In vivo interactions of the Acanthamoeba TBP gene promoter

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Received as resubmission January 22, 2004; Accepted January 29, 2004

ABSTRACT

Transcription of the TATA box binding protein (TBP) gene in Acanthamoeba castellanii is regulated by TATA box binding protein promoter binding factor (TPBF), which binds to an upstream TBP promoter element to stimulate transcription, and to a TATA proximal element, where it represses transcription. In order to extend these observations to the in vivo chromatin context, the TBP gene was examined by in situ footprinting and chromatin immunoprecipitation (ChIP). Acanthamoeba DNA is nucleosomal with a repeat of ~160 bp, and an intranucleosomal DNA periodicity of 10.5 bp. The TBP gene comprises a 220 bp micrococcal nuclease hypersensitive site corresponding to the promoter regulatory elements previously identified, flanked by protected regions of a size consistent with the presence of nucleosomes. ChIP data indicated that TPBF is associated with the TBP, TPBF and MIL gene promoters, but not to the CSP21, MIIHC, 5SrRNA or 39SrRNA promoters, or to the MIL gene C-terminal region. Binding by TPBF to the TPBF and MIL gene promoters was confirmed by in vitro assays. These results validate the in vitro model for TBP gene regulation and further suggest that TPBF may be autoregulated and may participate in the regulation of the MIL gene.

INTRODUCTION

Eukaryotic gene expression by RNA polymerase II (pol II) requires the recruitment of general transcription factors (TFs) such as TFIID, TFIIB, TFIIF, TFIIE, TFIIF and TFIIH (1–4), and sequence-specific regulatory factors, which act either as activators or as repressors (5–7). The architecture of pol II-transcribed gene promoters reflects this modularity in that a typical promoter contains a core element and upstream regulatory elements (8–11). Sequence-specific regulatory proteins bound to the upstream regulatory elements are thought to communicate with the general TFs through protein–protein interactions and control the activities of the basal transcription machinery.

TATA box binding protein (TBP) is a general TF that is an essential component of complexes TFIID (12,13), TFIIB (14) and TIF/SL1 (15), which are required for pol II, III and I transcription, respectively (16,17). In TATA-less promoters, TBP may have a structural role (18–20), whereas in some pol II promoters and pol III promoters that contain a TATA box, TBP binds the TATA box and nucleates formation of the transcription initiation complex (2,21,22).

In Acanthamoeba castellanii, TBP gene expression is controlled by the activator TBP Promoter Binding Factor (TPBF) (23–27). TPBF is recruited to the TBP promoter element (TPE) located between −94 and −72 of the TBP promoter (23), and activates TBP gene transcription. TPBF is thought to enhance the recruitment of the general TFs such as TFIID to the core promoter element (26), and may dictate the orientation of TBP on the TATA box (27,28), facilitating productive assembly of the transcription machinery. TPBF also binds with a 10-fold lower affinity to a negative element, the nTPE, located immediately downstream of the TATA box. At relatively high TPBF concentration, TPBF binds to the nTPE and represses TBP gene transcription (26). While the interaction of TPBF and the TBP gene promoter has been well studied in vitro, little is known about their interactions in vivo.

Eukaryotic DNA is packaged as chromatin, the central component of which is the nucleosome, a complex between a histone octamer and DNA (29–31). The topological complexity of DNA within the nucleosome and the presence of histones have a profound impact on transcription (32). For example, both the basal and regulatory factors may compete with histones for access to the DNA template (33,34). Consequently, nuclease hypersensitive sites are often found between nucleosome arrays at the 5’ end of genes, and generally reflect open segments that facilitate or result from the recruitment of TFs to the promoters (29,35,36).

