therefore, the C-terminal domain of TBP is sufficient to interact productively with the pol III transcription apparatus.

TBP Associates Specifically with a Component of TFIIIB

The above results do not distinguish whether TBP recognizes protein alone or whether it recognizes a protein–DNA complex. We therefore tested whether TBP associates with pol III factors in the absence of DNA. The C-terminal domain of TBP was expressed in bacteria as a glutathione S-transferase (GST) fusion protein and affinity purified using glutathione–Sepharose beads. The resultant GST–TBP beads were then used as an affinity matrix to assay for DNA-independent interactions with components of the class III transcription apparatus. Beads linked to GST alone were used as a control matrix.

A PC-C fraction containing TFIIIC was passed down columns of beads linked to GST or GST–TBP. The flow-through fractions were then tested in a complementation assay with PC-B; this allows TFIIIC activity to be measured, since all the other required factors (TFIIIB, TBP, pol III) are present in the complementing PC-B fraction. The flow-through fractions from GST and GST–TBP columns displayed similar levels of TFIIIC activity (Figure 5A). In the converse experiment, PC-B fraction containing TFIIIB was passed down GST or GST–TBP columns and the flow-through fractions were tested in a complementation assay with PC-C; this allows TFIIIB activity to be measured, since all the other required factors (TFIIIC, TBP, pol III) are present in the complementing PC-C fraction. The TFIIIB activity of the GST–TBP flow-through fraction was dramatically reduced relative to that of the GST flow through (Figure 5B). The GST–TBP flow-through fraction was found to be depleted of TFIIIB activity when assayed at several concentrations and with several templates (data not shown). This result shows that TBP can interact with and retain a component of TFIIIB. The polypeptide compositions of the GST and GST–TBP flow-through fractions were virtually indistinguishable, as judged by silver staining, indicating that the retention of TFIIIB activity is a specific effect (data

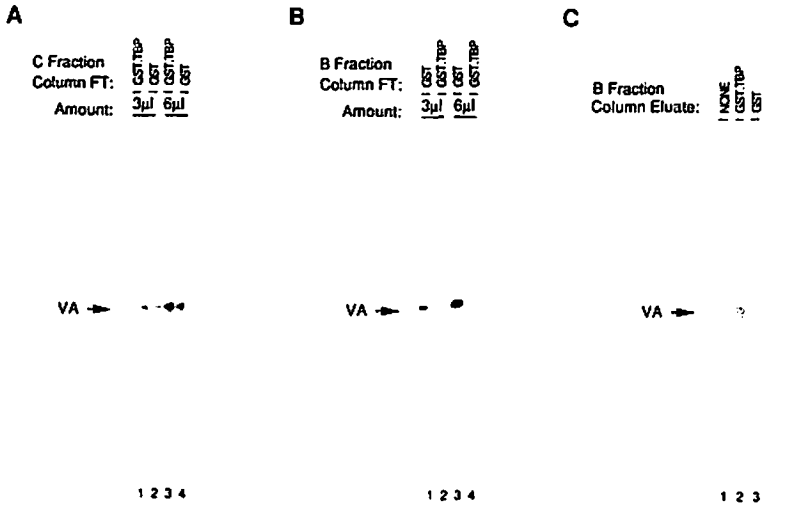

Figure 5. Immobilized TBP Specifically Interacts with TFIIIB

(A) TFIIIC activity flows through GST–TBP and GST columns. pBRVA$_1$ template (1 µg) and 2 µl of PC-B were preincubated for 15 min at 30°C with 3 µl (lane 1) or 6 µl (lane 3) of the PC-C flow-through (FT) fraction from a GST–TBP column, or with 3 µl (lane 2) or 6 µl (lane 4) of the PC-C flow-through fraction from a GST column. Nucleotides were then added and transcription was allowed to proceed for 60 min at 30°C.

(B) TFIIIB activity is specifically depleted by GST–TBP, but not GST, columns. pBRVA$_1$ template (1 µg) and 2 µl of PC-C were preincubated for 15 min at 30°C with 3 µl (lane 1) or 6 µl (lane 3) of the PC-B flow-through fraction from a GST column, or with 3 µl (lane 2) or 6 µl (lane 4) of the PC-B flow-through fraction from a GST–TBP column. Nucleotides were then added and transcription was allowed to proceed for 60 min at 30°C.

(C) TFIIIB activity is detectable in the B fraction eluate from GST–TBP, but not GST, columns. pBRVA$_1$ template (1 µg) and 2 µl of PC-C were preincubated for 15 min at 30°C with 6 µl of buffer (lane 1), 6 µl of the PC-B eluate from a GST–TBP column (lane 2), or 6 µl of the PC-B eluate from a GST column (lane 3). Nucleotides were then added and transcription was allowed to proceed for 60 min at 30°C.

not shown). The fact that TFIIIC activity is not retained further illustrates the specificity of the interaction.

We attempted to recover TFIIIB from the columns by washing at elevated salt concentrations. However, since GST is linked to the matrix noncovalently, we were unable to employ harsh elution conditions. With washes of up to 1 M KCl it was possible to recover some TFIIIB activity from GST–TBP columns, but the recovered activity was weak and variable. Figure 5C shows an experiment in which the PC-B eluates from GST–TBP and GST columns were used to reconstitute transcription in the presence of PC-C. TFIIIB activity was detected in the eluate from a GST–TBP column, but not in that from a GST column. However, this activity was very weak and reconstituted transcription was barely above background. The poor recovery of TFIIIB from GST–TBP columns may partly be due to dilution and/or inactivation, but may also indicate that much of the activity is retained on the column under these relatively mild elution conditions. Some protein–protein interactions involving TBP are stable to 2 M urea (Comai et al., 1992). Nevertheless, the specific and dramatic depletion of TFIIIB activity from the PC-B fraction by the GST–TBP column demonstrates that the C-terminal domain of TBP can associate with a component of TFIIIB in the absence of TFIIIC or a class III gene.

