Linker Scanning Analysis of TBP Promoter Binding Factor DNA Binding, Activation, and Repression Domains*

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Li Chen and Erik Bateman‡

From the Department of Microbiology and Molecular Genetics, Markey Center for Molecular Genetics, University of Vermont, Burlington, Vermont 05405

The transcription activator TATA box-binding protein promoter-binding factor (TPBF) is both an activator and repressor of TBP gene expression in Acanthamoeba. TPBF bears little similarity to previously characterized families of factors. In order to identify domains that are involved in DNA binding, activation, and repression, we constructed several alanine linker scanning mutants and tested them for their ability to function in a variety of assays. The DNA binding domain comprises a large 100-amino acid domain within the central third of the protein, suggesting that DNA recognition is accomplished by interactions derived from several structural units within this domain. Surprisingly, transcription activation and repression are impaired by mutations within either of two discrete amino acid sequences located on either side of the DNA binding domain. These data suggest that TPBF activation and repression are accomplished by interactions with the same target. Since TATA elements can function bidirectionally, and in solution TPBF can bind to TATA elements in either orientation, we propose that TPBF functions in part by orienting TBP or TFIIID correctly on the TATA box.

Eukaryotic promoters for genes transcribed by RNA polymerase II are frequently modular, containing discrete sequences that interact with specific proteins such as transcription activators, repressors, or components of the basal transcription machinery such as TBP (reviewed in Refs. 1–10). The arrangement of such elements varies widely, depending on whether a given gene is inducible, housekeeping, or developmentally regulated. However, there is no universal theme akin to that which can describe promoters for Escherichia coli RNA polymerase (11).

Transcription activators and repressors function by stimulating or inhibiting the activity of the basal machinery. A large number of activators have been described and frequently can be categorized on the basis of clear structural motifs (12–16), for example helix-turn-helix, helix-loop-helix, leucine zipper, or homeodomain. Activators function in part by interacting with other proteins, including basal factors, acetylases, or deacetylases, in each case increasing the local concentration of groups is reminiscent of those made by p53 or NFκB (37, 38). TPBF is tetrameric in solution and when bound to DNA. Deletion analyses demonstrated that the C-terminal region containing a perfect 4-3 repeat of hydrophobic amino acids is necessary for tetramerization and that the N-terminal 122 amino acids are not necessary for DNA binding (36).

In this report we describe the construction and assay of a series of alanine linker scanning mutants of TPBF. The DNA binding domain occupies the central 100 amino acids of TPBF, in which all substitutions abolish binding. The activation domain maps to two short amino acid segments flanking the DNA binding domain. Surprisingly, these same regions are also crit-
for mutant LS262/266, polymerase chain reaction products were diluted 100 ng of protein and 50,000 cpm of end-labeled probe were incubated at 30 °C for 30 min in 15 μl of DNA binding buffer (20 mM HEPES, pH 7.5, 50 mM KCl, 7.5 mM MgCl₂, 0.2 mM EDTA, 10% glycerol, 0.5 mM DTT) containing 100 ng of poly(dG-dC) and 0.2 mM FMSF. An aliquot was loaded onto a 4% non-denaturing polyacrylamide gel. The gels were run at 4 °C, 300 V, for 2 h.

In the experiment testing the stability of the protein-TPE complexes discussed above. At selected time points, aliquots were taken out from the reactions and loaded onto a gel running at 4 °C, 150 V, so that the electrophoresis for each sample ranged from 20 to 180 min.

Chemical Cross-linking of Proteins—200 ng of each mutant protein was incubated with 0.005% glutaraldehyde at room temperature in DNA binding buffer without DTT. After 30 min, the reactions were quenched with 50 mM lysine, and samples were resolved on an 8% SDS-polyacrylamide gel. Proteins were detected by Western blotting.

Strip Purification of Anti-TPBF Antibody—1 mg of purified recombinant TPBF was fractionated on a 10% SDS-polyacrylamide gel and electrophoretically transferred to a nitrocellulose membrane (0.5 A, 1.5 h). The membrane was stained with 0.2% Ponceau S in 1% acetic acid. The band was cut out, and the remaining stain was removed using PBS (1.5 mM KH₂PO₄, 150 mM NaCl, 5.2 mM Na₂HPO₄, pH 7.2). The strip was blocked in 20 ml of PBS containing 5% milk and 0.1% Nonidet P-40 (Sigma) for 30 min, followed by 3 rounds of washing with 20 ml of PBS, each for 5 min. After an overnight incubation with 3 ml of crude rabbit anti-TPBF serum at 4 °C, the strip was removed and washed 3 times with 20 ml of PBS. Bound antibodies were eluted by incubation with 1 ml of 0.1 M glycine, pH 2.5, 100 mM NaCl at 4 °C for 30 min and immediately neutralized to pH 7.0 with 6 M NaOH.

