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A role for the TATA-box-binding protein component of the transcription factor IID complex as a general RNA polymerase III transcription factor

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ABSTRACT **The major class of vertebrate genes transcribed by RNA polymerase (EC 2.7.7.6) III, which includes 5S rRNA genes, tRNA genes, and the adenovirus VA genes, is characterized by split internal promoters and no absolute dependence upon specific upstream sequences. Fractionation experiments have shown that transcription of such genes requires two general RNA polymerase III-specific factors, TFIIIB and TFIIIC. We now demonstrate that a third general factor is also employed by these genes. This is the TATA-box-binding protein originally identified as being a component of the general RNA polymerase II transcription factor TFIID. This protein is involved in the transcription by RNA polymerase III of every template tested, even though the promoters of VA and most vertebrate tRNA and 5S rRNA genes do not contain recognizable TATA elements.**

The TATA-box-binding protein (TBP) is a component of transcription factor IID (TFIID), a general factor involved in transcription by RNA polymerase (EC 2.7.7.6) II that serves to nucleate the formation of preinitiation complexes (refs. 1–3 and reviewed in ref. 4). TATA sequences are also important for transcription of the U6 small nuclear RNA (snRNA) (5–11), 7SK (12), and EBER2 (13) genes by RNA polymerase III; in the case of U6 this has been shown to reflect a requirement for TBP (9–11, 14). However, the promoters of these genes are located largely or entirely upstream of the transcribed region and also contain binding sites for other RNA polymerase II-associated factors (5–22). In contrast, most genes transcribed by RNA polymerase III (class III genes) in vertebrates lack TATA motifs, have predominantly internal promoters, and exhibit no absolute dependence on specific upstream sequences; these include the adenovirus VA genes and many tRNA, 5S rRNA, and B2 genes (reviewed in refs. 16, 19, and 23–25; refs. 26 and 27 and our unpublished results). Early fractionation experiments demonstrated that at least two general factors, separable by phosphocellulose (PC) column chromatography, are required for the transcription of such genes (28, 29). Although these factors, TFIIIB and TFIIIC, have since been substantially purified (30–41), in no case has pure complementing protein been used, leaving open the possibility that additional essential factors present in both PC fractions may have escaped detection. Furthermore, attempts to reconstitute tRNA transcription with yeast or human TFIIIB after gel purification to homogeneity proved unsuccessful (31, 34), perhaps due to the loss of an essential component. Substantially purified yeast TFIIIB was found to contain significant amounts of the RNA polymerase II factor TBP (9), which was shown to be required for transcription of the TATA-dependent U6 snRNA gene by RNA polymerase III (9–11, 14). Indeed, TFIID activity fractionates extremely heterogeneously; although most of it is found in the 0.6–1.0 M KCl PC-D fraction (1, 2, 28, 42), significant amounts also occur in the 0.1–0.35 M PC-B fraction, from which TFIIIB was characterized, and the 0.35–0.6 M PC-C fraction, from which TFIIIC was characterized (9–11, 14). Therefore, the fractionation and purification studies published to date have not excluded a role for the TFIID complex, or components thereof, in the transcription of genes with internal promoters. We have tested this possibility directly, using specific depletion and reconstitution as an alternative to conventional fractionation. We find that TBP is involved in the transcription of a range of class III genes with internal promoters and no TATA boxes. As such, it may be considered as part of the general RNA polymerase III transcription apparatus.

MATERIALS AND METHODS

Plasmid and Oligonucleotide DNAs. The $tRNA^{Glu_6}$, 5S rRNA, VA_I, $tRNA^{Leu}$, B1, and B2 templates are plasmids pGlu6, pXbs, $pBRVA_I$, pLeu, TP1, and pAG38, respectively, which are detailed in ref. 43 and 44. The H2b octamer oligonucleotide contains sequences of the human histone H2b promoter (45) from −58 to −38: 5′-GCTTATGCAAATAAGGTGAAG-3′. The ML TATA oligonucleotide contains the TATA box region of the adenovirus major late promoter (3) from −40 to −15: 5′-CGATAAGGGGGGCTATAAAAGGGGGTGGGGAT-3′. The ML TATA mutant oligonucleotide is the same as above except for two base changes (underlined) in the TATA box: 5′-CGATAAGGGGGGCTAGAGAAGGGGGTGGGGAT-3′. The mouse β-globin (mβG) TATA oligonucleotide contains the TATA box region of the mβG gene (46) from −38 to −13: 5′-CGATGCAGAGCATATAAGGTGAGGTAGGAT-3′. The mβG TATA mutant oligonucleotide is the same as above except for two base changes in the TATA box: 5′-CGATGCAGAGCATAGCAGGTGAGGTAGGAT-3′. The human β-globin (hβG) TATA oligonucleotide represents the TATA box region of the hβG gene (47): 5′-GGGCTGGGCATAAAAGTCAGGGCAGAGCCATC-3′. The hβG TATA mutant oligonucleotide is the same as above except for four base changes in the TATA box: 5′-GGGCTGGGCATCGCCGTCAGGGCAGAGCCATC-3′. The B-block oligonucleotide is as described previously (43).

