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TATA-Binding Protein Is Limiting for both TATA-Containing and TATA-Lacking RNA Polymerase III Promoters in *Drosophila* Cells

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We have investigated the role of the TATA-binding protein (TBP) in modulating RNA polymerase (Pol) III gene activity. Epitope-tagged TBP (e-TBP) was both transiently and stably transfected in *Drosophila* Schneider S-2 cells to increase the total cellular level of TBP. Analysis of the transcripts synthesized from cotransfected tRNA and U6 RNA genes revealed that both types of RNA Pol III promoters were substantially stimulated by an increase in e-TBP in a dose-dependent manner. Furthermore, a TBP-dependent increase in the levels of endogenous tRNA transcripts was produced in the stable line induced to express the e-TBP. We further determined whether the ability of increased TBP to induce RNA Pol III gene expression was due to a direct effect of increased TBP complexes on RNA Pol III gene promoters or an indirect consequence of enhanced expression of RNA Pol II genes. A TBP expression plasmid (e-TBP332), containing a mutation within the highly conserved carboxy-terminal domain, was both transiently and stably transfected into S-2 cells. e-TBP332 augmented the transcription from two RNA Pol II gene promoters indistinguishably from that observed when e-TBP was expressed. In contrast, e-TBP332 was completely defective in its ability to stimulate either the tRNA or U6 RNA gene promoters. In addition, increasing levels of a truncated TBP protein containing only the carboxy-terminal region failed to induce either the tRNA or U6 RNA gene promoter, whereas it retained its ability to stimulate an RNA Pol II promoter. Thus, the TBP-dependent increase in RNA Pol II gene activity is not sufficient for enhanced RNA Pol III gene transcription; rather, a direct effect on RNA Pol III promoters is required. Furthermore, these results provide the first direct evidence that the amino-terminal region of TBP is important for the formation or function of TBP-containing complexes utilized by TATA-less and TATA-containing RNA Pol III promoters. Together, these studies demonstrate that TBP is limiting for the expression of both classes of RNA Pol III promoters in *Drosophila* cells and implicate an important role for TBP in regulating RNA Pol III gene expression.

RNA polymerase (Pol) III is responsible for the transcription of many small cellular and viral RNAs which are untranslated. There are three major classes of promoters that dictate the expression of the genes transcribed by RNA Pol III (43). The tRNA and 5S RNA classes of promoters each contain intragenic promoter elements and generally lack TATA sequences upstream of the gene. Both of these types of promoters require TFIIIB and TFIIIC components to reconstitute transcription in vitro, while the 5S RNA gene additionally uses TFIIIA. The U6 RNA class of promoters require elements that reside exclusively upstream of the gene and include TATA and proximal sequence elements. For the vertebrate systems, this class of promoter requires TFIIIB, TATA-binding protein (TBP), and a distinct multisubunit complex referred to as SNAPc or PTF (18, 29, 44). Although these three genes are representative of the three classes of promoters, there are other RNA Pol III genes that contain features distinct from these classes (for a review, see reference 43) or contain a combination of elements from more than one class of promoters (34). All RNA Pol III promoters appear to require TFIIIB for transcription. In mammalian systems, this component consists of TBP and at least two additional TBP-associated factors (TAFs) (32). One of the human TAF subunits has been identified and cloned (37). Since there is evidence that the SNAPc complex also contains TBP (29), it appears that the TATA-containing U6 RNA promoter uses TBP in a distinct manner compared to the TATA-less tRNA and 5S RNA promoters.

TBP has been shown to be necessary for the transcription of all cellular genes. The gene encoding TBP has been cloned from a variety of organisms (19), and the structure of the protein has been resolved by crystallography (3, 27). The carboxy-terminal domain of approximately 180 amino acids is highly conserved in all organisms and has been shown to contain the DNA binding function (21). Additionally, amino acids that are essential for transcription and are likely involved in the interaction of TBP with other TAFs have been identified in this region (2, 4, 7). TBP is recruited to RNA Pol III TATA-containing promoters through its interaction with DNA, whereas it is recruited to TATA-less promoters via protein-protein interactions with other DNA-bound factors (25, 39). The role of the amino-terminal region of TBP in the formation of RNA Pol I, II, or III transcription initiation complexes is still not clear. This region varies significantly in both length and sequence between different species. Previous studies have used antibodies specific for the amino-terminal region of human TBP to elucidate its role in transcription initiation (23, 26). Studies in yeast have suggested that the amino-terminal region of TBP may be important for transcription of RNA Pol III genes, since human TBP failed to complement a defective yeast TBP and restore RNA Pol III gene expression (6).

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RNA Pol III gene expression has been previously shown to be modulated by specific viral transactivator proteins. RNA Pol III promoters have been shown to be up-regulated by the adenovirus E1A protein (15, 20) and the simian virus 40 (SV40) large T antigen (41). In both cases, the mechanism for induction was shown to involve an increase in the amount of TFIIIC activity. Several other regulatory events have been shown to involve changes in the activity of TFIIIB. The differentiation of F9 embryonic carcinoma stem cells into endoderm results in a down-regulation of RNA Pol III genes and decreased TFIIIB activity (40). Similarly, down-regulation of these genes in growth-arrested cells was also found to be a result of decreased TFIIIB activity (33). Repression of RNA Pol III transcription at mitosis is mediated by a decrease in TFIIIB activity, which may involve alterations in the one or more of the TBP-associated components (17, 38). The human T-cell leukemia virus-encoded Tax protein has been shown to stimulate RNA Pol III promoters through an increase in the concentration of TFIIIB complexes (16). Although the transcription components that modulate these regulatory events have been identified, the specific subunits within these complexes that are altered in these responses have not yet been defined.

Transactivation of RNA Pol III gene promoters by the hepatitis B virus product, X, is mediated by an increase in TFIIIB activity, and a corresponding increase in the cellular levels of TBP is observed (36). Likewise, the induction of RNA Pol III gene expression by the phorbol ester tetradecanoyl phorbol acetate (13) was also shown to be a result of increased TFIIIB activity and increased cellular TBP levels (14). These studies suggest that in certain regulatory events, class III genes may be controlled by modulating the levels of cellular TBP to increase the number of functional TFIIIB complexes.

