An upstream promoter element of the *Acanthamoeba castellanii* TBP gene binds a DNA sequence specific transcription activating protein, TPBF

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ABSTRACT

We have characterized a positive-acting element in the upstream portion of the *Acanthamoeba* TBP gene promoter. The 27 bp element (TPE), located within the promoter between –97 and –70, stimulates transcription in an orientation independent fashion and tolerates modest changes in its distance from the TATA box. The TPE does not, however, function synergistically nor when positioned 3000 bp 5' or 260 base pairs 3' of the transcription start site. The TPE binds a DNA sequence-dependent factor, TPBF, which we have partly purified. TPBF was characterized using *in vitro* transcription, DNase I footprinting, methylation interference and electrophoretic mobility shift assays. TPBF does not have a counterpart in HeLa cells, but nonetheless strongly stimulates transcription of the *Acanthamoeba* TBP gene in mammalian extracts. Our results also suggest that there are additional positively and negatively acting elements within the TBP gene promoter, for which a model is presented.

INTRODUCTION

Eukaryotic gene expression is supported and regulated by a wide variety of proteins whose collective function is to stimulate or repress transcription from a given promoter. In the case of genes transcribed by RNA polymerase II, the protein factors fall into two groups: regulatory factors, such as Sp1 and CTF; and the basal factors which include TFIID, TFIIB and RNA polymerase II (1–4). Promoters for RNA polymerase II structurally reflect this variety, and generally consist of one or more upstream elements, a TATA box and, in some cases, an initiator. Each of these elements serves to bind either a regulatory factor, or in the case of the TATA box, TFIID. Regulatory factors that affect the rate of transcription are generally believed to act through direct protein interactions with one or more of the basal factors (5–7).

TBP (a component of TFIID) is involved in the formation of the preinitiation complex by RNA polymerases I, II and III

(8). In the case of RNA polymerase II promoters, TBP appears to recognize and bind to the TATA box and thereby direct subsequent binding by TFIIB and RNA polymerase II (9–12). While its recently described role in initiation by polymerases I and III (8,13–15) is less clear, TBP is an essential component of each transcription system, and is a possible target for upstream activator proteins (12,16).

Examination of TBP cDNA from a wide variety of organisms (12,17), shows that it contains a highly conserved C-terminal 180 amino acid domain and a non-conserved N-terminal domain of varying length. Extensive analysis of its biochemical properties and of a wide range of point and deletion mutants, shows that the C-terminal domain contains amino acids necessary for DNA interaction, and for interactions with other proteins (18). The crystal structure of TBP predicts a wide range of potential protein-protein contacts between TBP and members of the core factors such as TFIIB or RNA polymerase II, as well as regulatory factors, for example E1a (19), ATF (20) or GAL4 and its commonly used derivatives (16). Similar interactions can be expected for the components of RNA polymerase I and III factors, where TBP is part of multi-subunit complexes (8,13). The regulation of TBP gene expression may thus have important consequences for global regulation of transcription by RNA polymerases I, II and III.

The DNA-binding properties of recombinant and native TBP have been examined in several studies which, collectively, suggest that TBP binds DNA rather promiscuously, and can functionally interact with numerous DNA sequences (21–23). The broad specificity of TBP-DNA interaction is due at least partly to the mechanism of TBP-DNA interaction, which occurs primarily via the DNA minor groove (24,25). TBP binding is nonetheless sensitive to a variety of point mutations to the consensus sequence TATAAA (26,27). In the case of TATA-less promoters, TBP is thought to interact with specific DNA sites by virtue of additional proteins (28). By analogy to bacterial and phage repressors (7), these interactions with other proteins as well as non-TATA box DNA sequences will determine the amount of TBP required in the cell at a given time. It seems possible that

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regulation of TBP expression is important in order to prevent random or unbalanced interactions with DNA or the other ligands with which it can interact.