A Pol III Factor Coimmunoprecipitates with TBP

Antibodies raised against TBP have been found to coimmunoprecipitate factors required for TBP recruitment to TATA-less class I and II promoters; as a result, addition of recombinant TBP to an extract that has been immunodepleted with anti-TBP antibodies does not restore transcription of such genes (Pugh and Tjian, 1991; Comai et al., 1992). We tested whether this is also the case for the VA$_1$ gene. Polyclonal anti-TBP antibodies that had been purified to >95% homogeneity by TBP affinity chromatography were used to immunodeplete HeLa nuclear extract of TBP and associated factors, as previously (Pugh and Tjian, 1991). Western blotting of the immunodepleted and mock-depleted extracts using the anti-TBP antibody demonstrated that a substantial proportion of the endogenous TBP in the extract had been removed (Figure 6A). The immunodepleted extract was found to be severely compromised in its ability to transcribe 5S rRNA, VA$_1$, tRNA, and B2 genes relative to the mock-immunodepleted control (Figure 6B). This was the case when assayed at a range of template concentrations (Figure 6C). These observations provide further support for our previous conclusion that TBP is required for TATA-independent transcription by mammalian pol III (White et al., 1992a, 1992b).

We tested the ability of cloned TBP to restore the capacity of a TBP-immunodepleted extract to transcribe the VA$_1$ gene. However, addition of purified recombinant TBP did not activate VA$_1$ transcription by the TBP-depleted extract (Figure 7A). In contrast, basal pol II transcription of a TATA-containing template in the same TBP-immunodepleted extract was efficiently restored by the addition of recombinant TBP (Figure 7B), as reported previously (Pugh and Tjian, 1991). Amounts of TBP that have no effect upon VA$_1$ transcription in the immunodepleted extract produce strong activation when added to the heat-treated system used above (Figure 1A and White et al., 1992a). These results therefore suggest that anti-TBP antibodies remove a factor besides TBP that is required for transcription of VA$_1$, but not for TATA-directed pol II transcription. Coimmunoprecipitation of this factor with TBP is likely to reflect a direct interaction in solution.

Immunodepletion with Anti-TBP Antibodies Removes TFIIIB but Not TFIIIC

We compared the levels of TFIIIB and TFIIIC in mock-depleted and TBP-depleted extracts to determine whether

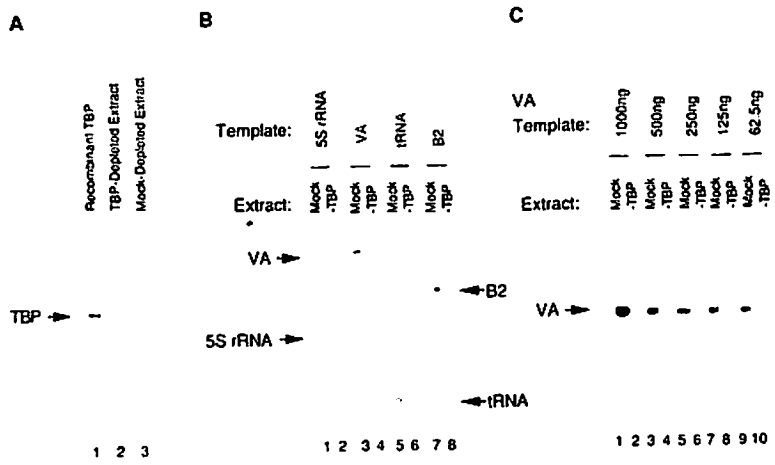

Figure 6. Extracts Immunodepleted Using Anti-TBP Antibodies Have Diminished Capacity for Pol III Transcription

(A) Comparison of TBP levels in TBP-depleted and mock-depleted extracts. Ten nanograms of recombinant TBP (lane 1), 4 μl of TBP-depleted extract (lane 2), and 4 μl of mock-depleted extract (lane 3) were analyzed by Western immunoblotting using anti-TBP antibody.

(B) TBP-depleted extracts have reduced capacity to transcribe class III genes. Templates (1 μg) pXbS (lanes 1 and 2), pBRVA$_t$ (lanes 3 and 4), pLeu (lanes 5 and 6), and pTB14 (lanes 7 and 8) were preincubated for 15 min at 30°C with 4 μl of mock-depleted (lanes 1, 3, 5, and 7) or TBP-depleted (lanes 2, 4, 6, and 8) extract. Nucleotides were then added and transcription was allowed to proceed for 40 min at 30°C.

(C) TBP-depleted extracts have reduced capacity to transcribe the VA$_t$ gene when assayed over a range of template concentrations. Four microliters of mock-depleted (lanes 1, 3, 5, 7, and 9) or TBP-depleted (lanes 2, 4, 6, 8, and 10) extract was preincubated for 15 min at 30°C with 1 μg (lanes 1 and 2), 500 ng (lanes 3 and 4), 250 ng (lanes 5 and 6), 125 ng (lanes 7 and 8), or 62.5 ng (lanes 9 and 10) of pBRVA$_t$ template. Nucleotides were then added and transcription was allowed to proceed for 40 min at 30°C.

one of these factors is coimmunoprecipitated with TBP. TFIIIC levels were measured directly using a gel retardation assay. The probe was an oligonucleotide containing the B-block internal promoter sequence of a B2 gene; this oligonucleotide has been shown previously to bind TFIIIC and to compete for VA$_t$ transcription (White et al., 1989). Under the conditions of the assay, a single complex is formed between TFIIIC and this probe (Figure 8A). The complex is efficiently competed by the homologous B-block oligonucleotide, but not by control oligonucleotides corresponding to part of the murine sarcoma virus enhancer or the TATA box region of the adenovirus major late promoter. Furthermore, a single base change in the B-block sequence at a residue conserved in all functional eukaryotic tRNA genes severely reduces the ability of the oligonucleotide to compete for formation of this complex. These competition experiments establish the specificity of the B-block–TFIIIC complex detected in this assay. Comparison of the TBP-depleted and mock-depleted extracts shows equal levels of this complex in each case (Figure 8A, lanes 6 and 7). Therefore, TFIIIC is not coimmunoprecipitated with TBP. This result, plus the failure of GST–TBP columns to retain TFIIIC, suggests that TBP and TFIIIC do not associate directly in solution.