In this study, we have investigated the possibility that the TBP subunit of the TFIIIB complex is the limiting component for the expression of RNA Pol III gene expression in *Drosophila* cells. We have therefore determined how these promoters respond to increasing cellular levels of TBP.Transient expression of either the TATA-containing U6 RNA gene or the TATA-less tRNA gene is substantially stimulated in a dose-dependent manner when increased cellular levels of TBP are produced in *Drosophila* S-2 cells by cotransfection of a *Drosophila* TBP cDNA. Furthermore, when a TBP gene is stably introduced into S-2 cells, overexpression of TBP results in a significant stimulation of a transiently transfected tRNA gene and an increase in the endogenous $tRNA^{Arg}$ gene transcripts. To further determine whether TBP is directly limiting at these RNA Pol III gene promoters, we have constructed a mutant *Drosophila* TBP protein which contains a single amino acid change at position 332 within the second direct repeat motif of the carboxy-terminal region. This TBP mutant is completely defective in its ability to stimulate either of the class III promoters, yet it retains its ability to stimulate two TATA-containing RNA Pol II promoters. These results indicate that the TBP-mediated increase in the expression of RNA Pol II genes is not sufficient for the enhanced expression of RNA Pol III genes. Furthermore, an amino-terminally deleted TBP, which enhanced the expression of an RNA Pol II promoter, failed to stimulate either the U6 RNA or the tRNA gene promoter. Thus, the amino-terminal region serves an important role for the assembly or function of TBP complexes for both TATA-containing and TATA-less class III promoters in vivo. Together, these studies demonstrate that TBP is limiting for both types of RNA Pol III promoters and that the increased expression of the RNA Pol III genes is due to a direct increase of TBP complexes at these promoters.

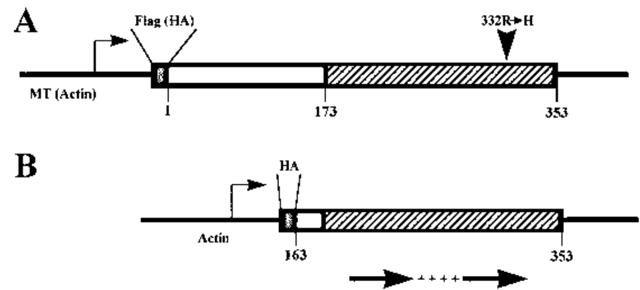

FIG. 1. Schematic representation of constructs used for expression of wild-type and mutant *Drosophila* TBP forms. The boxed regions depict the coding sequence used. The evolutionarily conserved carboxy-terminal region (amino acids 173 to 353) is shown in the striped region, the shaded region represents sequences encoding the epitope tags. The direct repeats and basic region are depicted below as arrows and plus signs, respectively. (A) Full-length TBP constructs. pTBP contains the TBP cDNA under the control of the *Drosophila* actin 5C distal promoter; pH-TBP additionally contains the HA epitope coding sequence at the 5′ end of the cDNA; pF-TBP contains the Flag epitope coding sequence at the 5′ end of the cDNA, and expression is under the control of the metallothionein (MT) promoter; pH-TBP332 and pF-TBP332 contain a single amino acid change in the carboxy-terminal region of TBP as shown. (B) Amino-terminal deletion TBP construct. The pH-ΔNTBP construct contains the HA epitope and amino acids 163 to 353 of the TBP cDNA. Expression is under the control of the actin 5C promoter.

MATERIALS AND METHODS

Recombinant plasmids. The constructs used are depicted in Fig. 1. The expression vector, pTBP, previously designated Act-TFIID (5), contains a *Drosophila* wild-type TBP cDNA driven by the *Drosophila* actin 5C distal promoter. The H-TBP expression vector, previously designated Act flu-TFIID (5), contains a TBP cDNA that has a 10-amino-acid sequence (flu epitope) from influenza virus hemagglutinin 1 (HA 1) (11) inserted at the 5′ end of the coding region. The pH-ΔNTBP vector, previously designated Act-flu-TFIIDΔNde (5), contains a cDNA encoding an amino-terminally deleted TBP which is fused with the flu epitope at the amino terminus. pTBP, pH-TBP, and pH-ΔNTBP were gifts from James Manley (5). To construct pH-TBP332, changing the arginine residue to a histidine residue at amino acid position 332 in TBP, PCR was used. The reverse oligonucleotide primer, 5′-TAGATCTCCTG**GT**GCACCTTTGCTCC-3′, and the forward primer, 5′-GATGTGAAGTTCCC(C, T, or A)AT(C or T)(C or G)G-3′, were used to synthesize a cDNA containing the nucleotide changes designated in boldface. The PCR product was digested with *Bst*XI and *Bgl*II and ligated to pH-TBP that had been digested with the same enzymes. DNA sequence analysis confirmed the presence of the desired base pair changes. To construct the pF-TBP and pF-TBP332 expression vectors which contain sequences encoding the nine-amino-acid Flag epitope (MDYKDDDDK) at the 5′ end, PCR was used to amplify the open reading frame of either the wild-type TBP or mutant TBP cDNA by using the following primers: 5′- CGGGATCCA TGGACTACAAGGACGATGACGATAAGATGGACCAAATGCTAAGC C-3′ (sense) and 5′-ACGCGTCGACTTATGACTGCTTCTTGAACTT-3′ (antisense). The PCR products were digested with *Bam*HI and *Sal*I. These fragments were gel purified and ligated into a plasmid, pMT, containing the metallothionein promoter (1) which had been digested with *Bam*HI and *Sal*I.

Plasmid pArg-maxi is a derivative of a *Drosophila* $tRNA^{Arg}$ gene in a pBluescript pSK^+ (pSK) vector (Stratagene) that contains an additional 12-bp insert between the internal promoter regions (9). pU6-maxi was constructed by ligating a 12-bp *Bam*HI linker into the *Eco*NI site at nucleotide 462 within the coding sequence of the *Drosophila* U6 RNA gene, pDU6-2 (8). This insert was excised from a pUC vector with *Eco*RI and ligated into pSK. Plasmid pADH-Luc, containing a luciferase coding sequence driven by the alcohol dehydrogenase (ADH) promoter, was constructed by digesting pADH-AP2 (42) with *Nco*I and *Bam*HI and ligating the resultant vector fragment to the luciferase gene that had been isolated from pT7-Luc-A50, which is a pSK-based plasmid with a luciferase gene insert (12). The pSV40-Luc plasmid (pGL2-Promoter; Promega) is a luciferase reporter plasmid driven by the SV40 minimal promoter. pAct-CAT, is a chloramphenicol acetyltransferase (CAT) reporter plasmid driven by the *Drosophila* actin 5C promoter (4).