Changes in chromatin organization and transcription are often correlated and involve both chemical modification of histones and chromatin restructuring. For example, acetylation of histones is often found within actively transcribed chromatin regions, while hypoacetylation is correlated with transcriptional silencing (37,38). The discoveries that TAFp250 (39) and some transcription coactivators such as GCN5 (40,41) have histone acetyltransferase activity indicated that modification of histones is a key element in transcription regulation (42,43). Furthermore, in yeast, nucleosome remodeling by the SWI/SNF complex is closely linked to transcription events (44), so that DNA sequences

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Nucleic Acids Research, Vol. 32 No. 4 © Oxford University Press 2004; all rights reserved
originally occupied by histones are transiently exposed for transcription initiation and elongation.

In this paper, we have analyzed the structure of the TBP gene by chromatin immunoprecipitation (ChIP) and by in situ footprinting with micrococcal nuclease (MNase). We found that *Acanthamoeba* DNA is nucleosomal, but that the TBP gene promoter is contained within a nucleosome-free region surrounded by positioned nucleosomes. ChIP assays demonstrate that TPBF is bound to the TBP gene promoter in support of the model of TBP gene regulation suggested by *in vitro* assays (26). We also observed the presence of TPBF at other promoters suggesting that TPBF may also regulate expression of other genes, and in particular that TPBF may be autoregulated.

MATERIALS AND METHODS

Oligonucleotide primers

The oligonucleotides used in this study were chemically synthesized by SIGMA-Genosys and the sequences are listed in Table 1.

Isolation of a clone containing the upstream region of the TBP gene

An *A. castellani* genomic DNA library (45) was screened using a TBP gene specific probe generated by PCR with primers TBP-149U and TBP+33B, which is complementary to the sequence starting from -149 or +33 of the TBP gene, respectively. A 1.3 kb Pst I fragment from one positive clone was identified by Southern hybridization (46), and subcloned into pSK(-) and sequenced. This fragment covers ±1033 to ±1252 of the TBP gene, and is reported in the second version of GenBank accession number M93340. A BLAST search showed that this upstream sequence is not homologous to any other gene in the NCBI database.

In vivo MNase footprinting

*Acanthamoeba* cultures were grown to 5–10 × 10⁶ cells/ml and nuclei were isolated from 1.2 × 10¹⁰ cells as described previously (47). Cells were collected by centrifugation and washed once in 40 ml of lysis buffer I [10 mM HEPES (pH 7.5), 15 mM KCl, 2 mM MgCl₂, 0.5 mM EDTA and 1 mM dithiorthreitol], resuspended in 20 ml of lysis buffer II (lysis buffer I containing 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM benzamidine, 0.1 mM *N*-tosyl-L-phenylalanine chloromethyl ketone, 2 µg pepstatin A/ml, 2 µg leupeptin/ml), and homogenized until 95% of cells were broken. The nuclear pellet was collected by centrifugation at 1600 g, 4°C for 20 min, and washed twice with 10 ml of nuclei storage buffer [40% glycerol, 40 mM HEPES (pH 7.9), 280 mM KCl, 20 mM MgCl₂ and 2 mM dithiorthreitol], once with 10 ml of MNase digestion buffer [10 mM HEPES (pH 7.5), 2.5 mM CaCl₂, 2.5 mM MgCl₂] and resuspended in MNase digestion buffer. The nuclei were then divided into six equal aliquots and digested in 1 ml MNase digestion buffer with 0, 20, 50, 100, 200 or 500 U of MNase at room temperature for 10 min (48). The reaction was stopped by adding 1.2 M ammonium acetate (pH 8.6), 4 mM EGTA, 4 mM EDTA, 0.5% SDS, 0.2 mg proteinase K/ml, and incubated at 50°C for 90 min. Unbuffered potassium acetate was added to the mixture to a concentration of 200 mM. The mixture was chilled on ice for 10 min, then centrifuged at 14000 g for 10 min. The supernatant was phenol/chloroform extracted and ethanol precipitated. The pellet was incubated in 200 µl of 20 µg ribonuclease A/ml at 37°C for 1 h, phenol/chloroform extracted, ethanol precipitated and resuspended in 200 µl of TE (10 mM Tris–HCl pH 8.0, 1 mM EDTA). For naked DNA controls, 20 µl of undigested DNA sample was digested with 1, 2, 4 or 10 U MNase/ml in 200 µl of MNase digestion buffer at room temperature for 10 min. The reaction was stopped and the DNA samples were prepared as described above, and resuspended in 20 µl of TE. Then 20 µl of either digested nuclei or naked DNA samples (~30 µg DNA per sample) were digested with either Pst I or XhoI, phenol/chloroform extracted, ethanol precipitated and fractionated on 1.2% agarose gels (Fig. 2, left panels). Southern hybridization was performed with PCR-generated, α-³²P-labeled probes. Primers TBP-pstU and TBP-pstB were used to amplify probe 1, TBP-u+37 and TBP-b+225 for probe 2, TBP+542 and TBP-b+742 for probe 3, and TBPu+542 and TBPb+742 for probe 4 (Fig. 2C).