Protein Extracts and Fractions. HeLa nuclear extract was prepared according to the method of Dignam *et al.* (48), followed by partial depletion of glycosylated proteins by wheat germ agglutinin chromatography (49). Phosphocellu-

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Abbreviations: TFIID, transcription factor IID; TFIIIB, transcription factor IIIB; TFIIIC, transcription factor IIIC; TBP, TATA-box-binding protein; PC, phosphocellulose; hβG, human β-globin; mβG, mouse β-globin; snRNA, small nuclear RNA; ML, major late.

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lose column fractions were prepared and analyzed as previously described (28, 43). Bacterially expressed cloned human TBP (50) was kindly provided by B. F. Pugh and R. Tjian. 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.

***In Vitro* Transcription.** Transcription reaction mixtures contained 10 μg of nuclear extract and 1 μg of template, and the reactions were performed and the products were processed as previously described (43). Selective heat inactivation was for 15 min at 47°C by the method of Nakajima *et al.* (2).

RESULTS

Specific Depletion of TATA-Binding Factors Inhibits Transcription of Class III Genes. TFIID was specifically sequestered from a HeLa cell extract and so made unavailable for transcription by the addition of saturating amounts of its cognate DNA binding site. Transcription of a range of class III genes was found to be dramatically inhibited by preincubating the extract with TATA-containing oligonucleotides prior to the addition of template and nucleotides (Fig. 1). Fig. 1*A* shows this to be the case for the $tRNA^{Glu6}$ gene and TATA sequences from the adenovirus ML (3), the mβG (46), and the hβG (47) promoters, all of which direct transcription by RNA polymerase II. That this effect is specific to the TATA sequence was demonstrated by comparison with the levels of transcription in the presence of an equal amount of a control, oligonucleotide [the octamer element of the histone H2b promoter (45)] or of ML and β-globin oligonucleotides in which the TATA boxes had been mutated (TATAAAA → TAGAGAA for ML, CATATAA → CATAGCA for mβG, and CATAAAA → CATCGCC for hβG).

Every class III gene tested has shown the same response as $tRNA^{Glu6}$. This is illustrated in Fig. 1*B*, in which a 5S rRNA gene, the VA_I gene, a $tRNA^{Leu}$ gene, and members of the B1 and B2 rodent-specific middle-repetitive gene families were used as templates. In each case, transcription was severely inhibited by the ML TATA sequence relative to the point-mutated TATA control. The mβG and hβG TATA motifs also inhibited transcription of each of these templates (Fig. 2*B* and data not shown). The preincubation step alone and a TATA mutant oligonucleotide had little adverse effect upon transcription (data not shown). Comparable results were obtained when whole cell extracts from HeLa cells or mouse fibroblasts were used instead of the HeLa nuclear extract, and at a range of template concentrations (data not shown). The genes tested in Fig. 1 are transcribed by RNA polymerase III, as indicated by α-amanitin sensitivity experiments, have internal promoters, and lack TATA boxes. Nevertheless, these data demonstrate the involvement of a TATA-binding factor in their transcription.

Cloned TBP Restores RNA Polymerase III Transcription to Extracts That Have Been Specifically Depleted of TATA-Binding Factors. Human TBP binds to the TATA box of the ML promoter (2–4, 50, 51), and the double point mutation used here has been shown to abolish binding (51). This implies that TBP, or a factor with similar or identical DNA-binding specificity to TBP, is involved in the transcription of these class III genes. We tested these alternatives directly by using cloned human TBP (50) expressed in bacteria and purified to >95% homogeneity. Addition of this protein produced a marked (up to 15-fold) stimulation of VA_I gene transcription after specific depletion of the HeLa extract of TATA-binding factors by preincubation with the ML TATA oligonucleotide (Fig. 2*A*). The same response was obtained when a β-globin TATA sequence was used to deplete the extract of TATA-binding factors (Fig. 2*B*, lanes 5 and 6). Comparable results have been obtained when tRNA or B2