Stable and transient transfections. Transient transfections were performed by using a calcium phosphate precipitation technique (10). *Drosophila* Schneider S-2 cells were maintained at 25°C in Schneider medium (Gibco) containing 10% fetal bovine serum (Gemini Bioproducts). For transfections, cells were plated at 0.5×10^6 to 1.0×10^6 cells per ml. For each transfection assay, 2.5×10^6 to 5×10^6 cells were cotransfected with 2 μg of a reporter plasmid, 2 μg of pAct-CAT (for measuring transfection efficiencies), and different concentrations of a TBP expression plasmid as designated. The final DNA concentration was maintained

at 20 μg by using pSK. The medium was changed 24 h posttransfection, and cells were harvested after an additional 24 h. To estimate the number of cells transfected, we have independently cotransfected a β-galactosidase reporter gene into S-2 cells. Subsequent staining of the cells in situ (24) and quantifying the number of blue cells showed that approximately 15% of the S-2 cell population is transfected (data not shown).

The pF-TBP and pF-TBP332 stable cell lines were obtained by calcium phosphate transfection of *Drosophila* S-2 cells with 5 μg of either pF-TBP or pF-TBP332, 5 μg of pCO-Hygro, containing the hygromycin resistance gene under the control of the copia promoter (31), and 10 μg of pSK vector. Five milliliters of S-2 cells in T25 Corning flasks was transfected at a density of 10^6 cells per ml. Twenty-four hours after transfection, the medium was replaced with medium containing hygromycin B (Boehringer Mannheim) to 250 μg/ml. The cells were selected by passage every 3 days in hygromycin B-containing medium for 6 weeks. Cell lines were tested for F-TBP or F-TBP332 expression by induction with copper sulfate for 48 h followed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and Western blot (immunoblot) analysis of whole cell lysates.

RNase protection assays. RNA was extracted by using TRIzol (Gibco) as instructed by the vendor. RNase protection assays were carried out by using an RPA II kit (Ambion). The isolated RNAs (0.1 μg of each sample) were hybridized with an excess of ^{32}P-labeled antisense transcript at 45°C overnight. The antisense transcript was generated from either pArg-maxi or pU6-maxi, using a Maxiscript kit (Ambion). The DNA was linearized and then transcribed with T7 RNA polymerase, and the transcript was labeled with [^{32}P]CTP (specific activity, >600 Ci/mmol; ICN). The resultant riboprobe was treated with DNase I and ethanol precipitated. For each reaction, 0.5×10^6 to 1×10^6 cpm was used. RNase digestion conditions for the pArg-maxi transcript used a mixture of 2.5 U of RNase A per ml and 100 U of RNase T_1 per ml per reaction. For the pU6-maxi transcript, the digestion conditions were 100 U of RNase T_1 per ml per reaction. Digestion was carried out at 37°C for 30 min. The reaction was terminated by adding 300 μl of stop buffer and 200 μl of ethanol, and the RNA products were precipitated and resuspended in 8 μl of RNA loading dye and electrophoresed on 8% polyacrylamide–8 M urea gels. The gel was exposed to X-ray film for 30 to 120 min at −80°C, and the resultant autoradiographs were quantitated with a Bioimage Scanner.

Western blot analysis. Crude whole cell lysates were prepared by repeated freeze-thaw cycles of cells resuspended in 0.25 M Tris (pH 7.5). Protein concentrations were determined by using a Bio-Rad assay. Equal amounts of all protein samples were loaded onto SDS–10% polyacrylamide gels and electrophoresed. The separated proteins were blotted onto nitrocellulose (Schleicher & Schuell), using the semidry method described by Kyhse-Andersen (22). Total cellular TBP was probed with anti-*Drosophila* TBP polyclonal antibodies, and the eIF-2α polypeptide was probed with polyclonal antibodies against *Drosophila* eIF-2α as previously described (36). The TBP polypeptides that were tagged with the flu epitope were detected with a monoclonal antibody against the epitope (provided by James Manley, Columbia University). The nine-amino-acid Flag epitope was detected with M5 monoclonal antibodies (Kodak). Horseradish peroxidase-linked antibodies (Vectastain) and enhanced chemiluminescence reagents (HRPL kit; National Diagnostics) were used to detect bound antibodies.

Luciferase and CAT assays. Luciferase activity was detected by using a luciferase assay kit (Promega). Briefly, approximately 8×10^6 transfected cells were resuspended in 300 μl of lysis buffer and incubated at room temperature for 10 min. One hundred microliters of luciferin reagent was added to each sample containing equal amounts of protein, and the resultant luminescence was measured after 30 s, using a Moonlight 2000 luminometer. For CAT activity measurements, 2×10^6 cells were used per sample. Briefly, cell pellets were resuspended in 90 μl of 0.25 M Tris (pH 7.5) followed by repeated freeze-thaw cycles. Extracts were diluted 1:50 to 1:500 in 0.25 M Tris (pH 7.5), and 5-μl aliquots of the diluted extracts were used to assay for CAT activity as previously described (30). Products were analyzed by thin-layer chromatography and quantitated by scanning the resultant autoradiograms with a Bioimage Scanner.