Chromatin immunoprecipitation (ChIP)

ChIP was performed as described previously (49), with the following modifications: 5 × 10⁶ *Acanthamoeba* cells were collected and resuspended in 25 ml growth medium, fixed in 1% formaldehyde at room temperature for 15 min, and the reaction stopped by adding glycine to a final concentration of 125 mM. Cells were washed twice with 10 ml of lysis buffer I, once with 3 ml of sonication buffer [50 mM HEPES–KOH (pH 7.5), 150 mM NaCl, 1 mM EDTA, 0.1% sodium deoxycholate, 1% Triton X-100], resuspended in 3 ml of

<table>
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<th>Table 1. Oligonucleotide sequences</th>
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<tr>
<td>TBP-149U 5'-AGGCTCTATCTTCTGTGAAGCTG-3'</td>
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<tr>
<td>TBP+33B 5'-AAATTCGTAGCCGAAAGCAGAAG-3'</td>
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<tr>
<td>TBP-pstU 5'-GCACCTGCCCAGCAGCACAAG-3'</td>
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sonication buffer and incubated for 10 min on ice. The chromatin pellet was collected by centrifugation at 14,000 g for 5 min at 4°C, resuspended in sonication buffer to a final volume of 1.5 ml, and sonicated eight times for 30 s each. The lysate was then clarified by centrifugation at 14,000 g for 15 min, and stored at −80°C. To immunoprecipitate TPBF-associated chromatin, 100 μl of anti-TPBF antiserum (25) or pre-bleed rabbit serum was incubated with 20 μl of Affi-Gel protein A beads (Bio-Rad) at 37°C for 2 h. The beads were then triple-washed with 1 ml of 150 mM NaCl, 20 mM Tris–HCl (pH 8.0), 0.1% SDS, 1% Triton X-100, 2 mM EDTA and 2 mM phenylmethylsulfonyl fluoride at room temperature for 5 min each. Then 200 μl of cell lysate was adjusted to 275 mM NaCl and incubated with beads overnight at 4°C. The beads were washed twice with 1 ml of sonication buffer, twice with 1 ml of sonication buffer with 500 mM NaCl, once with 1 ml of 250 mM LiCl, 10 mM Tris–HCl (pH 8.0), 1 mM EDTA, 0.5% NP-40 and 0.5% sodium deoxycholate, and once with 1 ml of TE. The bound fraction was recovered by incubating beads in 200 μl of TE with 0.5% SDS, 250 μg proteinase K/ml at 50°C for 1 h, and then at 65°C for 5 h to reverse the crosslinks. After adding 5 μg carrier tRNA, the supernatant was phenol/chloroform extracted, ethanol precipitated and dissolved in 100 μl of TE. To prepare whole cell extract DNA, 200 μl of cell lysate was incubated with 1.2 M ammonium acetate (pH 8.6), 4 mM EGTA, 0.5% SDS, 0.2 mg proteinase K/ml at 50°C for 1 h and 65°C for 5 h. The mixture was centrifuged at 13,000 g for 10 min, and the supernatant was phenol/chloroform extracted twice, ethanol precipitated, resuspended in 100 μl of 50 μg ribonuclease A/ml at 37°C for 30 min, phenol/chloroform extracted, ethanol precipitated, and resuspended in 100 μl of TE. Then 1 μl of immunoprecipitated DNA or 1:75-diluted whole cell extract DNA was used as template in a 26-cycle PCR (or 30 cycles for CSP21, myosin II and TPBF promoters), and fractionated on a 1.5% agarose gel. The promoters of TBP, TPBF, CSP21 (50), 5S rRNA (51), 26S rRNA (52), myosin heavy chain gene MIL (53), myosin II (54) and the C-terminus of MIL were examined using primers specified in Figure 3A.