As explained above, reconstitution of VA$_t$ transcription in an extract that has been heated at 47°C for 15 min requires the addition of TBP and PC-C (White et al., 1992a). This reflects the fact that TBP and TFIIIC are heat labile, whereas TFIIIB is relatively stable under these conditions. It is therefore possible to assay TFIIIB activity in the immunodepleted extracts by heat treating them first and then providing excess TBP and TFIIIC exogenously; such an assay allows direct comparison of TFIIIB levels between extracts under conditions in which the levels of the other known factors are constant and in excess. VA$_t$ transcription is not observed using TBP and PC-C alone (Figure 8B). Using this system, we found that TFIIIB activity in the TBP-depleted extract is considerably less than that in the mock-depleted extract. This suggests that a component of TFIIIB is removed by immunoprecipitation with anti-TBP antibodies. An alternative explanation is that TFIIIB becomes less heat stable in the absence of TBP. Either explanation implies an interaction between these two factors.

As a control for specificity, we compared levels of class II factors between TBP-depleted and mock-depleted extracts. The activities of basal pol II factors were measured by heat treating the extracts and assaying in the presence of excess exogenous TBP to provide comparable assay conditions to those used above. In such a system the TBP-depleted and mock-depleted extracts display similar levels of basal pol II transcription (Figure 8C). This suggests that none of the pol II general factors, apart from TBP itself, is efficiently removed by immunoprecipitation with anti-TBP antibodies. This conclusion is supported by the fact that TATA-directed pol II transcription in a TBP-depleted extract can be fully reconstituted by the addition of TBP alone (Figure 7B). These control experiments demonstrate that the decrease in TFIIIB activity observed following immunoprecipitation with affinity-purified anti-TBP antibodies is a specific effect. Further evidence of specificity is provided by the observation that TFIIIC does not coprecipitate with TBP. The fact that TFIIIB activity is diminished by immunodepletion of TBP provides strong evidence that these factors associate in solution.

A TFIIIB Fraction Specifically Restores the Ability of a TBP-Immunodepleted Extract to Transcribe the VA$_t$ Gene

The previous experiments imply that the failure of the TBP-depleted extract to transcribe the VA$_t$ gene even when TBP has been added back to the system is due to the loss of TFIIIB activity. If so, then one would predict that addition of PC-B to the immunodepleted extract will be sufficient to restore transcription, since PC-B contains both TFIIIB and TBP. Figure 9A shows that this is indeed the case. Addition

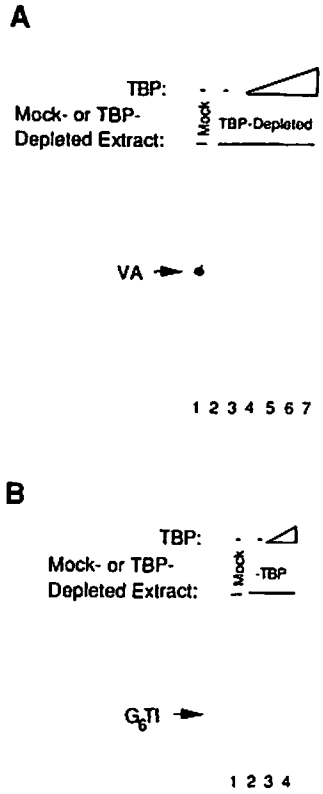

Figure 7. TBP Alone Does Not Restore VA$_1$ Transcription to an Extract Immunodepleted Using Anti-TBP Antibodies

(A) Effect of TBP addition upon VA$_1$ transcription by pol III in a TBP-depleted extract. pBRVA$_1$ (1 μg) was preincubated for 15 min at 30°C with 4 μl of mock-depleted (lane 1) or TBP-depleted (lanes 2–7) extract in the presence of the following amounts of TBP: lanes 1 and 2, none; lane 3, 2.5 ng; lane 4, 5 ng; lane 5, 10 ng; lane 6, 15 ng; and lane 7, 20 ng. Nucleotides were then added and transcription was allowed to proceed for 40 min at 30°C.

(B) Effect of TBP addition upon basal transcription of G$_6$TI by pol II in a TBP-depleted extract. G$_6$TI (1 μg) was preincubated for 15 min at 30°C with 4 μl of mock-depleted (lane 1) or TBP-depleted (lanes 2–4) extract in the presence of the following amounts of TBP: lanes 1 and 2, none; lane 3, 5 ng; and lane 4, 10 ng. Nucleotides were then added and transcription was allowed to proceed for 40 min at 30°C.

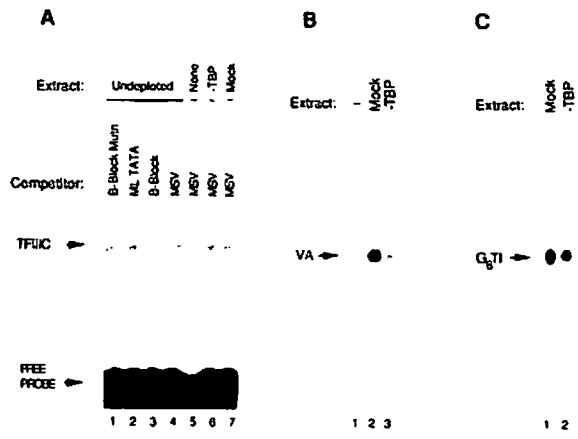

Figure 8. Immunodepletion with Anti-TBP Antibodies Specifically Reduces TFIIIB Activity

(A) TFIIIC levels are unaffected by immunodepletion with anti-TBP antibodies. One hundred nanograms of B-block mutant (lane 1), major late TATA (lane 2), B-block (lane 3), or murine sarcoma virus (lanes 4–7) oligonucleotides was preincubated for 15 min at 30°C with 2 μg of poly(dI–dC)–poly(dI–dC) and 4 μl of undepleted (lanes 1–4), TBP-depleted (lane 6), or mock-depleted extract (lane 7), or no extract (lane 5). Two nanograms of end-labeled B-block oligonucleotide probe was then added and the incubation was continued for a further 15 min at 30°C before the reaction samples were analyzed by gel retardation assay.