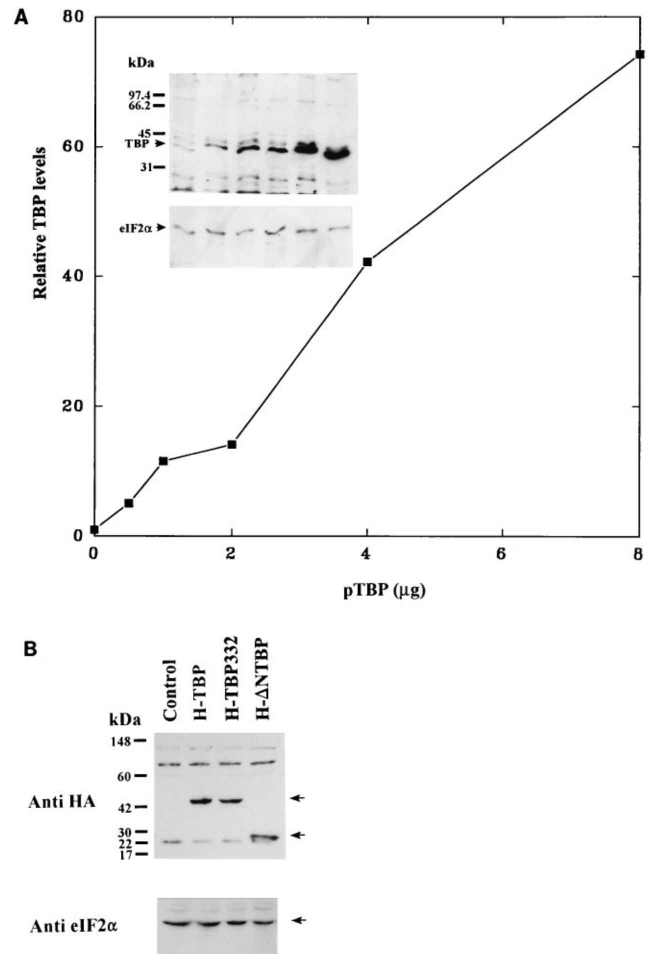

FIG. 2. Expression of wild-type and mutant TBPs. (A) TBP is overproduced in S-2 cells transiently transfected with pTBP. As described in Materials and Methods, cells were transiently transfected with increasing amounts of pTBP (as indicated on the graph), extracts were prepared, equal amounts of protein were subjected to SDS-PAGE, and Western blot analysis was used to determine the amount of TBP produced. The blot in the top panel was probed with anti-*Drosophila* TBP antibodies, whereas the blot in the bottom panel was probed with anti-*Drosophila* eIF-2α antibodies. *Drosophila* TBP is represented by two polypeptides with apparent molecular masses of 38 and 39 kDa, and eIF-2α is 38 kDa. The amount of TBP present in each lane was quantified by densitometry. The relative amounts shown in the graph were normalized to correct for variations in transfection efficiencies by measuring CAT activity resulting from the expression of a cotransfected CAT reporter gene. (B) Mutant e-TBPs can be stably produced by transient transfection. Cells were transfected with 4 μg of the plasmids shown above the lanes, and equal amounts of protein from the resultant extracts were subjected to Western blot analysis. The top panel depicts a blot probed with the anti-HA antibodies. The bottom panel represents a blot probed with anti-eIF-2α antibodies. The arrows indicate the positions of the TBP and eIF-2α polypeptides.

RESULTS

Increased transient expression of TBP stimulates both TATA-containing and TATA-less RNA Pol III promoters. To determine whether variations in cellular TBP levels could affect RNA Pol III gene activity, a TBP expression plasmid (pTBP) was first transiently transfected into *Drosophila* Schneider S-2 cells to increase the concentration of TBP. The pTBP plasmid contains a *Drosophila* TBP cDNA, and transcription of the gene is driven by the actin 5C distal promoter. Increasing amounts of pTBP were transfected into S-2 cells. Lysates were prepared from the transfected cells, and Western blot analysis was used to determine the amount of TBP, using polyclonal antibodies directed against *Drosophila* TBP (Fig. 2A). We routinely observed two cross-reacting polypeptides with apparent molecular masses of 38 and 39 kDa. The most prevalent 38-kDa band represents unphosphorylated TBP, whereas the minor, more variable 39-kDa band represents a phosphorylated form of TBP (20a). By increasing the amount of pTBP in the transfection assays, a corresponding increase in the amount of total cellular TBP was obtained (inset, upper panel). In contrast, the eIF-2α polypeptide remained unchanged (inset, lower panel). Thus, consistent with previous studies, TBP can be stably overproduced by transient expression (5).

The effect of increased TBP levels on the transcription of

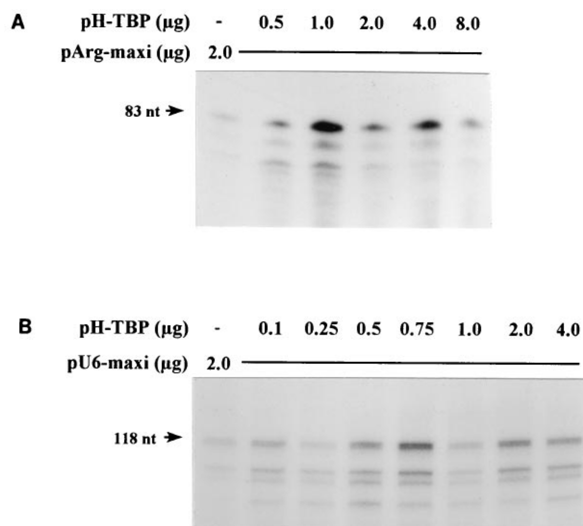

FIG. 3. Increased cellular levels of H-TBP enhance transcription from tRNA and U6 RNA promoters. Cells were cotransfected with increasing amounts of pH-TBP and either pArg-maxi (A) or pU6-maxi (B). RNA was isolated from the cells, and 0.1 μg of each sample was used for RNase protection assays as described in Materials and Methods to determine the amount of transcript produced from the transfected genes. For the pArg-maxi transcript, the size of the protected fragment is 83 nucleotides (nt). For the pU6-maxi transcript, the size of the protected fragment is 118 nucleotides. The amount of labeled product was quantified from the resultant autoradiograph by densitometry.

both tRNA and U6 RNA gene promoters was next examined. To distinguish TBP produced from the endogenous and transfected genes, we used a TBP expression plasmid that had a flu epitope inserted at the 5′ end of the coding region of the TBP cDNA (pH-TBP). A plasmid containing a $tRNA^{Arg}$ gene promoter (pArg-maxi) was cotransfected with increasing amounts of pH-TBP. In addition, a plasmid containing a CAT reporter gene driven by the actin 5C promoter was cotransfected to determine the relative transfection efficiencies. The actin 5C promoter was not stimulated in response to increased TBP (data not shown). RNA was extracted from each set of transfected cells, and an RNase protection assay was used to determine the amount of RNA transcript synthesized from the tRNA gene promoter (Fig. 3A). As shown, a dose-dependent increase in the transcription of the pArg-maxi gene was observed. The maximal tRNA gene expression was observed at approximately 1 μg of pH-TBP. Based on Western blot analysis, this corresponded to an approximate 12-fold increase in cellular TBP levels (Fig. 2A). An additional peak of activity was also reproducibly seen at 4 μg of pH-TBP. When more than 4 μg of pH-TBP was used for transfection, a decreased response to TBP was observed. An average of results from three independent experiments demonstrated that increased levels of TBP stimulated the tRNA gene promoter approximately 15-fold (Table 1).