**Electrophoretic mobility shift assay**

The 5’ region of the MIL gene was obtained by PCR using the anti-TPBF immunoprecipitated DNA as the template and oligonucleotides MILu1 and MILb3 as primers. The HindIII and EcoRI digested PCR product was cloned into the pSK(−) vector and sequenced. Probe myoTPE was obtained by PCR using the cloned 5’ region of the MIL gene as the template and oligonucleotides MILu1 and MILb4 as primers. The PCR product was digested with HindIII to remove the flanking sequence. Assays were performed with purified recombinant TPBF, as previously described (25,27).

**In vitro DNase I footprinting**

A probe was generated by PCR with primer TPBF-UT539 and end-labeled primer TPBF-284B. Various amounts of recombinant TPBF were incubated with 50 ng of probe in conditions specified in the above electrophoretic mobility shift assay, and were subjected to digestion by 1 U of DNase I for exactly 1 min at room temperature. Reactions were stopped and analyzed as previously described (23).

In order to determine the helical pitch within the nucleosome, the DNase I digestion patterns of DNA or DNA within nuclei were compared. DNase I cuts preferentially between nucleosomes but also introduces single-stranded cuts to the exposed minor groove of DNA wrapped around the nucleosome (48). A DNA sample from DNase I digested nuclei was end-labeled with [γ-32P]ATP and fractionated on a polyacrylamide gel, resulting in a 10.0 ± 0.5 bp ladder (Fig. 1B). As expected, purified DNA digested with DNase I results in a smear. These data indicate that Acanthamoeba DNA is packed as nucleosomes with an average repeat length of ~160 bp. The DNA within a nucleosome has a periodicity of 10.0 ± 0.5 bp per turn. While this arrangement of DNA within the nucleosome was expected, these experiments had not been previously reported for Acanthamoeba, and were a necessary prelude to the experiments described below.

**In situ structure of the TBP gene promoter**

In order to determine the overall accessibility of the TBP gene promoter in nuclei, and to determine whether nucleosomes are present in the vicinity of the TBP gene, we mapped MNase cleavage sites by indirect end-labeling (48). MNase digested genomic DNA samples were digested with PstI and detected using probe 1 or probe 3. Samples digested with XhoI were hybridized with probe 2 or probe 4. Each probe is complementary to sequences that start from the restriction site and extend ~200 bp toward the region being examined (Fig. 2C).

In all cases, the nuclear digests produce a pattern of cutting which is distinct from that obtained with naked DNA (Fig. 2). In particular, a 220 bp hypersensitive region and protection which is distinct from that obtained with naked DNA is nucleosomal.

**RESULTS**

Acanthamoeba DNA is nucleosomal

As a prelude to examining the in vivo structure of the TBP gene, we examined the previously uncharacterized organization of Acanthamoeba genomic DNA within nuclei. Samples derived from MNase digested nuclei or naked DNA were fractionated on a 1% agarose gel. DNA isolated from the digested nuclei produced ladders with an ~160 bp repeat, reflecting the size of a nucleosome and linker sequences. In contrast, a smooth smear was produced by the digested naked DNA samples (Fig. 1A).