Immunodepletion of TPBF from Acanthamoeba Nuclear Extract—150 μg of strip-purified antibodies were coupled to 0.5 ml of CNBr-activated Sepharose 4B resin (Amersham Pharmacia Biotech) according to the manufacturer's recommendation. The column was washed with 20 ml of HEPES, pH 7.5, 0.05 M or 0.1 M DTT, and immunodepleted TPBF was eluted with incubation for 10 min with 200 ng of mutant protein and 200 ng of pTPE-Acanthamoeba nuclear extract, 25 mM HEPES, pH 7.5, 0.02 mM EDTA, 2% glycerol, 12.5 mM MgCl₂, 20 mM KCl, 30 mM potassium acetate, 2 units of RNase (Life Technologies, Inc.), and 0.4 ml of each NTP. 150 ng of pGML26 was also included in the incubation as an internal control. In the negative control lane, TPBF protein is substituted by same volume of Buffer A. Reactions were stopped by adding 20 μl of 3 M sodium acetate, pH 5.2, and 0.1 M H₂O. Reaction mixtures were extracted with phenol-chloroform twice and ethanol-precipitated, and the resulting pellets were dissolved in 10 μl of annealing buffer (20 mM Tris-HCl, pH 8.3, 0.4 mM KCl) and 50,000 cpm of end-labeled primer RTA3, to detect downstream transcription (see Fig. 6). Mixtures were incubated at 65 °C for 10 min and gradually cooled to 42 °C. Reverse transcription was performed at 42 °C for 1 h in 40 μl containing 55 mM Tris-HCl, 8.3 mM MgCl₂, 100 mM KCl, 0.25 mM of each dNTP, 2 units of RNaseH, and 20 units of SuperScript II (Life Technologies, Inc.). Primer extension products were ethanol-precipitated and analyzed by electrophoresis on a 6% denaturing polyacrylamide gel in 1X TBE buffer. In the experiment shown in Fig. 7, reactions were set up as above except that 150 ng of pTATA1, which contains a TATA box (35), replaced the pGML26 as the internal control, and transcripts were annealed to RTA4 to detect upstream transcription.

RESULTS

Construction, Expression, and Purification of Linker Scanning Mutants—Linker scanning mutants in which TPBF regions rich in charged amino acids are replaced with alanine were constructed as described under “Materials and Methods.” The substitutions were made in the central portion of the protein, since previous studies demonstrated that the N-terminal 122 amino acids are not necessary for DNA binding and that the C-terminal region is necessary for tetramerization, and hence indirectly DNA binding (Fig. 1) (36). Each mutant was expressed in E. coli and purified to near homogeneity by Ni²⁺-Sepharose chromatography (Fig. 2). TPBF binds to nickel
by virtue of its naturally occurring histidine-rich N terminus (36).

Tetramerization of Linker Scanning Mutants—In order to determine whether substitution of alanine residues results in loss of structural integrity, rather than disruption of a specific function, we performed a cross-linking experiment to determine whether the various mutants could form tetramers. As shown in Fig. 3, all of the mutants are able to form tetramers as efficiently as the wild type protein, suggesting that they have retained their overall structure.

DNA Binding Activities—Electrophoretic mobility shift assays were used to determine whether the mutant TPBF proteins could bind to DNA containing a TPE, the TPBF recognition element. Linker scanning mutants ls87/96, ls104/110, ls121/125, ls147/156, d247/259, and ls262/266 were all able to bind DNA with avidity similar to wild type TPBF (Fig. 4). Linker scanning mutants ls147/156 through ls227/236 are completely inactive in the mobility shift assays (Fig. 4), suggesting that the DNA binding domain is contained within this region.

Mutant ls132/136 is able to bind DNA but less effectively and produces a smearing pattern beneath the shifted band. A similar pattern has been obtained using several independent preparations of this mutant. The DNA binding behavior of the ls132/136 mutant was further characterized. Preliminary experiments failed to show a significant difference between off rates when ls132/136 was compared with wild type TPBF (not shown). We therefore determined whether the complex between ls132/136 and DNA was unstable during electrophoresis. When complexes were electrophoresed for varying lengths of time, the ls132/136 mutant appeared to dissociate during electrophoresis, whereas the wild type TPBF complex is completely stable under these conditions (Fig. 5), confirming that the mutant complex is less stable than the wild type complex.