Since evidence suggests that at least the mammalian U6 RNA promoter may use a distinct TBP-containing complex for transcription (29, 35, 44), the effect of increasing TBP levels on transcription activity of this promoter was determined. A plasmid containing a U6 RNA gene promoter (pU6-maxi) was cotransfected with increasing amounts of pH-TBP, RNA was isolated, and an RNase protection assay was performed to quantify the amount of transcript produced. Consistent with results obtained for the tRNA gene promoter, a similar TBP concentration-dependent increase in transcription for the U6 RNA gene promoter was observed (Fig. 3B). Based on several independent experiments, the U6 RNA gene promoter was stimulated approximately fivefold. These results reveal that both TATA-containing and TATA-less RNA Pol III promoters are stimulated by increasing TBP levels in *Drosophila* S-2 cells.

Overproduction of TBP from a stably introduced gene enhances the expression of both a transiently transfected tRNA gene and endogenous tRNAs. To further substantiate that TBP is limiting for the transcription from RNA Pol III promoters, and to specifically examine whether the levels of endogenous tRNA gene transcripts could also be augmented with increased cellular TBP concentrations, we constructed a stable line that could be induced to overexpress an epitope-tagged TBP (e-TBP). The plasmid, pF-TBP, was constructed to contain a nine-amino-acid Flag epitope coding region fused at the amino terminus of the TBP cDNA, and the gene was placed under the control of the metallothionein promoter, which is inducible with copper (Fig. 1). S-2 cells were transfected with pF-TBP, and the pooled population of selected cells were first examined for the ability to overproduce e-TBP (Fig. 4A). Whole cell lysates were prepared from the stable pF-TBP cells that were either uninduced (lanes 1 and 5) or induced with 500 μM copper sulfate (lanes 2 and 6). Using Western blot analysis and a monoclonal antibody, M5, that recognizes the Flag epitope, we found that cross-reacting polypeptides were expressed only when the cells were induced with copper sulfate (compare lanes 5 and 6). To further determine whether the total TBP levels were affected by expression of F-TBP, Western blots were probed with polyclonal antibodies directed against *Drosophila* TBP. As shown in lanes 1 and 2, the cellular increase in TBP was a result of expression of the pF-TBP. In addition, overproduction of the F-TBP protein did not alter the steady-state levels of endogenous TBP.

The effect of increased expression of the stably integrated F-TBP gene on tRNA gene transcription was examined. The F-TBP stable line was transiently transfected with pArg-maxi, and cells were incubated with increasing amounts of copper sulfate to induce production of F-TBP. RNA was isolated from the transfected cells, and an RNase protection assay was used

TABLE 1. Enhancement of RNA Pol II and III promoter activity in S-2 cells transiently transfected with wild-type and mutant TBP genes

Promoter	Fold enhancement (mean ± SD)[a]		
	H-TBP	H-ΔNTBP	H-TBP322
tRNA (Pol III)[b]	15 ± 4.0	1.0 ± 0.2	1.1 ± 0.4
U6 RNA (Pol III)[b]	4.5 ± 1.5	0.7 ± 0.1	0.7 ± 0.5
ADH (Pol II)[c]	4.7 ± 0.6	3.6 ± 0.7	4.5 ± 1.0
SV40 (Pol II)[c]	3.3 ± 1.0	ND[d]	2.8 ± 0.7

[a] The data shown were derived from comparison of promoter activities in the absence and in the presence of a cotransfected TBP expression plasmid. At least three independent experiments were performed for each promoter. In all transfections, 2 μg of plasmid containing a CAT gene under the control of the actin 5C promoter was cotransfected. The resultant CAT activity was measured and used to normalize the relative promoter activities for transfection efficiencies. For the data shown, the CAT activities from each of the transfections varied less than 10%.

[b] Two micrograms of pArg-maxi (tRNA) or pU6-maxi (U6 RNA) was cotransfected without or with 0.75 μg of either the pH-TBP, pH-ΔNTBP, or pH-TBP332 expression vector, and the resultant RNA transcripts were analyzed by RNase protection assays as described in Materials and Methods.

[c] Two micrograms of pADH-Luc (ADH) or pSV40-Luc (SV40) was cotransfected without or with 0.5 μg of either the pH-TBP, pH-ΔNTBP, or pH-TBP332 expression vector, and the resultant luciferase activity was measured as described in Materials and Methods.

[d] ND, not determined.