(B) TFIIIB activity is reduced in TBP-immunodepleted extracts. PC-C (2.5 μl) and 5 ng of TBP were preincubated for 15 min at 30°C either alone (lane 1) or with 8 μl of mock-depleted (lane 2) or TBP-depleted (lane 3) extract that had been heat treated for 15 min at 47°C. pBRVA$_1$ template (1 μg) and nucleotides were then added and transcription was allowed to proceed for 40 min at 30°C.

(C) Pol II basal factors other than TBP are not removed by immunodepletion with anti-TBP antibodies. Eight microliters of mock-depleted (lane 1) or TBP-depleted (lane 2) extract was heat treated for 15 min at 47°C and then preincubated for 15 min at 30°C with 5 ng of TBP. G$_6$TI template (1 μg) and nucleotides were then added and transcription was allowed to proceed for 40 min at 30°C.

of PC-B to the TBP-depleted extract strongly activates VA$_1$ transcription and is sufficient to reconstitute expression to levels occurring in the mock-depleted extract. This indicates that all the factors required for VA$_1$ transcription that are removed by immunoprecipitation with anti-TBP antibodies are present in the PC-B fraction.

To determine if the activity depleted from PC-B by GST–TBP columns is the same as that removed using anti-TBP antibodies, we tested whether the ability of PC-B to restore pol III transcription to the TBP-immunodepleted extract is removed by passage through a GST–TBP column. We found that VA$_1$ transcription in the TBP-depleted extract is reconstituted by the PC-B flow-through fraction from the control GST column but not by the same amount of flow-through fraction from the GST–TBP column (Figure 9B). This shows that a factor required for VA$_1$ transcription can be depleted either by immunodepletion with antibodies against TBP or by association with a GST–TBP affinity matrix. Although these two approaches clearly remove at least one component that is the same, the possibility remains that additional components may be uniquely depleted by one of these procedures.

Western blot analysis demonstrates the presence of significant amounts of TBP in the PC-B, PC-C, and PC-D fractions (data not shown). We compared the abilities of these fractions to activate VA$_1$ transcription in the TBP-immunodepleted extract after normalizing for TBP content, as determined by quantitative immunoblotting. Whereas PC-B again fully restored VA$_1$ expression, the same amount of TBP either alone or in the PC-D or PC-C fractions had little or no effect (Figure 9C). The TBP present in PC-D is, however, active for pol II transcription (data not shown). Therefore, a factor required to reconstitute VA$_1$ transcription in this system selectively elutes in the PC-B fraction. When these fractions are added to the mock-depleted extract, the strongest activation is obtained using PC-C (Figure 9D), consistent with previous reports that TFIIIC is rate limiting for VA$_1$ transcription in HeLa extracts (Yoshinaga et al., 1986). These data demonstrate that a PC-B-specific class III factor is coimmunoprecipitated with

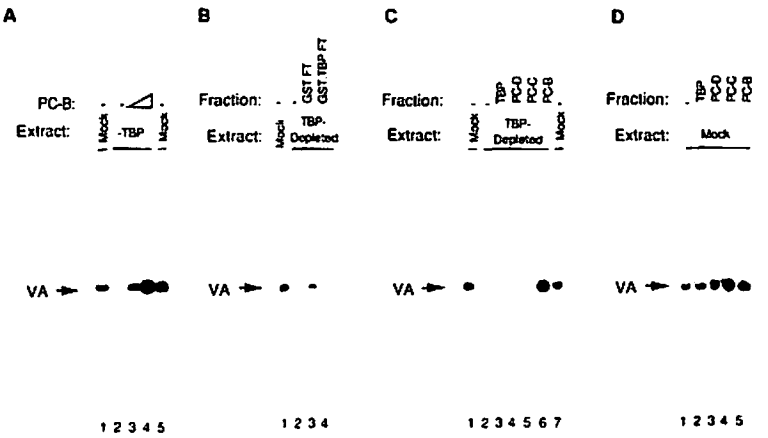

Figure 9. A PC-B-Specific Class III Factor Is Coimmunodepleted with TBP

(A) PC-B is sufficient to restore the ability of TBP-depleted extract to transcribe VA_1. Four microliters of mock-depleted (lanes 1 and 5) or TBP-depleted (lanes 2–4) extract was preincubated for 15 min at 30°C with 1 μl (lane 3), 4 μl (lane 4), or no (lanes 1, 2, and 5) PC-B. $pBRVA_1$ template (1 μg) and nucleotides were then added and transcription was allowed to proceed for 40 min at 30°C.

(B) GST–TBP columns specifically deplete PC-B of an activity required to reconstitute VA_1 transcription in TBP-immunodepleted extract. Four microliters of mock-depleted (lane 1) or TBP-depleted (lanes 2–4) extract was preincubated for 15 min at 30°C either alone (lanes 1 and 2) or with 4 μl of PC-B flow through from a GST (lane 3) or GST–TBP column (lane 4). $pBRVA_1$ template (1 μg) and nucleotides were then added and transcription was allowed to proceed for 40 min at 30°C.