**Transcription Activation**—TPBF linker scanning mutants were included in transcription reactions using TPBF-depleted nuclear extracts and a synthetic promoter containing a TPE upstream of a TATA box in order to locate the activation domain. As an internal control, transcription from the adenovirus

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**TABLE I**

**Linker scanning mutagenesis**

Each mutant was constructed by ligating polymerase chain reaction Fragment A and B to a pRSETB vector. Pairs of primers used in polymerase chain reaction amplification of each fragment are indicated. The wild type and mutant amino acid sequences in the mutated regions are also shown.

| Mutant proteins | Fragment 1 | Fragment 2 | Wild type amino acids | Mutant amino acids |
|-----------------|------------|------------|-----------------------|-------------------|
| ls87/96         | PTN        | PMS1B      | EYHHEEEEMEEH           | Ala<sub>10</sub>  |
| ls104/110       | PMS2A      | PMS2B      | HKRLMD                | Ala<sub>8</sub>   |
| ls121/125       | TM1A       | TM1B       | VMAS                  | Ala<sub>8</sub>   |
| ls123/136       | TM2A       | TM2B       | KKRK                  | Ala<sub>8</sub>   |
| ls142/146       | TM3A       | TM3B       | KVVRK                 | Ala<sub>8</sub>   |
| ls147/156       | PMS3A      | PMS3B      | KYVLMKAFR             | Ala<sub>8</sub>   |
| ls161/170       | PMS4A      | PMS4B      | DSKDGMVLDK            | Ala<sub>10</sub>  |
| ls185/194       | PMS5A      | PMS5B      | RIARNAMYR             | Ala<sub>10</sub>  |
| ls209/218       | PMS8A      | PMS8B      | NYREYIKIK             | Ala<sub>10</sub>  |
| ls227/236       | PMS7A      | PMS7B      | NYGYEKDLD            | Ala<sub>10</sub>  |
| d247/259S       | PMS8A      | PMS8B      | FDFFEELEKSGP           | Ser               |
| ls262/266       | TPBF-N9    | TPBF-C9    | GGSS                  | Ala<sub>8</sub>   |

**Fig. 1.** Diagram of TPBF showing the features deduced from the amino acid sequence and the positions of alanine substitutions. The activities manifested by each mutant as determined by the assays described later are shown to the right. ND, not determined.

**Fig. 2.** SDS-polyacrylamide gel electrophoresis analysis of mutant proteins produced in E. coli and purified by Ni<sup>2+</sup> chromatography. The positions of alanine substitutions are shown above each lane.
major late promoter was assayed using the same primer (upper band in Fig. 6). None of the linker scanning mutants which fail to bind DNA are able to activate transcription. The ls132/136 mutant, which is impaired in DNA binding, is only marginally effective in transcription activation. This defect can be incompletely overcome at higher mutant concentrations, indicating it is less effective as a consequence of impaired DNA binding. Two mutants, ls142/146 and d247/259S which retain full DNA binding activity, are impaired in their ability to activate transcription.

The results shown in Fig. 6 were obtained using 300 ng of each mutant or the wild type protein, with equivalent amounts of DNA binding activity. The defect in activation found for mutant d247/259S could not be overcome by the addition of more protein, indicating that saturating amounts were present. By contrast, mutant ls142/146 behaves anomalously, in that its defect in activation can be partially overcome by addition of more protein. Since the level of activation by ls142/146 never reaches that of wild type TPBF, we regard this mutant as being deficient in activation.

Transcription Repression—Wild type TPBF is able to repress transcription if its binding site is located in any of several positions within a promoter (32, 35). In one case, TPBF represses upstream-initiated transcription directed by TATA boxes, which otherwise function bidirectionally (35). In order to determine whether any of the linker scanning mutants were defective in repression, their ability to suppress upstream-initiated transcription was examined by in vitro transcription from the same promoter as above. As an internal control, a promoter containing only a TATA box was included in the reactions. As shown in Fig. 7, all the linker scanning mutants that fail to bind DNA or that bind DNA poorly (ls132/136 and ls147/156 through ls227/236) also fail to repress transcription. The mutant ls142/146 that is fully able to bind DNA is only weakly able to repress transcription (Fig. 7). Similarly mutant d247/259S is less effective than wild type TPBF in repressing transcription. In this assay, addition of more protein did not overcome the defect in repression by both of these mutants, demonstrating that the failure to repress is not due simply to incomplete occupancy of the TPE (not shown). The remaining four mutants, which bind DNA and activate transcription, repress transcription as effectively as the wild type TPBF.