In order to determine the helical pitch within the nucleosome, the MNase I digestion patterns of DNA or DNA within nuclei were compared. DNase I cuts preferentially between nucleosomes but also introduces single-stranded cuts to the exposed minor groove of DNA wrapped around the nucleosome (48). A DNA sample from DNase I digested nuclei was end-labeled with [γ-32P]ATP and fractionated on a polyacrylamide gel, resulting in a 10.0 ± 0.5 bp ladder (Fig. 1B). As expected, purified DNA digested with DNase I results in a smear. These data indicate that Acanthamoeba DNA is packaged as nucleosomes with an average repeat length of ~160 bp. The DNA within a nucleosome has a periodicity of 10.0 ± 0.5 bp per turn. While this arrangement of DNA within the nucleosome was expected, these experiments had not been previously reported for Acanthamoeba, and were a necessary prelude to the experiments described below.

**In vitro transcription and primer extension**

Constructs containing various TPBF promoter regions were generated by PCR and subcloned into pSK(−) and sequenced. Transcription and primer extension reactions were performed as previously described (27).
inferred by bands that are less sensitive to nuclease digestion, as illustrated by the dots in the center panels. Probe 3 cross-reacted with other sequences, as indicated by the non-specific bands in the undigested sample (Fig. 2A, right panel, fifth lane from the left), which partly masked the authentic signals. In addition to the upstream regions of protection, probe 4 revealed that a 160 bp region (Fig. 2B, right panel, indicated as ellipse V) in the coding region is also protected, suggesting the presence of another potential nucleosome. The positions of these putative nucleosomes are summarized in Figure 2C. Samples visualized by probes 1 and 2 (center panels) also reveal a nuclease sensitive region between nucleosomes II and III. This may be the result of DNA structural transition between two nucleosomes, or the effect of protein binding within this region. Higher resolution footprinting using DNase I was rather inconclusive, but tended to support the extended non-nucleosomal conformation of the promoter region of the TBP gene (not shown).

TPBF is associated with the TBP promoter in vivo

The footprinting assays described above suggest the presence of DNA-bound proteins at the TBP promoter as judged by hypersensitivity, but cannot identify which factor is bound. Therefore, ChIP assays were used to examine whether TPBF is associated with the TBP gene promoter in vivo. Whole cell extracts were prepared from either non-crosslinked or cross-linked cell samples and immunoprecipitated with anti-TPBF antiserum. Using TBP gene specific primers TBP-149U and TBP+33B (Fig. 3A), a positive PCR signal was detected in the immunoprecipitated sample, while in non-crosslinked or pre-bleed serum samples, only background signals were observed (Fig. 3B). We also tested the Acanthamoeba CSP21, 5S rRNA, 26S rRNA and myosin II heavy chain gene promoters as negative controls. The cyst-specific protein CSP21 is expressed in early stages of encystment (50), 5S rRNA transcription is controlled by pol III, and 26S rRNA is transcribed by Pol I. None of these genes contains a discernable TPE, and therefore are not expected to recruit TPBF and none of them produced a ChIP signal. This critical result represents a strong validation of the method, and we infer that in vivo TPBF is bound to the TBP promoter, but not to the other promoters, which lack a TPE.

TPBF is associated with its own promoter

Examination of the TPBF gene promoter identified several TPE-like elements upstream of the transcription start site (W. Huang and E. Bateman, unpublished observation, Fig. 4A), and electrophoretic mobility shift assays suggested that TPBF could bind to the upstream region of its own promoter. The ChIP assays shown in Figure 3B demonstrate clearly that TPBF is indeed bound to this upstream region in intact cells.
In order to confirm directly that the TPE-like elements can bind TPBF, DNase I footprinting experiments were performed. These experiments demonstrate that TPBF stably binds to several specific regions of the TPBF promoter (Fig. 4B), but not the rest of the upstream segment or the 5' end of the gene (data not shown). These protected regions resemble the TPE found in the TBP gene promoter. We infer that both in vivo and in vitro TPBF can bind specifically to its own promoter.