(C) A factor required to restore the ability of TBP-depleted extract to transcribe VA_1 is located selectively in PC-B. Four microliters of mock-depleted (lanes 1 and 7) or TBP-depleted (lanes 2–6) extract was preincubated for 15 min at 30°C with 5 ng of TBP (lane 3), 2.6 μl of PC-D (lane 4), 2.4 μl of PC-C (lane 5), 1.7 μl of PC-B (lane 6), or without addition (lanes 1, 2, and 7). The amounts of PC fractions included in lanes 4–6 each contained 5 ng of endogenous TBP, as determined by quantitative Western blot analysis. $pBRVA_1$ template (1 μg) and nucleotides were then added and transcription was allowed to proceed for 40 min at 30°C.

(D) Effect of adding PC fractions to mock-depleted extract. Mock-depleted extract (4 μl) was preincubated for 15 min at 30°C with 5 ng of TBP (lane 2), 2.6 μl of PC-D (lane 3), 2.4 μl of PC-C (lane 4), 1.7 μl of PC-B (lane 5), or without addition (lane 1). $pBRVA_1$ template (1 μg) and nucleotides were then added and transcription was allowed to proceed for 40 min at 30°C.

TBP. Of the proteins known to be required for VA_1 transcription, namely, TBP, TFIIIB, TFIIIC, and pol III, only TFIIIB is specific to PC-B. These results therefore provide further evidence that TBP associates with a component of TFIIIB in solution.

PC-B Contains Chromatographically Separable Populations of TBP

A TBP-containing complex named B-TFIID that can support pol II transcription is reported to elute from phosphocellulose between 40 mM and 350 mM KCl and to flow through DEAE–Sepharose when applied in 60 mM KCl (Timmers and Sharp, 1991). We loaded our PC-B fraction onto DEAE–Sepharose in 60 mM KCl and collected the flow through as well as three step fractions obtained by eluting at increasing ionic strength (Figure 10A). The fractions were then assayed for TFIIIB activity by testing for VA_1 transcription in the presence of PC-C. TFIIIB was found almost entirely in the high salt DE-1.0 fraction, with trace amounts in the intermediate steps and no activity detected

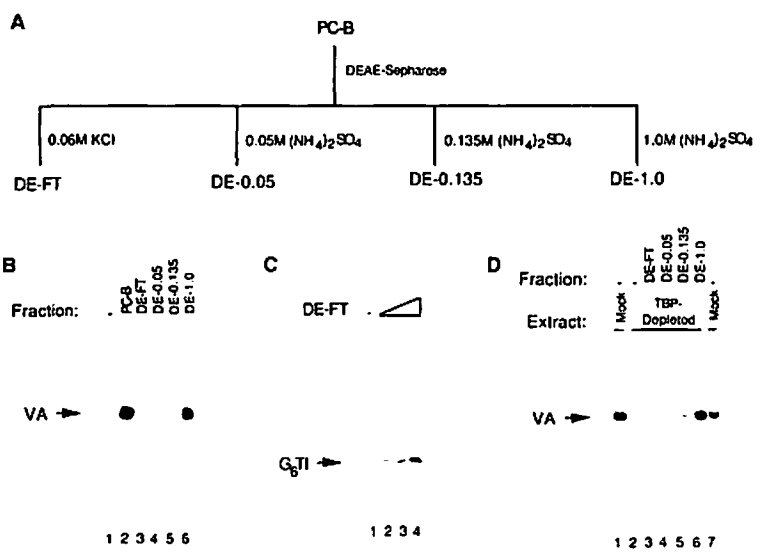

Figure 10. The Activity Required to Reconstitute VA_1 Transcription in a TBP-Immunodepleted Extract Cofractionates with TFIIIB on DEAE–Sepharose

(A) Fractionation scheme for chromatography of PC-B on DEAE–Sepharose.

(B) Assay for TFIIIB activity in DEAE–Sepharose step fractions. Two microliters of PC-C was preincubated for 15 min at 30°C either alone (lane 1), with 1 μl of PC-B (lane 2), or with 4 μl of DE flow through (DE-FT; lane 3), DE-0.05 (lane 4), DE-0.135 (lane 5), or DE-1.0 (lane 6). $pBRVA_1$ template (1 μg) and nucleotides were then added and transcription was allowed to proceed for 40 min at 30°C.

(C) DE flow through is able to activate pol II transcription in a heat-treated extract. HTNE (1.6 μl) was preincubated for 15 min at 30°C either alone (lane 1) or with 1 μl (lane 2), 2 μl (lane 3), or 4 μl (lane 4) of DE flow through (DE-FT). G_5TI template (1 μg) and nucleotides were then added and transcription was allowed to proceed for 40 min at 30°C.

(D) A factor required to restore the ability of TBP-depleted extract to transcribe VA_1 is located selectively in DE-1.0. Four microliters of mock-depleted (lanes 1 and 7) or TBP-depleted (lanes 2–6) extract was preincubated for 15 min at 30°C either alone (lanes 1, 2, and 7) or with 4 μl of DE flow through (DE-FT) (lane 3), DE-0.05 (lane 4), DE-0.135 (lane 5), or DE-1.0 (lane 6). $pBRVA_1$ template (1 μg) and nucleotides were then added and transcription was allowed to proceed for 40 min at 30°C.

in the DE flow-through fraction (Figure 10B). Western immunoblot analysis detected significant quantities of TBP in both the DE flow-through and DE-1.0 fractions (data not shown). The TBP in the DE flow through had not been inactivated in some way, since it was found to support pol II transcription in an HTNE (Figure 10C). It therefore appears that the DE flow-through fraction, which has been reported to contain B-TFIID (Timmers and Sharp, 1991), lacks some component(s) required for TFIIIB activity.

Since these data suggest that PC-B contains at least two distinct populations of TBP, it was important to confirm that the activity that restores pol III transcription to extracts that have been immunodepleted of TBP cofractionates with TFIIIB. Figure 10D demonstrates that this is the case. The DE-1.0 fraction, which contains TFIIIB (Figure 10A), activates VA_t transcription in the TBP-depleted extract, whereas DE flow through does not. This observation therefore provides additional support for our conclusion that the pol III activity that is immunodepleted by association with TBP is indeed TFIIIB.