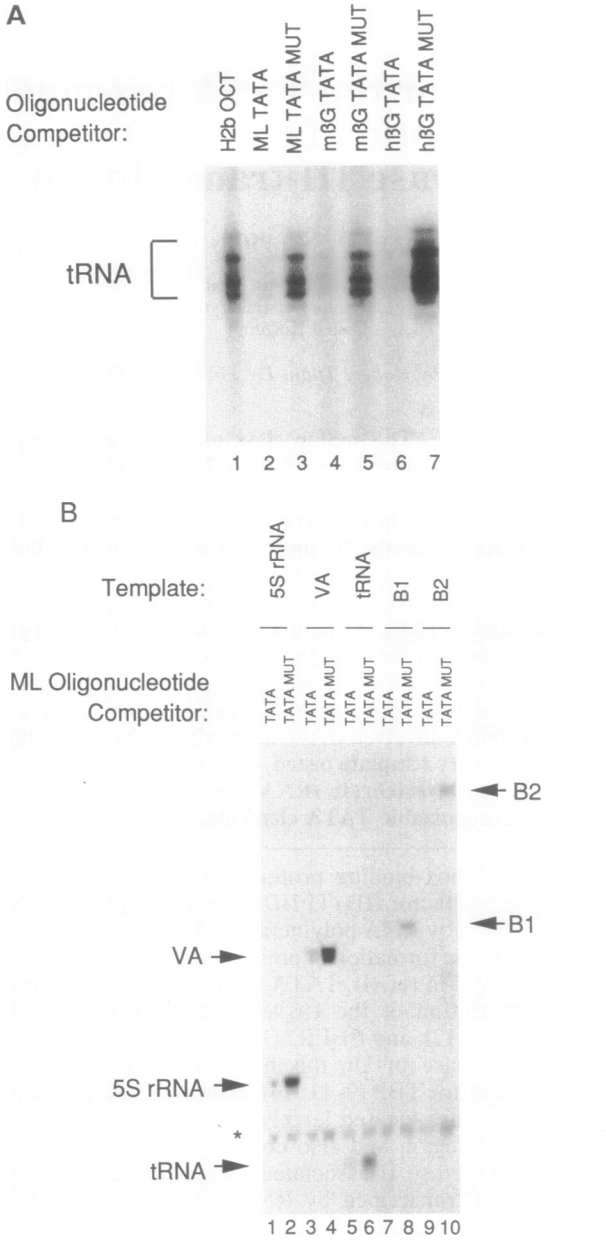

FIG. 1. Effect of oligonucleotide competitors upon RNA polymerase III transcription. (*A*) The $tRNA^{Glu6}$ gene was transcribed by HeLa extract that had been preincubated for 15 min at 30°C with 200 ng of H2b octamer (lane 1), ML TATA (lane 2), ML TATA mutant (lane 3), mβG TATA (lane 4), mβG TATA mutant (lane 5), hβG TATA (lane 6), or hβG TATA mutant (lane 7) oligonucleotides. (*B*) 5S rRNA (lanes 1 and 2), VA_I (lanes 3 and 4), $tRNA^{Leu}$ (lanes 5 and 6), B1 (lanes 7 and 8), and B2 (lanes 9 and 10) genes were transcribed by HeLa extract that had been preincubated for 15 min at 30°C with 200 ng of ML TATA (lanes 1, 3, 5, 7, and 9) or ML TATA mutant (lanes 2, 4, 6, 8, and 10) oligonucleotides. The origin of the additional band in all lanes of *B*, which is marked by an asterisk and does not respond to competition, is unclear. It is produced in the presence of α-amanitin at 100 μg/ml and is therefore not due to transcription by either RNA polymerase II or RNA polymerase III. End-labeling of endogenous small RNAs seems a likely possibility. The additional bands in lanes 9 and 10 of *B*, one of which comigrates with the asterisked band, are B2 transcripts that result from the use of alternative termination sites.

genes were used, or when a whole cell extract was used instead of the nuclear extract, and at a range of template concentrations (data not shown). Heat-treated TBP had no activity in this assay (data not shown). The stimulation by TBP was not a general response under conditions of low-level