Little is known about the *in vivo* expression of TBP. Despite several reports describing the chromatographic behaviour of native complexes containing TBP, it is not clear how abundant TBP is, nor how many distinct TBP complexes exist (8). One study indicated that human TBP expression may be autoregulated, since expression of epitope-tagged TBP from a transfected gene driven by a heterologous promoter reduced levels of endogenous TBP (29).

In *Acanthamoeba*, TBP is encoded by a unique gene which produces a single, rare mRNA (30). Analysis of the TBP gene promoter region shows that three regions are important for transcription initiation: an upstream positive acting element between -97 and -35, the TATA box (at -30), and the initiation region (31). Anti-TBP antibodies are inhibitory to transcription from the TBP gene promoter, and initiation can be competed by TATA-containing oligonucleotides, showing that the TATA box probably functions to bind TBP or TFIID (F.L. and E.B. unpublished data). The initiation region serves primarily to position the start site (31). By contrast, the large upstream stimulatory region (-97 to -35) is poorly defined.

In this report, we characterize a positive acting element within the upstream portion of the *Acanthamoeba* TBP gene promoter. We show that a promoter region between -97 and -70 (referred to as the TPE) functions in a manner analogous to upstream activating sequences from yeast or higher eukaryotes. The TPE binds a trans-acting stimulatory factor, TPBF. Surprisingly, partly purified TPBF strongly stimulates transcription of the TBP gene even when assayed in HeLa nuclear extracts, providing a convenient system for monitoring its purification and for eventually determining its site and mechanism of action.

MATERIALS AND METHODS

Oligonucleotides, plasmids and templates

Synthetic oligonucleotides containing all or a portion of the TPE contained both complementary strands of the following sequences.

TP27: 5' AACAACTGAGAAAAAACCAGGATCGG 3'

TP20: 5' AGCTGAGAAAAAACCAGGAT 3'.

The -66 and -42 5' deletions to the TBP promoter (Figure 1A) were constructed by sub-cloning appropriate blunt-ended restriction fragments derived from the TBP gene into the *Sma*I site of pSK(-). Orientations of positive clones were determined by restriction enzyme mapping and by DNA sequencing. All subclones used had the same orientation, with respect to the vector, as the parent plasmid. The promoter deletions to -97 and -35 have been described previously (31). Standard methods (32) were used in each case.

Subclones containing one, two, or three TPBF binding sites in different orientations, were obtained by ligating one, two, or three TP27 oligos into the single *Hind*III site of the -66 or -42 5' deletions (Figures 6 and 7). This procedure resulted in the presence of 26 base pairs of vector DNA between the TPE and the 5' end point of the deletions. The procedure for inserting the shorter, twenty base pair TP 20 oligo was the same. All clones were sequenced in order to determine the orientation of the ligated TPE.

The plasmid used to examine the effect of distance on the function of the TPE (Figure 7) was made by inserting a TP27

oligo into the single *Xba*I site of the -66 deletion. Digestion of the resulting plasmid with *Not*I generates a template in which the TPBF site is 260 bp downstream of +1 site, whereas digestion with *Bam*HI locates the TPBF site about 3 kb upstream of the promoter.

Templates used for *in vitro* transcription were generated by linearizing plasmids with *Bam*HI except in the case noted above. DNA concentrations were determined by spectrophotometry. Agarose gel electrophoresis and ethidium bromide staining, was used as an additional estimate of relative plasmid concentration.

For *in vitro* transcription experiments using an adenoviral major late promoter as an internal control, the template was a *Pvu*II fragment from pGML26 (F.L., unpublished). pGML26 contains adenovirus major late promoter and G-free sequences from -52 to +26 of pML(C₂AT)₁₉ (33), in plasmid pSK(-).