Discussion

This study has addressed the molecular mechanism whereby TBP is recruited to a class III gene without a consensus TATA box. It has shown that there is no stable direct recognition of the TATA-less VA_t gene by TBP. However, TBP can associate stably with the VA_t promoter in the presence of TFIIIC and TFIIIB. We provide evidence for a strong and specific interaction between TFIIIB and the evolutionarily conserved C-terminal domain of TBP. This association can occur in solution in the absence of TFIIIC or DNA. A stable interaction with TFIIIB can account for the ability of TBP to function at TATA-less class III promoters.

Template commitment assays show that TBP only interacts stably and productively with the VA_t gene if other class III factors are also present. Comparable results have been reported for the human rRNA promoter and for an artificial TATA-less class II template: in neither case does TBP alone bind to the DNA, but it does associate in the presence of other transcription factors (Pugh and Tjian, 1991; Comai et al., 1992). In the case of VA_t, a potential weak binding site (GATAAA) is found within the internal control region, overlapping the essential A-block element recognized by TFIIIC (reviewed by Geiduschek and Tocchini-Valentini, 1988; Gabrielsen and Sentenac, 1991; Wolffe, 1991). However, we were unable to detect direct TBP binding to this site. The fact that VA_t lacks a direct binding site for TBP does not preclude the possibility that some other apparently TATA-less class III genes might utilize cryptic sites in order to increase their affinity for TBP. However, it does indicate that such sites are not essential for genes with similar promoter arrangements to that of VA_t.

Although direct DNA binding is not required for TBP to function at a TATA-less class III gene, it remains possible that TBP does contact the DNA once it is assembled into a transcription complex. This might involve low affinity nonspecific contacts or altered DNA recognition properties induced by contacting other proteins. However, photocross-linking studies of complexes assembled on a yeast tRNA promoter have not detected a polypeptide of the size of TBP in proximity to the DNA (Bartholomew et al., 1990, 1991). Furthermore, a yeast TBP point mutant that is unable to bind a TATA element in vitro is still functional for transcription of 5S rRNA and $tRNA^{Leu3}$ genes (Schultz et al., 1992). Therefore the role of TBP in TATA-independent pol III transcription may not involve contacting DNA.

The demonstration that TBP is required for transcription of TATA-less class III genes (Cormack and Struhl, 1992; Schultz et al., 1992; White et al., 1992a, 1992b) did not necessarily mean that TBP interacts stably with such genes. A catalytic role for TBP was also compatible with the previous data. The template commitment experiments in this study indicate for the first time that TBP is sequestered into a class III transcription complex in the absence of a TATA box. This conclusion is strongly supported by the demonstration of a stable association between TBP and a class III general factor.

Genetic studies have shown that the C-terminal domain of TBP is important for pol III transcription (Cormack and Struhl, 1992; Schultz et al., 1992). We have demonstrated that it is, in fact, sufficient both for interacting with TFIIIB and for mediating transcription. The C-terminal domain is also sufficient for basal TATA-directed pol II transcription (Hoey et al., 1990; Horikoshi et al., 1990; Peterson et al., 1990; Lieberman et al., 1991; Reddy and Hahn, 1991; Zhou et al., 1991). The fact that the region of TBP that supports basal transcription is so conserved phylogenetically is consistent with the central role of TBP in nuclear transcription in both higher and lower eukaryotes (White and Jackson, 1992).

Several workers have postulated the existence of class III TAFs (Comai et al., 1992; Green, 1992; Sharp, 1992; White and Jackson, 1992). This suggestion has been based primarily upon analogy with the class I and II systems. The present study provides evidence that TBP associates stably in solution with a component of TFIIIB. The specificity of this interaction is established by several controls. TFIIIC is not retained by a GST–TBP column, nor is it coprecipitated by anti-TBP antibodies. Similarly, the pol II basal factors, apart from TBP, are not removed by immunodepletion with antibodies against TBP. In fact, the vast majority of polypeptides in the PC-B and PC-C fractions flow through GST–TBP columns (data not shown). We therefore conclude that the association between TBP and TFIIIB is highly specific.

The precise polypeptide composition of TFIIIB has not yet been conclusively determined. The only published purification of mammalian TFIIIB concluded that it is a single polypeptide of 60 kd (Waldschmidt et al., 1988). This conclusion is supported by an earlier study demonstrating that yeast TFIIIB activity also resides in a 60 kd polypeptide (Klekamp and Weil, 1986). However, Kassavetis et al. (1989) obtained preparations of yeast TFIIIB of much higher specific activity in which a 60 kd polypeptide is not a major component. The less homogeneous TFIIIB preparations of these workers contain at least two essential polypeptides, of 70 kd and 90 kd (Bartholomew et al., 1991; Kassavetis et al., 1991). Since the evidence that the

60 kd polypeptide is part of, or tightly associated with, TFIIIB seems compelling, Kassavetis et al. (1991) have suggested that it may be identical to their 70 kd component or a proteolyzed form of their 90 kd component, and that the low activity of the earlier preparations might reflect the loss of a complementary component(s). Another recent purification found that yeast TFIIIB has a native mass of ~130 kd (Margottin et al., 1991). Drosophila TFIIIB is reported to have an apparent molecular mass of 260 kd, as determined by gel filtration (Johnson Burke and Soll, 1985). We consistently observe that the polypeptides specifically retained by GST-TBP, but not GST control, columns include one or more in the ~60–70 kd size range (data not shown). However, because the recovery of activity from these columns has so far been weak and variable, we are not yet in a position to decide whether this polypeptide(s) is associated with TFIIIB activity.