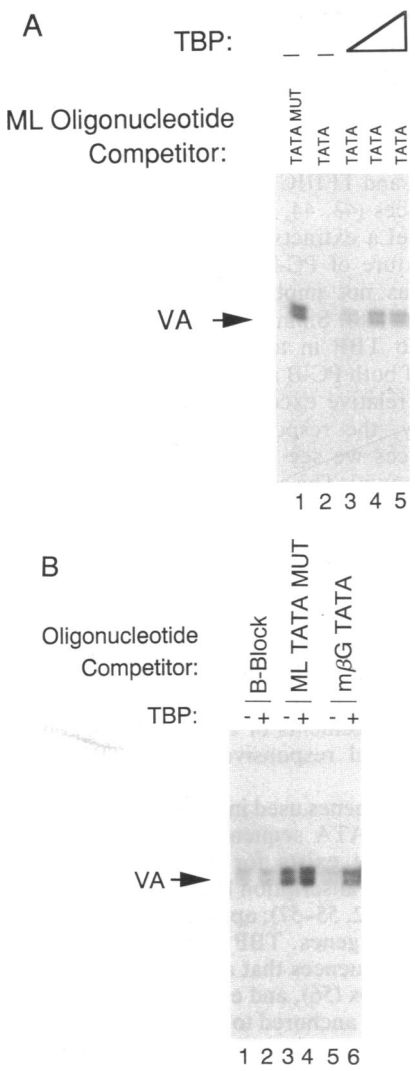

FIG. 2. Cloned TBP restores RNA polymerase III transcription to extracts depleted of TATA-binding factors by preincubation with TATA oligonucleotides. (*A*) The VA_I gene was transcribed in the presence of α-amanitin at 2 μg/ml by HeLa extract preincubated for 15 min at 30°C with the ML TATA mutant (lane 1) or the ML TATA (lanes 2–5) oligonucleotides. In lanes 3, 4, and 5, template was preincubated separately with 2.5, 5, and 7.5 ng, respectively, of >95% pure cloned human TBP for 15 min at 30°C before being added to the depleted extract. (*B*) VA_I was transcribed in the presence of α-amanitin at 2 μg/ml by HeLa extract that had been preincubated for 15 min at 30°C with B-block (lanes 1 and 2), ML TATA mutant (lanes 3 and 4), or mβG TATA (lanes 5 and 6) oligonucleotides. Template was separately preincubated for 15 min at 30°C with (lanes 2, 4, and 6) or without (lanes 1, 3, and 5) 8 ng of cloned TBP.