Fractionation of nuclear extract

Crude nuclear extract was prepared from a 15 liter culture of *Acanthamoeba* as reported previously (34). The nuclear extract was passed through a 20 ml phosphocellulose (Whatman) column equilibrated with buffer C (20 mM HEPES [pH7.4], 0.2mM EDTA, 10% glycerol, 0.2 mM PMSF, 1 mM benzamidine, 0.1 mM TPCK, 2 µg leupeptin/ml, 2 µg pepstatin/ml, 1 mM DTT) containing 100 mM KCl. Bound proteins were then eluted from the column with buffer C/600 mM KCl and dialyzed against buffer C containing 100 mM KCl. This PC600 fraction was then passed through a 15 ml DEAE cellulose (Whatman) column equilibrated with buffer C/100 mM KCl, and the bound proteins were eluted with buffer C/300 mM KCl. The DEAE 300 mM eluate is referred to as the PD300 fraction. The PD300 fraction was dialyzed against buffer C/100 mM KCl and loaded onto a mono S HR5/5 column (Pharmacia) equilibrated with buffer C/100 mM KCl. Proteins were eluted from the column with a 50 ml gradient of 100 mM to 1M KCl in buffer C at a flow rate of 1ml/min. 1 ml fractions were collected. TPBF activity was monitored by EMS assay, DNaseI footprinting or by *in vitro* transcription as described below. TPBF eluted from the mono S column between 160 mM and 200 mM KCl.

In vitro transcription and primer extension

Transcription reactions and primer extension analyses were performed essentially as described previously (31,34). Briefly, 100 ng of DNA template was incubated with 5 µl *Acanthamoeba* nuclear extract (25 µg protein) and nucleoside triphosphates in a 50 µl reaction at 30°C for 60 minutes. In the reactions using HeLa nuclear extract, 15 µl of protein was used in a total volume of 25 µl. In some experiments, reactions were supplemented with 1 µl of mono S fractions containing TPBF, as indicated.

For primer extension analysis of TBP gene transcription, a [³²P]-labeled TBP gene specific oligonucleotide was used (31); a pSK(-) oligonucleotide was used for primer extension of adenovirus major late transcripts [pSK(-) reverse primer, Stratagene].

Electrophoretic mobility shift (EMS) assays

In a typical assay, 1 µl of a 1:10 diluted mono S fraction was incubated with 2 µg poly(dIdC) on ice for 15 minutes in a total volume of 14 µl containing 20 mM HEPES (pH7.4), 50 mM KCl, 7.5 mM MgCl₂, 0.2 mM EDTA, 10% glycerol, and 0.5 mM DTT. Approximately 2 ng of probe was then added to the binding reaction and incubated at 30°C for 30 minutes. 5 µl of the reaction was analyzed at 4°C on a 4% polyacrylamide

Figure 1. *In vitro* transcription of TBP promoter deletion mutants. **A.** Diagram of deleted templates used for transcription assays. The boxed regions represent the TBP gene and promoter, thin lines represent vector sequences. The positions of the TATA box and the primer used for primer extension are shown. The approximate activity of each construct, and the lane number are indicated. **B.** Primer extension analysis of *in vitro* transcription products produced from the deleted templates. TBP indicates the TBP gene transcript. AdMLP indicates the adenovirus major late promoter transcript, which served as an internal control.

(acrylamide:bis=40:1) gel containing $0.25 \times$ TBE. The gel was pre-run at 250 V for 60 minutes at 4°C before loading the sample. In the competition assay, the indicated amount of unlabeled DNA oligos were added together with the labeled probe. An 81 bp DNA fragment containing TBP promoter sequence from -44 to -97 was used as the probe for EMS assays.

DNase I footprinting

A 318 bp fragment containing TBP promoter sequences between -167 to +124 was synthesized by PCR, in which one primer was labeled. DNA binding reactions containing about 5 ng of labeled probe and various amounts of mono S fraction were performed as described above for the EMS assays. 1 μl of DNase I (BRL), diluted to a final concentration of 80 $\mu\text{g}/\text{ml}$, was added and digestion was allowed to proceed for 30 to 60 seconds at room temperature. Digestions were stopped by adding 25 μl of

Figure 2. Fractionation and EMS analysis of a TBP promoter binding factor [TPBF]. An *Acanthamoeba* nuclear extract was fractionated through the steps shown in **A**. Proteins were eluted from the various columns at the concentrations of KCL (M) indicated. **B.** EMS analysis of TPBF in fractions eluted from the mono S column.