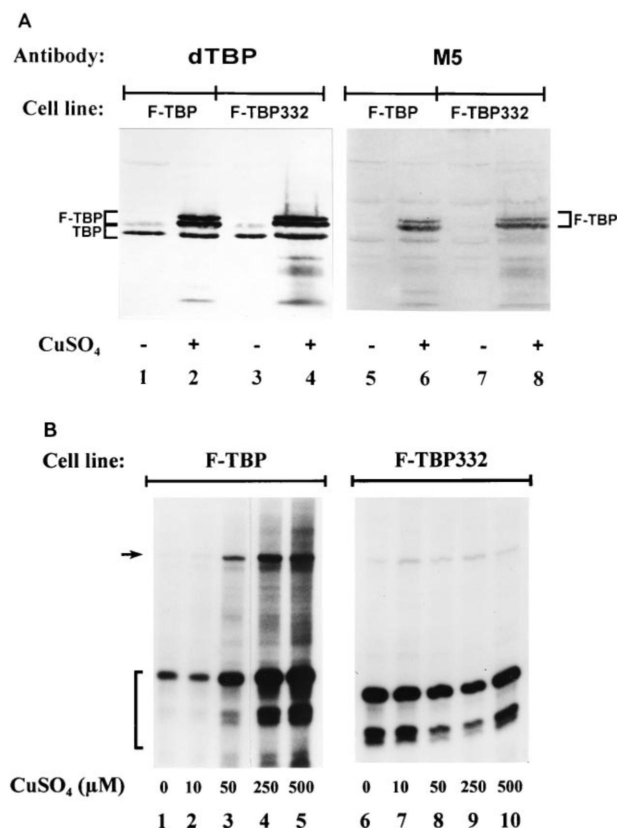

FIG. 4. F-TBP and F-TBP332 expression in stable cell lines and resultant effect on tRNA gene expression. (A) F-TBP and F-TBP332 are expressed upon induction with copper. F-TBP and F-TBP332 stable lines were constructed as described in Materials and Methods. The cell lines were either induced (+) or not induced (−) with copper sulfate as shown, whole cell extracts were prepared, and Western blot analysis was performed as described in Materials and Methods, using equal amounts of protein from all extracts derived from the F-TBP cells (lanes 1, 2, 5, and 6) and F-TBP332 cells (lanes 3, 4, 7, and 8). The blots were probed with either anti-*Drosophila* TBP antibodies (dTBP) (lanes 1 to 4) or anti-Flag antibodies (M5) (lanes 5 to 8). (B) Effect of F-TBP and F-TBP332 expression on a transiently transfected tRNA promoter and endogenous tRNA levels. pArg-maxi was transiently transfected into either the F-TBP or the F-TBP332 cell line, and the cells were induced with the amount of copper sulfate shown at the bottom. RNA was isolated, and RNase protection assays were carried out as described in Materials and Methods. The resultant autoradiographs depict protected pArg-maxi transcripts generated from the transfected gene (designated by the arrow) and endogenous half-tRNA products that hybridize with the pArg-maxi probe (designated by the bracket).

to quantify the amount of pArg-maxi transcript produced. As shown in Fig. 4B (lanes 1 to 5), a substantial increase in transcription of the pArg-maxi gene was obtained in a manner that was proportional to the amount of copper used to induce the expression of the F-TBP gene. The maximum level of stimulation (approximately 100-fold) occurred when the cells were induced with 250 μM copper sulfate. The pArg-maxi-derived riboprobe used to detect the transcripts generated from the transfected tRNA gene also hybridizes with the endogenous $tRNA^{Arg}$ transcripts, producing half-sized RNA species. Thus, we were able to examine potential changes in the amount of endogenous $tRNA^{Arg}$ gene transcripts generated. As shown (Fig. 4B), a significant increase in the accumulation of tRNA transcripts was observed with increased production of the F-TBP protein. Together, these results suggest that TBP is limiting for the expression of both transiently transfected and endogenous tRNA genes.

Expression of a mutant TBP protein that is specifically defective in stimulating RNA Pol III gene activity reveals that increased TBP levels directly affect RNA Pol III promoters. The results above are consistent with the notion that TBP is limiting for tRNA and U6 RNA classes of promoters in *Drosophila* S-2 cells. However, increased cellular levels of TBP could also indirectly stimulate RNA Pol III gene expression. It has been previously shown that endogenous TBP levels are limiting for certain RNA Pol II promoters in *Drosophila* cells (5). If the expression of limiting RNA Pol III transcription factor subunits is enhanced by the increase in TBP, the cellular levels of these components could be increased. This could indirectly result in enhanced expression of the RNA Pol III promoters. Therefore, we examined whether increases in cellular TBP were directly or indirectly responsible for stimulating RNA Pol III gene activity.

Several temperature-sensitive mutations in *Saccharomyces cerevisiae* TBP that are specifically defective for the synthesis of tRNAs and 5S RNAs have been identified, whereas the transcription of RNA Pol II promoters is unaffected (7). One of these mutations occurred at amino acid position 220 in the carboxy-terminal region of *S. cerevisiae* TBP and changed a conserved arginine to histidine residue. Based on these results, we constructed analogous mutation in the *Drosophila* TBP gene that changed amino acid residue 332 in the pH-TBP construct (pH-TBP332) (Fig. 1). The ability of the resultant H-TBP332 protein to stimulate transcription from two TATA-containing RNA Pol II promoters was tested (Table 1). Cells were transiently transfected with a plasmid containing a luciferase cDNA driven by either the *Drosophila* ADH promoter (pADH-Luc) or the SV40 minimal promoter (pSV40-Luc). When cells were cotransfected with increasing amounts of either pH-TBP or pH-TBP332, the ADH promoter was stimulated approximately 4.5-fold. Likewise, transcription from the SV40 promoter was enhanced approximately threefold when either the H-TBP or H-TBP332 protein was expressed. The maximal stimulation of both RNA Pol II promoters was obtained when approximately 0.5 μg of either the pH-TBP or pH-TBP332 was transfected (data not shown). In addition, Western blot analysis of lysates derived from the pH-TBP and pH-TBP332-transfected cells showed that similar amounts of these proteins were produced (Fig. 2B). Thus, the H-TBP332 protein completely retained its ability to stimulate transcription from RNA Pol II promoters.

The ability of H-TBP332 to enhance transcription from the RNA Pol III promoters was next tested. As shown, independent of the amount of pH-TBP332 used in the transient transfection assays, no increase in transcription was observed from either the tRNA gene promoter (Fig. 5A) or the U6 RNA gene promoter (Fig. 5B). In addition, a stable transformant was constructed that allowed overproduction of a Flag epitope-tagged TBP332 protein (F-TBP332) driven by the metallothionein promoter in the S-2 cells. The selected cells were pooled and transiently transfected with pArg-maxi. The cells were subsequently incubated with increasing amounts of copper, and RNA was harvested for RNase protection assays (Fig. 4B). Irrespective of the amount of copper used for induction of the F-TBP332 protein, no significant increase in transcript levels was observed from the transfected pArg-maxi gene or the endogenous $tRNA^{Arg}$ genes (Fig. 4B, lanes 6 to 10). The amount of F-TBP332 protein produced in these cells was similar to the level of TBP protein produced from the F-TBP stable transformant (Fig. 4A; compare lanes 6 and 8). Expression of the endogenous TBP protein was also unaffected by the increased production of the F-TBP332 protein (Fig. 4A, lanes 3 and 4). These results demonstrate that this single amino acid