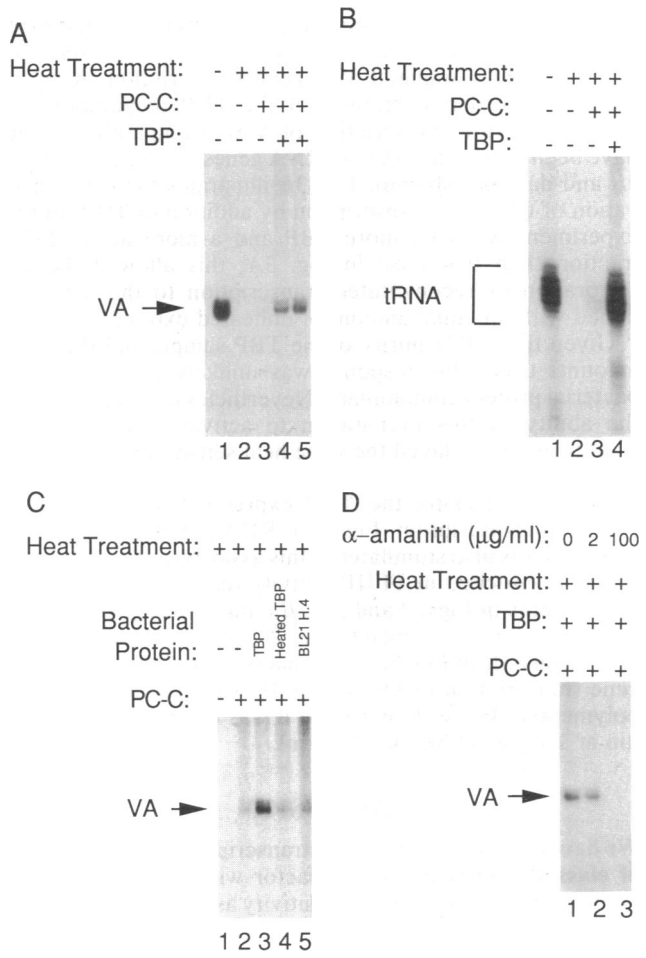

FIG. 3. Cloned TBP restores RNA polymerase III transcription to heat-inactivated extracts. (*A*) VA_I was transcribed in the presence of α-amanitin at 2 μg/ml by either untreated (lane 1) or heat-treated (lanes 2–5) HeLa extract in the presence (lanes 3–5) or absence (lanes 1 and 2) of 4 μg of a PC-C fraction. Lanes 4 and 5 contained 5 and 7.5 ng of cloned TBP, respectively. (*B*) The $tRNA^{Glu6}$ gene was transcribed in the presence of α-amanitin at 2 μg/ml by either untreated (lane 1) or heat-treated (lanes 2–4) HeLa extract in the presence (lanes 3 and 4) or absence (lanes 1 and 2) of 3.8 μg of a PC-C fraction. Lane 4 contained 10 ng of cloned TBP. (*C*) VA_I was transcribed in the presence of α-amanitin at 2 μg/ml by heat-inactivated HeLa extract. PC-C (2 μg) was present in lanes 2–5. Eight nanograms of cloned TBP (lane 3), 8 ng of heat-inactivated cloned TBP (lane 4), or 8 ng of the equivalent fraction from bacteria lacking the TBP expression vector (lane 5) was also included. (*D*) VA_I was transcribed by heat-inactivated HeLa extract, 2 μg of PC-C, and 8 ng of cloned TBP in the presence of α-amanitin at 0 (lane 1), 2 (lane 2), or 100 (lane 3) μg/ml.

transcription because TBP had no effect upon extract preincubated with a B-block internal promoter oligonucleotide to deplete TFIIIC (Fig. 2*B*, lanes 1 and 2). Nor did TBP activate transcription in an extract preincubated with a TATA mutant oligonucleotide (Fig. 2*B*, lanes 3 and 4) or in the absence of oligonucleotide (data not shown). Thus, TBP is not normally rate limiting for transcription of VA_I in HeLa cell extracts. However, when it became rate-limiting after specific depletion, a dramatic inhibitory effect was observed on the transcription of a variety of templates by RNA polymerase III. That this inhibition was overcome by the addition of cloned TBP suggests that TBP is the depleted component. However, an alternative explanation is also possible. Since the TATA-binding factor from the HeLa extract was not physically removed from the reaction, but only sequestered by preincubation with a TATA oligonucleotide, the subsequent addition of TBP may simply displace an undefined TATA-binding factor from the oligonucleotide, allowing the latter factor to stimulate RNA polymerase III transcription. To distinguish between these alternatives we tested the effect of cloned TBP in the absence of oligonucleotides, using an extract depleted of endogenous TBP by mild heat treatment (2).

Cloned TBP Restores RNA Polymerase III Transcription to an Extract Depleted of TBP by Heat Inactivation. As previously reported (10), heat treatment abolishes transcription of class III genes (Fig. 3*A*, lanes 1 and 2). As well as TBP (2, 10, 50), a component of TFIIIC is inactivated in this way (10); we therefore included in our reconstituted system a PC-C fraction that contains TFIIIC activity (28) (Fig. 3*A*, lanes 3–5). This was sufficient to restore weak transcription, presumably

due to the presence of some TBP in this fraction. Addition of cloned TBP to the heat-treated extract in the presence of PC-C further stimulated VA_I gene transcription by up to 21-fold. This demonstrates that the TBP preparation can directly activate transcription of VA_I. Comparable results have been obtained with two tRNA genes and a B2 gene (Fig. 3*B* and data not shown). Fig. 3*B* illustrates the direct activation of $tRNA^{Glu6}$ transcription by addition of TBP. In this experiment we used more TBP and a more active PC-C fraction than that used in Fig. 3*A*; this allowed the full restoration of reconstituted transcription to the level obtained with an equal amount of unheated extract.

Given the >95% purity of the TBP sample and the small amounts used, this response was unlikely to be due to a bacterial protein contaminant. Nevertheless, we verified that the ability of this preparation to activate class III gene transcription displayed the same heat sensitivity as TBP and that the corresponding protein fraction from the same bacterial strain lacking the TBP expression vector did not stimulate transcription (Fig. 3*C*). RNA polymerase III transcription was also stimulated in this assay by a PC-D fraction, which is enriched in TFIID activity (data not shown). The experiments in Figs. 2 and 3 *A*, *B*, and *C* were performed in the presence of α-amanitin at 2 μg/ml to inhibit RNA polymerase II. In Fig. 3*D* we formally prove that the class III gene transcription restored by TBP was mediated by RNA polymerase III, by showing that it was inhibited by α-amanitin at 100 μg/ml but not 2 μg/ml.