H_2O and 50 μl of $2 \times$ STOP buffer (0.6M NaOAc [pH5.2], 0.2% SDS, 200 μg yeast tRNA/ml). The DNA was extracted with phenol/chloroform, precipitated with ethanol, and analyzed on a 6% polyacrylamide gel containing 8M urea.

Methylation interference assays

Labeled DNA was treated with dimethyl sulfate (35,36), purified and used for EMS assays with the mono S TPBF fraction. Bands corresponding to free and TPBF-bound DNA were eluted from the gel and cleaved with piperidine. The pattern of methylated bases in each population were analyzed on a denaturing 8% polyacrylamide gel.

RESULTS

Deletion analysis of the TBP gene promoter identifies an upstream transcription activating element

Deletion analysis of the TBP gene promoter previously showed that a region between -97 and -35 had a stimulatory effect on transcription, whereas a deletion into the TATA box completely abolished activity (31), due to interference with TFIID binding. That the TATA binding protein, as well as the TATA box, are necessary for transcription of the TBP gene was also shown by antibody and oligonucleotide inhibition studies (F.L. and E.B., unpublished data). Here, we have constructed mutations in order to more precisely define the position and role of the putative upstream regulatory element. The endpoints of the promoter deletions tested in this study are -97, -66, -44 and -35

Figure 3. DNase I footprinting of TPBF. Increasing amounts of mono S purified TPBF were incubated with a TBP promoter fragment prior to DNase I footprinting, as detailed in Materials and Methods. For both strands, additions were as follows. Lane 1, no protein; lanes 2–4, 0.4 μ g, 1.0 μ g and 2.0 μ g TPBF respectively; lane 5, 2.0 μ g TPBF and 30 ng TP27 oligonucleotide as competitor. The boxes indicate the TPBF-protected regions on the coding and non-coding strands.

relative to the transcription start site. The activities of the deleted templates, as compared to a template with wild type activity (–97 deletion), were assayed by *in vitro* transcription.

The results (Figure 1) show that the template deleted to –66 is about five-fold less active than the –97 deletion, and that the –44 deletion is roughly two-fold less active than either the –66 or –35 deletion mutants. In all cases the activity of the adenovirus major late promoter, which served as an internal control, is unchanged.

These experiments suggest the presence of activating and repressing sequences within the TBP gene promoter. However, since the strongest activating region is between –97 and –66, we have focused on the function of DNA element(s) within this window.

Identification and fractionation of a protein, TPBF, that binds to the TBP gene promoter

Because previous work has shown that stimulatory promoter elements function by interacting with one or more protein factors (see Introduction), we anticipated that *Acanthamoeba* extracts would contain an activity, or activities, which would bind to the TBP gene promoter between positions –97 and –66. Footprinting or band shift experiments using crude nuclear

extracts were inconclusive, due to the presence of an endonuclease and several non-specific DNA binding proteins. We therefore fractionated an *Acanthamoeba* nuclear extract by chromatography on phosphocellulose, DEAE cellulose and mono S columns (Figure 2). Analysis of DNA binding by electrophoretic mobility shift assay, shows that a TBP promoter DNA binding activity elutes from the mono S column at approximately 160 mM KCl. Comparison between the mobility of the complex and that of a TBP-DNA complex suggest that a relatively large protein is responsible for the shift (not shown). We have dubbed this DNA binding fraction as TPBF (TBP Promoter Binding Factor).