DISCUSSION

A summary of the structural elements of TPBF inferred from this study is shown in Fig. 8. The C-terminal domain consists of a perfect leucine zipper containing hydrophobic residues that follow perfectly the 4-3 rule (39). This region is predicted to contain 9 helical turns and is required for tetramerization (36). The formation of a tetramer is also consistent with studies on coiled coils, in which perfect leucine zippers form a hollow tetrameric coiled-coil, whereas substitutions with polar amino acids at some positions result in formation of a dimeric coiled-coil (40).

The TPBF DNA binding domain is contained within the...
central 100 amino acids as evidenced by the observation that alanine substitutions within this region abolish DNA binding. Additionally, N-terminal deletions that lack amino acids 1–122 are able to bind DNA (36). The central 100 amino acids contain several hydrophobic amino acids interspersed among basic amino acids, consistent with the extensive array of DNA phosphate contacts made by TPBF (31). One group of basic amino acids outside of this central domain, amino acids 132–136 (KKRRK), appears to contribute to the stability of TPBF DNA contacts in that their removal does not completely abolish DNA binding. These amino acids presumably contribute to protein DNA contacts, thereby stabilizing the complex. Interestingly, these basic amino acids also strongly resemble a nuclear localization sequence (41).

Large DNA binding domains, as defined by mutagenesis and crystallography, have been reported for NFκB and p53 (37, 38). In these examples the DNA-binding surface is derived from multiple parts of the protein, disruption of any one of which abolishes the conformation of the others. It seems probable that this will be the case for TPBF.

An unexpected outcome of these experiments is that amino acids 142–146 (QVKRH) and 247–259 (FDFFEELEEKSFGP) are involved in both activation and repression of transcription, but their removal has no effect on DNA binding as assessed by mobility shift assays. These regions immediately flank the central DNA binding domain. Since mutation in either impairs activation and repression, it is possible that they are juxtaposed in the intact structure.

Comparison of these sequences to those of other activation domains is hampered by the fact that there is no consensus for such sequences. Although activation sequences have been proposed to comprise acidic amino acids arranged on one face of an amphipathic helix (17), not all activator sequences are acidic. Treizenberg and co-workers (42) have pointed out that interactions between the VP16-activating domain and its targets are mediated by essential hydrophobic residues, rather than acidic ones, which function somewhat redundantly. Essential hydrophobic amino acids also occur within the activation domains of RelA, GCN4, glucocorticoid receptor, or p53 (43–46). Although one might envision protein interactions to utilize hydrophobic interactions, this is not a universal requirement since activation can be mediated by homopolymeric glutamine or proline (47), the CTD of RNA polymerase II, as well as the N terminus of human TBP (48). Similarly, comparison of these TPBF sequences to others used in repression also indicates a remarkable functional plasticity (30).

Activators and repressors are thought to function by recruiting or impeding recruitment of one or more components of the basal transcription apparatus, including the general factors, histone acetylases and deacetylases (23, 29, 30). Clearly the mechanisms used can vary, depending on the particular factor, but most studies argue that protein contact between an activator and its target occurs (23). The model activator VP16 has been shown to interact with TBP (49, 50), TFIIB (51), or TBP-associated factor II110 (52). Each of these interactions has the
potential to direct formation of a productive, uniquely oriented preinitiation complex. Therefore, TPBF may act as an activator by recruiting TFII D or TFII B to the promoter. On the other hand, TPBF may act as a repressor using three distinct possible mechanisms. First, in the natural TBP promoter, TPBF appears to displace TBP from the TATA box. Second, TPBF sites positioned downstream of a TATA box, as in the constructs used here, might simply produce a roadblock to elongating polymerase. However, the fact that ls142–146 can bind DNA argues against that simple mechanism. Finally, the activation domain may function by positioning TFII D correctly on the TATA box, with which it would otherwise interact symmetrically (35, 53). By specifically positioning TFII D (or another factor such as TFII B), TPBF will prevent (repress) upstream transcription.

Because the same amino acids of TPBF mediate both activation and repression, an immediate inference from these observations is that the activating and repressing domains of TPBF achieve opposite effects on transcription by contacting the same target. We have previously demonstrated that one of the functions of TPBF is to determine the polarity of transcription by repressing upstream-directed transcription but promoting correctly initiated transcription downstream of a TATA box (35). The availability of mutant TPBF proteins will assist in determining the mechanisms used in activation and repression.

In summary, TPBF provides an otherwise symmetric promoter with directionality, and this function is accomplished by two discrete regions on either side of the DNA binding domain. In this regard, the eukaryotic promoters studied here are quite similar to those of E. coli, in which the bipartite arrangement of the −35 and −10 sequences provides asymmetry and determines promoter strength (11).

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