In order to determine whether TPBF could activate or repress transcription from its own promoter, the promoter constructs shown in Figure 4C were used for in vitro transcription. In no case does removal of the upstream TPE-containing sequences have an effect on transcription. However, removal of the sequences between +66 and +46 results in a large increase in transcription efficiency (compare Fig. 4C, lanes 3 and 6), suggesting the presence of a repressor element unrelated to TPBF binding.

These results suggest that, under the present assay conditions, binding of TPBF to its own promoter does not have an effect on transcription. In addition, removal of TPBF from nuclear extracts by immunopurification did not alter the efficiency of transcription from the TPBF promoter (not shown).

Surprisingly, in the ChIP assays we additionally detected a positive PCR signal using the MIL gene promoter specific primers, but not with primers matching the 3' end sequences of the MIL gene or the myosin II gene promoter (Fig. 3B). The size of the MIL gene is ~5 kb, much larger than the average 500 bp fragment resulting from sonication, suggesting that there is a TPBF binding site in the proximity of the 5' end of the MIL gene. Two partially overlapping TPE-like sequences were subsequently identified between ±265 and ±241 and between ±246 and ±226 of the MIL gene. These sequences are similar to the TPE in the TBP promoter (Fig. 5A).

In order to confirm that these sequences could bind TPBF, we used an electrophoretic mobility shift assay in which a double-stranded myoTPE oligonucleotide, matching the sequence from ±281 to ±226 of the MIL gene, was co-incubated with various amounts of recombinant TPBF (Fig. 5B). Within the range 50–200 ng, TPBF bound to myoTPE at least equally as well as to the TPE. With an increase in protein amount, a larger complex formed, suggesting the binding of two TPBF tetramers. These results not only further validate the ChIP assays, but also suggest that TPBF may participate in MIL gene expression.
DISCUSSION

The present study was initiated in order to validate models of the TBP gene promoter based upon in vitro transcription and footprinting assays, and to examine the possible involvement of chromatin structure in TBP gene expression. In addition, it had been hypothesized (55) that the TPBF gene promoter is autoregulatory and could participate in a reciprocal mode of gene regulation with the TBP gene. Here we show directly that TPBF is associated with both the TBP gene and the TPBF gene promoters in vivo, and can bind in vitro, but were unable to demonstrate an effect of TPBF on transcription from its own gene. In addition, the results demonstrate that the core TBP promoter is largely nucleosome free, but is flanked by nucleosomes both upstream and downstream of the promoter.

The nucleosomes found in the upstream region of the TBP gene promoter appear to be stably positioned since randomly placed nucleosomes would result in a smeared protection pattern. Nucleosomes positioned with respect to DNA sequence have been previously described, for example, in the MMTV GRE (56,57) or the heat shock protein 26 gene promoters (58). Nucleosome positioning can arise as a result of several non-exclusive mechanisms. Nucleosomes can be excluded from certain sequences, for example those which contain runs of adenine (59), such as that which occurs in the TPE of the TBP gene. Nucleosomes may additionally have sequence preferences (60,61) based on in vitro nucleosome assembly assays, although it is not clear how strong such sequence preferences can be (62). In vivo analysis of the human HPRT gene suggested that nucleosomes are randomly positioned in non-induced promoters indicating that sequence preference alone is insufficient to determine the position of nucleosomes in vivo (63). DNA binding proteins that limit the translational position of nucleosomes have been described (64,65). In the present case, it is possible that TPBF could function as a boundary protein in addition to its direct role as a transcription activator. This point is particularly important, since in vitro TPBF has no apparent effect on transcription of its own gene, even though it clearly binds the promoter both in vivo and in vitro. The in vitro assays almost certainly do not recapitulate the situation found in a chromatin complex, and it is feasible that TPBF plays a role in maintaining the TPBF gene in an open configuration within which other regulatory mechanisms can operate. In contrast, the role of TPBF as an activator and repressor at the TBP gene promoter has been well documented (24,26,27).