Since we have used relatively crude fractions in our experiments, the possibility remains that the activity we have shown to interact with TBP does not correspond to a factor purified by other groups. Because of the current uncertainty concerning the precise molecular composition of TFIIIB, the factor may, at present, be best described as an activity. The established criteria for defining an activity as TFIIIB are that it should be a general class III factor, be specific to the PC-B fraction, and be necessary to reconstitute transcription in the presence of the PC-C fraction. We have shown that TBP interacts with a factor that meets these criteria, and therefore feel justified in referring to this factor as TFIIIB. TBP is required for transcription of all class III genes tested (Cormack and Struhl, 1992; Schultz et al., 1992; White et al., 1992a; White et al., 1992b). This is also true for TFIIIB but not for TFIIIC, which has recently been shown to be inessential for in vitro transcription of U6 snRNA genes (Lobo et al., 1991; Margottin et al., 1991; Waldschmidt et al., 1991). Therefore a conserved interaction between TBP and the pol III transcription machinery is likely to be made with TFIIIB. Yeast TFIIIB has been resolved into two essential components, and although both components are required for tRNA transcription, it has been suggested that one of them could be dispensable for U6 transcription (Kassavetis et al., 1991). Human TFIIIB may also consist of more than one component. If there are multiple components, the question arises as to which component is called "TFIIIB," or whether they all are. The clear precedent in the pol III system is to refer to each component as TFIIIB. Thus, the chromatographically separable components of yeast TFIIIB have been named B′ and B″ (Kassavetis et al., 1991). Similarly, the human PC-C fraction has been resolved into two chromatographically separable factors that have been named TFIIIC1 and TFIIIC2 (Yoshinaga et al., 1987). To a large extent, the issue is semantic rather than scientific. Components that form part of the native complex may be dissociable under certain conditions. Indeed, only one essential component would have to be exclusive to the B fraction for TFIIIB activity to be specific to PC-B, as originally defined. TFIIIB may perhaps be thought of as a TBP-containing complex, analogous to TFIID in the class II system. TBP and TFIIIB copurify through multiple stages of fractionation in both yeast (Margottin et al., 1991; G. A. Kassavetis, personal communication) and human (B. Moorefield and R. G. Roeder, personal communication) systems, consistent with a strong interaction between these factors in the absence of DNA. Further purification and cloning are clearly required to establish the precise composition of the class III general factors.

Transcription complex assembly on VA and tRNA genes begins with promoter binding by TFIIIC and is followed by TFIIIB recruitment (Lassar et al., 1983; Fuhrman et al., 1984; Carey et al., 1986; Dean and Berk, 1988; Kassavetis et al., 1989, 1990; reviewed by Geiduschek and Tocchini-Valentini, 1988; Gabrielsen and Sentenac, 1991; Wolffe, 1991). We have shown that TBP does not interact stably with naked VA_I DNA, with TFIIIC in solution, or with a complex formed between TFIIIC and VA_I. Since TBP can be stably assembled into a complex involving TFIIIB, TFIIIC, and DNA, and since TBP and TFIIIB can associate in the absence of TFIIIC or DNA, it is likely that TBP recruitment to a TATA-less VA_I gene occurs either concurrently with or subsequent to TFIIIB binding to the TFIIIC–DNA complex.

Although TFIIIB does not bind DNA independently (Fuhrman et al., 1984; Carey et al., 1986; Klekamp and Weil, 1986; Waldschmidt et al., 1988; Kassavetis et al., 1989), the yeast factor has been shown to interact with DNA in an apparently sequence-independent fashion once recruited via TFIIIC (Braun et al., 1989; Kassavetis et al., 1989, 1990, 1991; Bartholomew et al., 1991). Treatment of the assembled yeast transcription complex with heparin or high salt concentrations strips TFIIIC from the promoter but leaves TFIIIB bound (Kassavetis et al., 1989, 1990, 1991; Bartholomew et al., 1991). Comparable interactions are not observed in mammalian systems, where TFIIIB is preferentially removed by high salt (Carey et al., 1986). The heparin- or salt-stripped yeast complex involving promoter-bound TFIIIB in the absence of TFIIIC is fully competent to direct multiple rounds of transcription initiation, leading to the conclusion that TFIIIB is the pol III initiation factor proper whereas TFIIIC is an assembly factor (Kassavetis et al., 1990). Strikingly, TBP is also retained with TFIIIB in the heparin-stripped initiation complex (G. A. Kassavetis, personal communication). This observation provides strong support for our conclusion that TFIIIB is involved in a stable functional interaction with TBP. It also implies that the role played by TBP in pol III transcription is likely to be closely associated with the initiation process.

There is a striking parallel between the mechanisms employed by the class I, II, and III systems to recruit TBP to a TATA-less promoter (Figure 11). For human class I genes, promoter recognition is achieved by a DNA-binding factor, UBF; TBP is then recruited as part of SL1, which does not bind DNA independently but interacts with the DNA–UBF complex (Comai et al., 1992). For a model TATA-less class II template, promoter recognition is by Sp1; TBP recruitment then involves protein–protein interactions between promoter-bound Sp1 and a factor, TFIID, that cannot bind this template independently (Pugh and Tjian, 1991). In the case of the VA_I class III gene, the promoter is bound directly by TFIIIC; TBP is then recruited

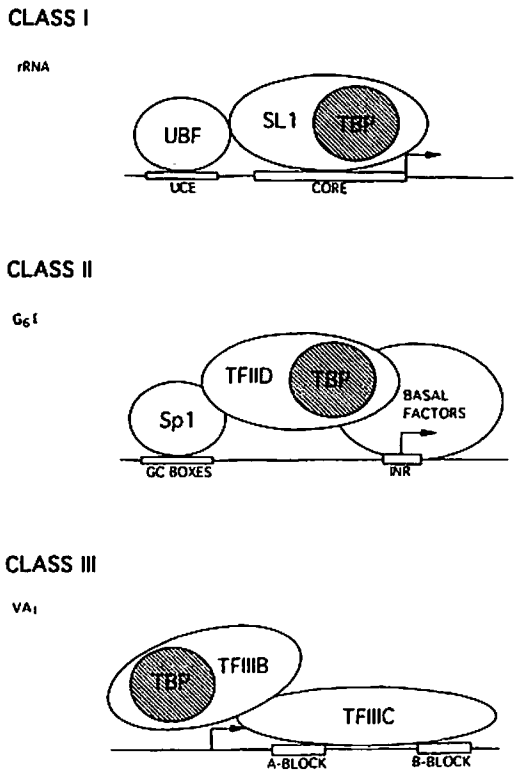

Figure 11. Schematic Comparison of the Recruitment of TBP to TATA-Less Promoters of Classes I, II, and III