Localization of the TPBF binding site

To better define the nucleotide sequence of the binding site for TPBF, DNase I footprinting experiments were done (Figure 3). The most active mono S fraction generates clear protected regions on both DNA strands roughly between –70 and –100, demonstrating that one or more factors interact with a specific region the TBP promoter. This region includes the sequences found to be important for transcription activation as described earlier. We have compared the elution profiles of promoter binding factors from the mono S column, using band shift and footprinting, and have found a good correlation between the two

Figure 4. Methylation interference assays of TPBF DNA binding. **A.** Methylated DNA containing a TPBF binding site was used for methylation interference assays as described in Materials and Methods. DNA corresponding to TPBF-bound and free forms after EMS assays are labeled B and F respectively. Arrowheads indicate the residues at which methylation decreases TPBF binding. **B.** Summary of the interference data. Positions that are sensitive to methylation are indicated by arrowheads. The positions of the oligonucleotides, TP27 and TP20 are also shown.

assays (not shown). This result demonstrates that the complex observed by band shift is the same as that examined by footprinting.

Methylation interference assays, using the mono S TPBF fraction, were also done in order to better define the DNA sequence in close contact with TPBF. Several positions between -94 and -72 were found to be sensitive to modification in the major DNA groove (Figure 4 A and B). In most cases, interference is incomplete, that is the bands are reduced in intensity, rather than being absent. The main importance of these results is that they define a relatively small twenty three base pair promoter region likely to be involved in factor binding. However, because the DNA-binding activity is contained within a fairly crude fraction, rather than being a pure protein, these experiments do not permit an unambiguous assignment of base contacts made by TPBF. We have also performed preliminary carbethoxylation interference experiments, which showed that TPBF binding is sensitive to carbethoxylation at several adenine residues (not shown). All of these residues lie within the twenty three base pair region defined by methylation interference. For present

Figure 5. Competition for TPBF promoter binding. TPBF was simultaneously incubated with a labeled TBP promoter fragment and various amounts of the indicated competitor oligonucleotide prior to EMS analysis. Lane 1, no competitor; Lanes 2-5, TP27 competitor; lanes 6-8, GAL_{UAS} competitor; lanes 9-11, TP20 competitor. Each oligonucleotide series contained 5 ng, 20 ng or 50 ng of competitor.

purposes, we refer to the complete binding site in the context of the promoter as the TPE (TBP Promoter Element). Its sequence is as follows:

which corresponds to nucleotides -94 to -72 of the TBP gene promoter (31).

The results of methylation interference experiments allowed us to design and construct a 27 base pair oligonucleotide that contains a complete binding site. This oligonucleotide includes an additional two base pairs at each end to avoid possible end-effects. In addition, we prepared a twenty base pair oligonucleotide that does not contain the complete TPBF site as defined by interference assays. Specifically, the twenty-mer lacks the G at -94 and the G at -72.

The TPE binds a DNA sequence-specific protein

The twenty seven base pair oligonucleotide (TP27) containing all bases contacted by TPBF was synthesized and tested for its ability to specifically compete for protein-DNA interactions between TPBF and the TBP gene promoter. As shown in Figure 5, the TP27 oligonucleotide completely eliminates binding by TPBF. In contrast, neither a 17 base pair GAL4 binding site, nor a truncated TP20 oligonucleotide that contains only the central twenty base pairs of the putative binding site competed for TPBF binding. However, if the TP20 oligomer is ligated to form multimers prior to competition assays, it serves as a very weak competitor, implying that the core sequence may operate, albeit poorly, if flanking DNA is present (not shown). Although the GAL and TP20 oligonucleotides are considerably shorter than the effective TP27 oligonucleotide, their shorter length is not the reason for their failure to compete, since a 29 bp oligonucleotide derived from the TATA box region of the adenovirus major late promoter also fails to compete (not shown).