The Acanthamoeba TBP gene can be regarded as a housekeeping gene, in that it is constitutively active in growing cells (66). It is therefore not necessary to suggest that the promoter hypersensitive region arises as a result of
inducible chromatin remodeling, for example as occurs in MMTV GRE, human HPRT, and yeast GAL and PHO genes (57,63,67). A better candidate for such remodeling is the *Acanthamoeba* CSP21 gene, which is completely repressed in growing cells and strongly induced as a consequence of nutrient depletion (50).

The results of *in situ* nuclease digestion assays additionally suggested the presence of a positioned nucleosome within the coding region of the TBP gene. While it is possible that this nucleosome is present in a subset of inactive loci, it may be necessary for pol II to bypass this potential roadblock. While there has been some dispute regarding the question of whether any RNA polymerase can elongate through a stable nucleosome (68), mechanisms by which this could be accomplished without nucleosome displacement have been proposed (58,69,70). *In vitro* chromatin assembly and transcription would provide an approach to address this issue in the context of the TBP gene, which is transcribed at relatively low levels *in situ*. Alternatively, because this last protected region is within the coding region, there is a possibility that it could be a paused polymerase molecule, as reported for the *Drosophila* heat shock genes (71).

ChIP assays described here firmly support the results of *in vitro* assays which demonstrated that the TBP gene is both positively and negatively regulated by TPBF (24,26,27). In addition, two other gene promoters were found to bind TPBF *in vivo* — the MIL gene and the TPBF gene. In both cases, TPE-like elements were identified in the promoters and mobility shift assays and DNase I footprinting demonstrated directly that these elements can avidly bind TPBF. The use of several negative control promoters further supports these results (Fig. 3). These observations provide solid evidence that TPBF is recruited to these two promoters in intact cells and that TPBF may regulate its own expression as well as that of the TBP gene.

A recently released set of data containing ~5 600 000 bases of *Acanthamoeba* genomic sequences (TIGR, http://tigrblast.tigr.org/er-blast/index.cgi?project=eha1), allowed us to con-

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**Figure 5.** Electrophoretic mobility shift assay of myoTPE and TPE. (A) Sequence comparison of the myoTPE from the MIL gene and the TPE from the TBP gene. MyoTPE contains two TPEs which partially overlap each other. (B) Approximately 5 ng of γ-32P-end-labeled double-stranded DNA probes were incubated with the indicated amounts of TPBF. Solid arrows point to the complex formed by the TPBF tetramer and the open arrow represents the binding of two TPBF tetramers.
duct a preliminary search for TPE-containing promoter regions in order to give an indication of the prevalence of the TPE in the genome. We identified just nine genes that contain one or more TPEs upstream of the probable transcription start site. These are: adenylate kinase, chromosome condensation protein, an ankyrin repeat protein, a signal recognition particle subunit, a histone deactylase, lumazine synthase, a low temperature/salt response protein, a GTP cyclohydrolase related protein and translation initiation factor IF5. Intriguingly, the IF5 promoter contains two TPE elements—one upstream and one within the N-terminal coding region, an arrangement similar to that found in the TBP gene. While these promoters have not been further characterized, it seems probable that TPBF will be involved in the regulation of a fairly discrete group of genes, and this will be an important question to address as the genome sequence is completed.

A final demonstration of the effects of TPBF on expression of the TBP or TPBF genes in living cells will require transfection experiments using promoter constructs containing or lacking TPEs. Until recently, no reliable methods for transfection of Acanthamoeba existed, predating our in vitro and in situ approaches. Kong and Pollard recently described a reproducible method for Acanthamoeba transfection (72) and we have recently developed a stable transfection method (unpublished data), which should permit an extensive analysis of the role of TPEs in vivo.

ACKNOWLEDGEMENTS

We are grateful to Dr David S. Pederson for critical comments on the manuscript and to Dr Jeff Bond for assistance with database searches. We also thank the Vermont Cancer Center sequencing facility for sequencing. Supported in part by a database searches. We also thank the Vermont Cancer Center on the manuscript and to Dr Jeff Bond for assistance with

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REFERENCES