DISCUSSION

We have demonstrated that the transcription of a broad range of class III genes involves a factor with the same TATA-binding specificity and heat sensitivity as the TBP component of the TFIID complex and that can be functionally substituted for by TBP. The most likely explanation is that this factor is TBP, and that TBP is, therefore, a general RNA polymerase III transcription factor.

This general role for TBP in RNA polymerase III transcription had not been anticipated because many of the promoters concerned do not contain TATA boxes. Furthermore, biochemical fractionation experiments failed to implicate TFIID in the transcription of genes such as 5S rRNA, tRNA, and VA_I (9, 10, 14, 28–41). Most of these studies began with step fractionation on PC, which separates TFIIIB and TFIIIC from each other, and also from the bulk of TFIID (28). However, TFIID activity fractionates very heterogeneously, and significant amounts are found in both the PC-B and PC-C fractions (9–11, 14, 42, 50). Therefore, a requirement for TBP would have been missed during purification of either of these factors unless the complementing fraction had also been purified away from TBP. In the exceptional case of U6 snRNA genes, which do not need a complementing TFIIIC fraction, extensive purification of TFIIIB demonstrated the necessity of TBP; the same studies did not reveal any TBP requirement for tRNA transcription, presumably because of the need in this case to include a TFIIIC fraction (9, 14). Precedent for the existence of additional polymerase III factors is provided by the discovery in silkworm extracts of two other factors, TFIIID and TFIIIR, each of which cofractionates with both TFIIIB and TFIIIC at early stages of factor separation (52, 53).

During investigation of the TATA-dependent human U6 gene, slight activation of a control $tRNA^{Met}$ gene by TBP was incidentally observed (10). In this case, using crude bacterial extracts containing cloned TBP, the tRNA response was variable and no greater than 2-fold, whereas strong activation of U6 transcription was obtained. We typically obtain 15- to 20-fold stimulation of VA_I transcription by TBP in two different assays (Figs. 2 and 3*A*), and we have observed similar effects with two tRNA genes, a 5S rRNA gene, and a B2 gene (Fig. 3*B* and data not shown). However, to see such effects it is necessary to reduce the effective concentration of TBP, using an approach such as oligonucleotide depletion or heat inactivation. This is because TBP is normally in relative excess for the transcription of class III genes with internal promoters, and TFIIIC is the rate-limiting factor under most circumstances (43, 44, 54). This is true not only of unfractionated HeLa extracts but also of reactions reconstituted with a mixture of PC-B and PC-C; this is why the PC-D fraction was not implicated in the original fractionation experiments (28). Simmen *et al.* (10) tested the response of $tRNA^{Met}$ to TBP in an unfractionated extract or in the presence of both PC-B and PC-C, situations in which TBP is already in relative excess and TFIIIC is rate limiting; not surprisingly, the response was negligible. Under similar circumstances we see no stimulation of VA_I transcription (data not shown). The same experimental conditions allowed a dramatic response to TBP by the U6 gene, creating the impression that TBP is involved in the transcription of U6 but not tRNA genes (10, 14). This difference reflects the fact that U6 transcription *in vitro* does not require TFIIIC (9, 11, 14), which is the limiting component for the transcription of tRNA and VA genes. As such, U6 transcription is free to respond to the addition of TBP, which is apparently the rate-limiting factor for transcription of this gene (10). Thus, the distinct promoter arrangements of U6 and tRNA genes account for their differential responsiveness to TBP in the previous study.

The class III genes used in the present work do not contain recognizable TATA sequences and yet clearly respond to TBP. Precedent exists for a TBP requirement for RNA polymerase II transcription from certain promoters that lack TATA boxes (42, 55–57); apparently, this is also the case for polymerase III genes. TBP appears capable of specifically recognizing sequences that are quite dissimilar to a conventional TATA box (56), and evidence has also been presented that TBP can be anchored to TATA-less promoters by means of protein–protein interactions with an undefined tethering activity (42, 57). The molecular mechanism by which TBP interacts with transcription complexes assembled onto the internal promoters of "classical" class III genes is not yet known.

Note Added in Proof. It has recently been shown (58) that TBP is also a component of the RNA polymerase I transcription factor SL1, and it therefore appears that TBP is required for transcription by all three RNA polymerases.

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