TBP is recruited to the class I human rRNA promoter as part of SL1, which recognizes a complex formed between UBF and DNA (Comai et al., 1992). TBP recruitment to the model class II template G_6I involves recognition by TFIID of a complex formed between Sp1 and DNA (Pugh and Tjian, 1991). TBP is recruited to the class III VA_1 promoter in association with TFIIIB, which recognizes a complex formed between TFIIIC and DNA. Promoter elements are indicated. Abbreviations: UCE, upstream control element; INR, initiator element.

via protein–protein interactions involving a factor, TFIIIB, that does not bind DNA independently. This conservation of the mechanism of TBP recruitment between the three classes of nuclear genes parallels the conserved central role of TBP in eukaryotic nuclear transcription (White and Jackson, 1992).

Experimental Procedures

Templates and Oligonucleotides

The VA_1, $tRNA^{Leu}$, B2, and 5S rRNA templates are plasmids pBRVA1, pLeu, pTB14, and pXbs, respectively, which are detailed by White et al. (1989, 1990). The G_6TI template is described in Pugh and Tjian (1990). The B-block, B-block mutant, and murine sarcoma virus oligonucleotides are described in White et al. (1990). The major late TATA oligonucleotide is described by White et al. (1992a).

Extracts and Proteins

HeLa nuclear extract had a protein concentration of 6.2 mg/ml, as estimated by Bradford assay using bovine serum albumin as standard, and was prepared according to the method of Dignam et al. (1983) and depleted of glycosylated proteins by wheat germ agglutinin affinity chromatography (Jackson and Tjian, 1989). Full-length TBP and the C-terminal domain of TBP were expressed in bacteria from the vectors pARhTFIID and pARhTFIID–180C, respectively (Peterson et al., 1990). Full-length TBP was purified to >95% homogeneity by the method previously described (Pugh and Tjian, 1991). The C-terminal domain of TBP (180.C) was prepared as previously (Peterson et al., 1990) and was ~10% pure. BL21 H.4 is a heparin–agarose column 0.2–0.4 M KCl step fraction of proteins from the BL21 strain of Escherichia coli carrying no expression vector.

Transcription and DNA Binding Assays

Transcription reactions were performed as previously (White et al., 1992a). G_6TI template was linearized by digestion with NdeI in order to produce discrete run-off transcription products. Gel retardation assays were conducted as previously (White et al., 1990).

GST Constructs and Columns

GST–TBP protein was expressed from construct pGEX–TFIID-C, which contains the coding sequence for amino acids 168 to 339 of human TBP, inserted into the SmaI site of pGEX-3X (Promega) in-frame with the GST gene (Hagemeier et al., 1992). GST and GST–TBP proteins were expressed in bacteria and were affinity purified using glutathione–Sepharose beads (Promega) according to the method of Hagemeier et al. (1992). Relative amounts of bound protein were estimated by Coomassie staining of SDS–polyacrylamide gels.

Columns contained 0.2 ml bed volumes of beads carrying equivalent amounts of protein. PC fraction (0.25 ml) was loaded at room temperature onto each column after equilibration in buffer A (20 mM HEPES [pH 7.9], 20% glycerol, 0.2 mM EDTA, 0.5 mM dithiothreitol) containing 0.1 M KCl. The flow through was collected and reloaded onto the column twice. Flow was then stopped, and PC fraction was incubated in the presence of the column matrix for 15 min at 30°C, with gentle agitation. Flow was then resumed at room temperature, and the first 0.25 ml to flow through was collected. This was used as the flow-through fraction described in the text. Matrices were then washed with 50 column volumes of buffer A containing 0.1 M KCl. Columns were next eluted with buffer A containing 1.0 M KCl. The first 0.3 ml to flow through was collected, dialyzed against buffer A containing 0.1 M KCl, and then used as the eluate fraction described in the text. Silver staining of SDS–polyacrylamide gels confirmed that flow-through fractions had similar protein concentrations.

Immunodepletions and Western Blot Analysis

Immunodepletion reactions employed rabbit polyclonal antibodies raised against gel-purified recombinant TBP and then affinity purified to >95% purity, and were carried out as previously (Pugh and Tjian, 1991). Mock depletions were carried out in the same way, but in the absence of antibody. Mock-depleted and TBP-depleted extracts were used at a protein concentration of 2 mg/ml, determined as above. Western immunoblot analysis was performed using the rabbit anti-TBP polyclonal antiserum in the procedure of Towbin et al. (1979), as described by Jackson and Tjian (1988).

Fractionation

Phosphocellulose fractions were prepared according to the method of Segall et al. (1980). The PC-B fraction (4.5 mg) was applied to an 8 ml DEAE–Sepharose FF (Pharmacia) column in the same buffer as used by Timmers and Sharp (1991), i.e., 60 mM KCl, 20 mM HEPES–KOH (pH 7.9), 20% glycerol, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride. After washing with 3 column volumes of this buffer, bound protein was step eluted with three sequential washes (2.5 column volumes each) of buffer B (20 mM HEPES–KOH [pH 7.9], 20% glycerol, 5 mM $MgCl_2$, 3 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride) plus 50 mM $(NH_4)_2SO_4$, 135 mM $(NH_4)_2SO_4$, and 1.0 M $(NH_4)_2SO_4$. Peak fractions were dialyzed into buffer A and used in the experiments shown in Figure 10.

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