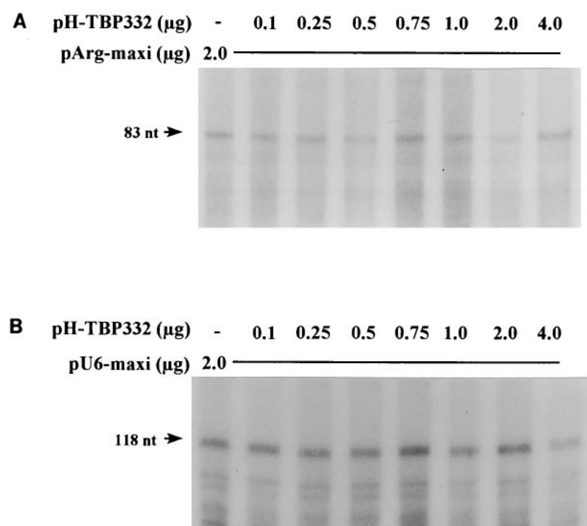

FIG. 5. Increased cellular levels of H-TBP332 do not stimulate transcription from either the tRNA or the U6 RNA promoter. Cells were cotransfected with increasing amounts of pH-TBP332 and either pArg-maxi (A) or pU6-maxi (B). RNA was isolated from the cells, and 0.1 μg of each sample was used for RNase protection assays as described in Materials and Methods to determine the amount of transcript produced from the transfected genes. For the pArg-maxi transcript, the size of the protected fragment is 83 nucleotides (nt). For the pU6-maxi transcript, the size of the protected fragment is 118 nucleotides. The amount of labeled product was quantitated from the resultant autoradiograph by densitometry.

change in *Drosophila* TBP at position 332 abolishes its ability to function in the transcription of both tRNA and U6 RNA classes of RNA Pol III promoters. Furthermore, the TBP-dependent stimulation of RNA Pol II promoter activity is not sufficient for the enhanced transcription from either of the RNA Pol III promoters. Together, these results reveal that the TBP-dependent increase in transcriptional activity of class III genes is due to a direct effect of TBP at the RNA Pol III promoters.

Stimulation of RNA Pol III gene promoters by increased TBP levels is dependent on the amino-terminal region of TBP. Since the results presented above demonstrated that TBP is limiting for transcription of RNA Pol III promoters in *Drosophila* cells, this system may be used to test mutant TBP proteins for the ability to stimulate class III promoters. To this end, the function of the amino-terminal region of TBP in the transcription of tRNA and U6 RNA gene promoters was examined. A construct containing the evolutionarily conserved carboxy-terminal domain of TBP fused to the flu epitope (pH-ΔNTBP [Fig. 1]) was expressed in S-2 cells by transient transfection. When either pArg-maxi or pU6-maxi was cotransfected with pH-ΔNTBP, no significant increases in transcription from these promoters was observed (Table 1), irrespective of the amount of H-ΔNTBP expressed (data not shown). However, deletion of the amino-terminal region of TBP did not abolish the ability of this protein to stimulate an RNA Pol II promoter (Table 1). The slightly reduced capacity of H-ΔNTBP, compared to H-TBP, to enhance transcription from the ADH promoter is likely a result of the slightly reduced accumulation of this truncated protein (Fig. 2B), consistent with previous observations (5). Thus, these results indicate that the amino-terminal region of TBP has a critical role in either the formation or the function of TBP complexes used for the transcription of both TATA-containing and TATA-lacking RNA Pol III promoters.

DISCUSSION

Our studies have demonstrated that in *Drosophila* S-2 cells, TBP is limiting for the expression of both TATA-containing and TATA-lacking RNA Pol III promoters. Transient expression of e-TBP resulted in an enhancement of transcription from both transiently transfected tRNA and U6 RNA gene promoters. When a stable line that could be induced to express increasing amounts of the e-TBP was constructed, a substantial increase in the transcription of a transiently transfected tRNA gene promoter was also observed. In addition, induction of TBP produced a corresponding increase in the endogenous tRNA transcripts. This finding suggests that transcription of the endogenous genes is also affected by the increase in cellular TBP. The TBP-dependent increase in RNA Pol III transcription is dose dependent. Reproducibly, we observed two peaks in both tRNA and U6 RNA promoter activities when increasing amounts of the TBP expression plasmid were transiently transfected (Fig. 3). The reason for this is not clear; however, it likely reflects the relative concentrations of both TAFs and other TBP-interacting proteins and the extent to which TBP is bound by these proteins. We were able to express the e-TBP in the stable line without altering the levels of endogenous TBP. However, the amount of the e-TBP produced upon copper sulfate induction diminishes gradually as the stable line is passaged over a period of several months in the absence of copper (data not shown). The reason for this change is not yet clear; however, it is possible that the small amount of e-TBP produced in the noninduced cells is deleterious, causing selection of clones that either do not contain the e-TBP gene or express e-TBP at lower levels. This phenomenon could have the same basis as the autoregulation of TBP observed in a stably transfected HeLa cell line, as reported by Zhou and coworkers (45). Interestingly, when the S-2 stable line that expresses the mutant e-TBP protein is continuously passaged, there is no alteration in the ability of this protein to be induced and overproduced (data not shown).