The TPE stimulates template activity in an orientation-independent manner

If the TPE indeed directs binding of a positive acting factor, then placing this site upstream of a minimal promoter should stimulate transcription. We therefore cloned the twenty seven base pair

Figure 6. The TPE functions in an orientation-independent manner. **A** A synthetic 27 base pair oligonucleotide was subcloned upstream of minimal TBP gene promoters as indicated in **A**. Double lines indicate TBP promoter sequences, single lines denote vector sequences. The oligonucleotide is indicated by TP27, and its orientation is shown with arrows. TP20 is the shortened, twenty base pair oligonucleotide derived from the TPE. **B**. Transcription analysis of templates containing TP27 or TP20. The promoters shown in **A** were assayed by *in vitro* transcription and primer extension as detailed in Materials and Methods. The templates assayed are shown above each lane.

TP27 oligonucleotide, corresponding to nucleotides -96 to -70, adjacent to TBP gene promoters deleted to either -66 or -42, and assayed the template activity by *in vitro* transcription (Figure 6). These templates contain a 26 base pair insert of vector DNA between the TPE and the 5' end of the deleted TBP promoter. One construct is therefore a linker scanning mutant, that replaces promoter DNA between -70 and -42 with vector DNA.

Figure 7. The TPE does not function synergistically nor over large distances. **A**. Structure and nomenclature of DNA constructs used to assay synergistic and distance effects of the TPE. The open boxes represent the TP 27 oligonucleotide cloned at the indicated positions relative to the TATA box. **B**. Templates T1, T2 and T3 were assayed by *in vitro* transcription and primer extension as described in Materials and Methods. **C**. Activities of templates T-3k and T+260, as compared to a wild type (WT) TBP promoter, or a TBP promoter -66 deletion (-66).

Figure 8. TPBF stimulates transcription in HeLa extracts. An *Acanthamoeba* TBP promoter containing an intact TPE (Lanes 1 and 2), or a deletion lacking the TPE (Lanes 3 and 4), were assayed in a HeLa cell nuclear extract. Mono S-purified TPBF was added to the reactions shown in lanes 2 and 4. Reactions were analyzed by primer extension.

In every construct, the oligonucleotide restored template activity to nearly wild type levels, demonstrating that the region identified by footprinting is functional in stimulating transcription. Moreover, the oligonucleotide was equally active when placed in opposing orientations (Figure 6), and is therefore orientation-independent. The 5' endpoints of the TP27 oligonucleotide in the -66a and -42a constructs are -119 and -95 respectively, as compared to -96 in the wild type template. The TPE can therefore tolerate small changes in its distance from the TATA box.

One other inference can be drawn from these data. Since transcription levels in the TPE-containing constructs never reach those of wild type templates, these experiments further suggest that there is a second stimulatory sequence between -70 and -44.

Interestingly, the TP20 oligonucleotide is also capable of enhancing transcription (Figure 6B, lane 8). Apparently, TP20 is capable of binding TPBF when placed in the appropriate sequence context. We note that at least one methylation-sensitive base (G -94) of the full length TPE is restored by cloning the TP20 oligonucleotide into the *Hind*III site of pSK(-).

The TPE is distance dependant

Because the ability of the TPE to stimulate transcription was found to be relatively insensitive to its location relative to the TATA box, at least over a 22 bp range, we tested whether it would function when located further away, by placing it ~3000 bp 5' or 260 bp 3' from the TATA box in the -66 dl TBP promoter, which otherwise lacks the TPE (Figure 7A). The TPE in these configurations is inactive, since there is no stimulation of transcription in either construct as compared to the -66 deletion alone (Figure 7B).

Figure 9. TPBF co-purifies with transcription activating activity, assayed in HeLa extracts. Fractions from the mono S elution shown in Figure 2 were assayed in a HeLa cell nuclear extract for their ability to stimulate transcription from a wild type TBP promoter containing the TPE. Conditions are the same as those described in the legend to Figure 8.

Figure 10. Model of the *Acanthamoeba* TBP gene promoter. The positions of the TPE, TATA box and the start site region shown to be important for promoter function are indicated, as are the proteins that interact with them. The areas labeled +ve? or -ve? are putative, additional positive and negative elements identified in this study, that have not been well characterized.

The TPE does not behave synergistically

Many DNA binding sites and proteins show synergistic activity when multiple copies are placed adjacent to a core promoter (37). We next tested if two or three copies of the TPBF binding site placed adjacent to a deleted TBP promoter, would stimulate transcription more efficiently than one copy. However, there is no additional stimulation by two or three copies of the TPE as compared to a single copy, when assayed in nuclear extracts (Figure 7C).

Because it was possible that TPBF is limiting in nuclear extracts relative to the number of TPE elements on the test templates, we supplemented assays with mono S purified and concentrated TPBF, but did not observe any further stimulation. Under these conditions, all of the TPE sites are occupied by TPBF as judged by mobility shift assays (not shown). We conclude that under the conditions used here the TPE does not behave synergistically.

TPBF stimulates transcription in HeLa cell extracts

Several components of the transcription apparatus are functionally conserved among eukaryotes, notably TBP, RNA polymerase, or some members of the leucine zipper class of activators (12,38,39). We therefore tested whether TPBF would function in a heterologous transcription extract from HeLa cells. We also asked whether HeLa extracts contained a similar activity. In addition, we wished to demonstrate unambiguously that TPBF could indeed activate transcription.

Wild type (−97 dl) and minimal (−66 dl) TBP promoters were assayed in HeLa extracts in the presence or absence of mono S-purified TPBF (Figure 8). Both versions of the TBP promoter are virtually inactive in HeLa extracts (Figure 8, lanes 1 and 3), but the adenovirus major late promoter, which served as an internal control, produces a modest signal. Addition of 1 μ L of TPBF (mono S fraction) produced a large increase in the amount of transcription from the TPE containing promoter fragment (Figure 8, lane 2). In contrast, the minimal promoter remained essentially inactive after addition of TPBF (Figure 8, lane 4). Transcription from the Ad2MLP internal control was unaffected by the addition of TPBF.

TPBF cofractionates with transcription stimulatory activity

Because HeLa extracts apparently lack a functional counterpart to *Acanthamoeba* TPBF, they provide a convenient system to assay chromatographic fractions for TPBF activity. In the experiment shown in Figure 9, fractions from the mono S column were added to HeLa extract and assayed for their ability to stimulate transcription from a TPE-containing promoter. The activity profile obtained parallels that measured by gel shift, and further supports the correlation between DNA binding and transcription activation.

DISCUSSION**An upstream region of the *Acanthamoeba* TBP promoter binds a trans-acting factor, TPBF**

The deletion analyses in this and a previous study (31) demonstrate that a region between −97 and −66 of the TBP promoter has a pronounced stimulatory effect on transcription. In addition, the promoter may also contain other elements whose actions are more subtle (Figure 10). We have partially purified a factor, referred to as TPBF that specifically binds promoter DNA in the stimulatory region identified by deletion analysis. The DNA-binding properties of TPBF allowed us to define the necessary binding site (the TPE) more precisely. The TPE is contained within a twenty seven base pair region, located between −96 and −70. Within this sequence, G residues within a twenty three base pair portion form apparent contacts with TPBF. The sequence of the TPE is unusual for a protein binding site for two reasons. First, it lacks obvious symmetry, and second, it contains a run of six consecutive adenine residues in the middle of the site. This latter feature is noteworthy since such runs may result in DNA bending (40). Not all bases within this site may be required for sequence-specific binding, since a shortened version of the TPE also functions within a plasmid context, suggesting that non-specific protein DNA contacts may contribute to DNA binding by TPBF. However, we note that at least one base contact is restored when the TP20 is cloned into the *Hind*III site of a deleted promoter, suggesting that the TPE may be able to tolerate

base changes at one or two positions, which would only disrupt a fraction of the contacts made by TPBF with DNA. This interpretation is consistent with the methylation interference experiments, which show that methylation of a given G residue reduces, but does not abolish binding by TPBF. When compared to sites for other DNA-binding proteins (41), the TPE is an unusually large element. However, we have preliminary evidence that a single protein is, in fact, involved in TPE binding (F.L. and E.B. unpublished data). Further chemical interference assays should precisely identify all contacts made by purified TPBF.