Previous studies have shown that increasing the cellular concentration of TBP in transient-expression assays alters RNA Pol II gene activity. In *Drosophila* L-2 cells, the transcription of cotransfected TATA-containing RNA Pol II promoters is enhanced whereas transcription from TATA-lacking RNA Pol II promoters is actually inhibited (5). Overexpression of TBP in mammalian cells has also been examined with respect to its consequence on the function of transcriptional activators (28). When tested with the same core RNA Pol II promoter, increased TBP expression was found to potentiate some transcriptional activators and inhibit others. Thus, changes in the cellular levels of TBP differentially affect RNA Pol II promoter activity. If the genes encoding the RNA Pol III transcription factor subunits are also affected by increased TBP levels, this could conceivably indirectly alter RNA Pol III gene activity. Therefore, we have examined whether TBP-mediated alterations in RNA Pol II promoter activity could account for the increase in RNA Pol III gene activity. We have constructed an e-TBP protein with a mutation that changes a highly conserved arginine residue to a histidine residue within the carboxy-terminal domain. When expressed by either transient or stable transfection, the wild-type and mutant TBP proteins accumulate to similar levels (Fig. 2B and 4A). This mutation has no effect on the ability of the resultant protein to stimulate RNA Pol II promoters, yet it completely abolishes the ability of the protein to enhance transcription from either of the RNA Pol III promoters. Thus, the enhancement of RNA Pol II gene expression by TBP is not sufficient for the increased RNA Pol III promoter activity observed. Interestingly, we observed no

change in either the amount of TBP required for maximal activity of the RNA Pol II promoters or in the level of induction when the RNA Pol III promoter-defective TBP protein is overexpressed. In the yeast system, however, expression of temperature-sensitive RNA Pol III-defective TBP proteins resulted in increased amounts of mRNA (7).

Cormack and Struhl (7) have previously shown that expression of a temperature-sensitive *S. cerevisiae* TBP mutation that changes a highly conserved arginine residue at position 220 to a histidine amino acid renders the protein specifically defective for the synthesis of tRNA and 5S RNA. Our studies have directly shown that the corresponding mutation at position 332 in *Drosophila* TBP is critical for transcription of both tRNA and U6 RNA promoters. The crystal structure of *Arabidopsis thaliana* TBP-2 has been resolved to 2.6-Å (0.26-nm) resolution (27). The residue at position 332 in *Drosophila* TBP lies within the second basic repeat of the carboxy-terminal domain and maps to an α helix on the convex surface of the saddle. This surface is thought to be involved in protein-protein interactions (7). We have recently determined why this mutation is defective for RNA Pol III transcription. Immunoprecipitation experiments using the two stable lines that express the wild-type and mutant proteins reveal that this mutation disrupts the ability of *Drosophila* TBP to stably interact with the *Drosophila* TFIIIB subunit, TAF_{III} 105 (33a).

Previous studies have suggested that tRNA and U6 RNA promoters, at least in mammalian systems, use distinct TBP-containing complexes (26). The TBP-containing complex designated SNAPc or PTF is specifically required for the transcription of U6 RNA gene promoters (29, 44). Evidence supports that TFIIIB is used by both tRNA and U6 RNA gene promoters, yet it is not clear whether the composition of the TFIIIB complex is different for these two classes of promoters. We do observe a difference in the level of the response of the two promoters to wild-type TBP, yet the amount of wild-type TBP required to produce the maximum response is not significantly different for the two promoters. Overall, we do not observe gross differences in the response of these promoters to the increase in wild-type or mutant e-TBP levels. Our previous studies have shown that TFIIIB is the limiting component for transcription in *Drosophila* S-2 extracts (14). Thus, it is likely that the TBP-dependent stimulation of tRNA and U6 RNA gene promoters reflects that TBP is limiting for the formation of TFIIIB complexes at both of these promoters.

The role of the poorly conserved amino-terminal domain of TBP in the transcription of class III genes is not known. Previous studies have used monoclonal antibodies directed against the amino-terminal region of human TBP and in vitro transcription assays to address the function of this region in RNA Pol III gene transcription. Lescure et al. (23) used several monoclonal antibodies against defined epitopes within the amino-terminal region of TBP. These antibodies were preincubated in cell extracts, and the transcriptional activities of tRNA and U6 RNA gene templates were examined. Only one antibody inhibited transcription from the U6 RNA gene promoter, whereas none of the antibodies inhibited tRNA gene transcription. However, in a similar study, Lobo et al. (26) found that transcription of both TATA-containing and TATA-lacking RNA Pol III promoters was inhibited when extracts were incubated with antibodies against undefined epitopes within the amino-terminal region. These studies suggest that certain regions within the amino-terminal region of TBP may be important for the function of preformed TBP-containing complexes, but they do not address whether the amino-terminal region is important for the formation of TBP-containing complexes used at these promoters. Studies in yeast have further suggested the importance of the amino-terminal domain of TBP in RNA Pol III gene expression. Human TBP was unable to complement the defects of yeast TBP mutants that are defective for RNA Pol III transcription (6). Since we have discovered that TBP is limiting for RNA Pol III promoters in *Drosophila* cells, we have begun to use this system to directly examine the role of the amino-terminal region of TBP in vivo. We transiently expressed a mutant TBP protein which contains only the evolutionarily conserved carboxy-terminal domain and found that it is still capable of stimulating an RNA Pol II promoter; however, deletion of this region prevents enhanced transcription of either the tRNA or U6 RNA promoter (Table 1). These results provide the first direct evidence that the amino-terminal region of TBP is essential in vivo for transcription of both TATA-containing and TATA-lacking RNA Pol III genes. In addition, these results provide further evidence that the TBP-dependent increase in RNA Pol II gene activity is not responsible for mediating the increase in RNA Pol III promoter activity.

Several studies have suggested that an increase in cellular TBP, due to the activation of cellular signaling events, may be responsible for mediating an increase in RNA Pol III gene expression. We have previously shown that the levels of TBP can be significantly increased by the activation of cellular protein kinases. Treatment of *Drosophila* S-2 cells with phorbol ester, a potent protein kinase C activator, induces the expression of endogenous RNA Pol III genes (13). Activation of these genes is mediated by an increase in TFIIIB activity, which is correlated with an increase in the cellular levels of TBP (14). Similarly, the transactivation of RNA Pol III genes by the hepatitis B virus X protein is dependent on the X-mediated activation of cellular protein kinases and results in an increase in both TBP and TFIIIB activity (36). This is not cell type dependent since similar results have been obtained for both mammalian and *Drosophila* cell lines that express the X protein (36). We have further determined that the activation of *ras* also induces RNA Pol III gene expression and elevates the cellular level of TBP (35a). Although other changes in the TFIIIB complex may also occur in response to the activated signaling pathway, our studies demonstrate that the increase in TBP, by itself, is sufficient to induce RNA Pol III gene activity. Furthermore, our results indicate that TBP acts directly at the RNA Pol III promoters. Thus, our studies provide new evidence that TBP plays an important role in regulating RNA Pol III gene activity.

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