When the deduced oligonucleotide containing the TPE was cloned upstream of deleted TBP promoters, it was able to stimulate transcription in an orientation-independent fashion, one of the properties of a eukaryotic enhancer. Furthermore, the fact that stimulation is equal in either orientation suggests that the protein is symmetric or perhaps binds as a dimer. However, as noted above, there is not any obvious symmetry to the binding site nor in the base contacts.

When the TP27 oligonucleotide is subcloned upstream of minimal templates, the 5' end points for the activating sequence are −119 and −95, as compared to −97 in the wild type template. Because each of these constructs are active, the function of this sequence is insensitive to modest changes in distance between it and the TATA box. However, the TPE is not able to activate *in vitro* transcription when placed 3000 bp 5' from the promoter, or 260 bp on the 3' side of the promoter. Moreover, multiple copies of the TPE do not appear to behave synergistically. Thus the similarity to eukaryotic enhancer elements is imperfect, at least under the *in vitro* conditions used here.

Whereas transcription stimulation by distant promoter elements has been generally far more pronounced *in vivo* (42), we were surprised at the lack of synergistic behaviour by the TPE, since other simple promoter elements such as the UAS_{GAL} function synergistically even *in vitro* (37). Synergism by promoter-bound factors has led to proposals about the nature of targets and mechanisms through which stimulatory factors act (43). The TPE appears to function maximally as a single copy, perhaps suggesting that TPBF interacts with a single target.

The observation that TPBF can function in a HeLa extract to stimulate transcription several-fold from an otherwise inefficient promoter shows that TPBF can interact efficiently with the HeLa basic transcription machinery. Because the TBP gene is poorly transcribed in HeLa extracts in comparison to the AdMLP, stimulation must reflect the ability of the TBP core promoter to interact with one of the general factors. Most importantly, this result provides evidence that DNA binding is correlated with transcription stimulation, but by no means proves that DNA binding and activation are conferred by a single protein.

TPBF has many of the properties expected of promoter binding proteins from yeast or higher eukaryotes, for example its ability to function in both orientations. We noticed that a region within the TPE corresponds to the binding site for C/EBP in the human *aP2* gene (44). While this does not prove they are related, it will be of interest to see if the *Acanthamoeba* TBP gene is regulated by a protein that has a developmentally important human counterpart. It is possible that TPBF contains more than one protein, either as a complex between heterologous subunits, or as a mixture of different factors that co-purify. We are currently using affinity chromatography to purify and clone TPBF, which should provide results that shed light on these questions.

Control of TBP gene expression

We have found that TBP mRNA is rare, in comparison to other messages such as actin mRNA (30). For this reason it was surprising that the TBP gene promoter is efficiently utilized by *Acanthamoeba* extracts *in vitro*, as compared to the adenovirus major late promoter. This latter result indicates that negative transcriptional controls, not apparent *in vitro*, operate within the cell. Moreover, we have not necessarily identified every promoter element. Indeed, the difference in activity between the -66, -42 and -35 deletions strongly suggests the existence of additional positive and negatively acting elements, as indicated in Figure 10. It is noteworthy, in regard to this point, that we have observed a promoter binding activity in cruder fractions (PC 0.6 M eluate, Figure 2) that produces a footprint extending from about -70 to -50, and which is distinct from TPBF (F.L. and E.B., unpublished observations). This factor can be resolved from TPBF by DEAE cellulose, and may prove to be a second stimulatory component of the TBP gene.

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