Mutations on the DNA-Binding Surface of TATA-Binding Protein Can Specifically Impair the Response to Acidic Activators In Vivo

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The TATA-binding protein (TBP) contains a concave surface that interacts specifically with TATA promoter elements and a convex surface that mediates protein-protein interactions with general and gene-specific transcription factors. Biochemical experiments suggest that interactions between activator proteins and TBP are important in stimulating transcription by the RNA polymerase II machinery. To gain insight into the role of TBP in mediating transcriptional activation in vivo, we implemented a genetic strategy in Saccharomyces cerevisiae that involved the use of a TBP derivative with altered specificity for TATA elements. By genetically screening a set of TBP mutant libraries that were biased to the convex surface that mediates protein-protein interactions, we identified TBP derivatives that are impaired in the response to three acidic activators (Gcn4, Gal4, and Ace1) but appear normal for constitutive polymerase II transcription. A genetic complementation assay indicates that the activation-defective phenotypes reflect specific functional properties of the TBP derivatives rather than an indirect effect on transcription. Surprisingly, three of the four activation-defective mutants affect residues that directly contact DNA. Moreover, all four mutants are defective for TATA element binding, but they interact normally with an acidic activation domain and TFIIB. In addition, we show that a subset of TBP derivatives with mutations on the DNA-binding surface of TBP are also compromised in their responses to acidic activators in vivo. These observations suggest that interactions at the TBP-TATA element interface can specifically affect the response to acidic activator proteins in vivo.

The mechanisms by which transcriptional activator proteins stimulate the RNA polymerase II (Pol II) machinery have been intensively investigated in vitro. Activators appear to function by affecting one or more steps in the assembly of general transcription factors into a preinitiation complex (5, 11, 14, 21, 32, 33, 47, 50). In principle, activators might recruit general factors to the promoter, stabilize protein-protein interactions within the preinitiation complex, and/or facilitate conformational changes in initiation factors. In vitro, activators can interact directly with the TATA-binding protein (TBP) (19, 42), TBP-associated factors (TAFs) that are components of the TFIID complex (11, 14), TFIIA (36), TFIIB (34), TFIIF (21), TFIH (54), and Pol II holoenzyme (13). In particular, many regulatory proteins such as VP16 (19, 42), p53 (39), and E1A (30) can interact with TBP. In addition, TBP mutants that are defective for activated transcription in vitro have impaired interactions with activation domains, TFIIA, TFIIB, and DNA (24). The physiological significance of these results remains to be clarified.

The molecular mechanisms of transcriptional activation in vivo are poorly understood. In yeast cells, activators can stimulate transcription by increasing recruitment of TBP to the promoter. First, accessibility of TBP to the his3 TATA element was shown to be a rate-limiting step that could be accelerated by the Gcn4 acidic activation domain (27). Second, direct recruitment of TBP to the promoter by physically connecting TBP to heterologous DNA-binding domains activates transcription (3, 26). Transient-transfection experiments in mammalian cells also indicate that TBP plays a role in transcriptional activation (6, 46). The hypothesis that activators stimulate TBP recruitment in vivo is attractive in light of observations in vitro that TBP binds very poorly to TATA elements in the context of chromatin (18, 53). What remains unclear, however, is the mechanism by which activators recruit TBP or other basal factors to the promoter.

To address this question, we have undertaken a genetic strategy to isolate yeast TBP mutants that are defective in the response to acidic activators but are otherwise normal for Pol II transcription in vivo. It is important to note that the above definition of activation-defective mutants does not involve the concept of basal transcription as defined in vitro. In principle, the basal transcription reaction involves the minimal set of purified general factors that is sufficient to direct accurate initiation from a promoter containing only a TATA and initiator element. In vivo, it is obviously impossible to generate such a situation because the cell contains all proteins involved in transcription (including activators) at physiological levels, and because chromosomally located promoters will always have sequences upstream of the TATA element that are potential weak binding sites for activator proteins. Thus, it cannot be determined whether the low level of transcription that is typically observed in vivo from promoters lacking upstream elements (45) reflects basal transcription as defined in vitro or is due to unknown activators binding to cryptic sequences upstream of the TATA element. Similarly, it cannot be determined whether transcription in the absence of a known activator reflects basal transcription or involves an unknown activator. For these reasons, we define an activation-defective TBP derivative as having a defect in the response to multiple acidic activators but conferring normal levels of transcription from other Pol II promoters in vivo.

Our strategy exploits the unique property of an altered-specificity TBP derivative (TBP\textsuperscript{m3}) to bind and function at

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promoters bearing the mutant TATA element, TGTTAAA (43). TBP contains a concave surface that interacts with TATA promoter elements and a convex surface that mediates interactions with the yeast GCN4 (16), a plasma membrane transporter that specifically transcribes transcriptional defects (23, 25). Because most models for activation invoke protein-protein interactions between activators and general factors, we screened TBPm3 mutant libraries that were biased to the convex surface. In this report, we characterize TBP derivatives that are specifically defective in the response to acidic activators. Surprisingly, most of the activation-defective TBP mutants affect residues that directly contact DNA, and all of them are unable to bind TATA elements in vitro. TBP derivatives, previously characterized as having DNA-binding and transcriptional activation defects in vitro (24), are also compromised in their responses to acidic activators in vivo. These results suggest that interactions at the TBP-TATA element interface can specifically affect transcriptional activity.

MATERIALS AND METHODS

Strains and DNAs. Saccharomyces cerevisiae yML1 was derived from ΔCUP1-S1 (31) by gene replacement of the HIS3 locus with Sc3765 (12), which contains a TGTTAAA TATA element and no activator-binding site, and the integrated 5′ XbaI- and 3′ AgeI-SacI fragment from parental TBP m3 (31). Analysis of mutants obtained in the TBPm3 context was performed in strains derived from KY320 (4) in which the HIS3 locus was replaced with TGTTAAA-containing alleles Sc3765 (no Gcn4-binding site; strain yML2), Sc3735 (with a Gcn4-binding site; strain yML3), or Sc3660 (four Gal4-binding sites; strain yML4). Analysis of wild-type TBP context was performed by plasmid shuffling into strain BY2Δ (7). To assess Ac1 activation, the HIS3 locus of BY2Δ was replaced with a derivative that contains a single Ac1-binding site upstream of the TATATA TATA element (20) to give strain yML5. For the genetic complementation experiment detailed in Fig. 5, the HIS3 locus of BY2Δ was replaced with allele Sc3660 (4) to generate strain yML7.

TBP libraries generated by regional codon randomization (9) were reconstructed in the TBPm3 context as follows. Libraries N1, N2, and N3 were converted by subcloning an Xbal-BamHI fragment containing the three mutations conferring altered specificity (43). Libraries N4, N5, and N6 were constructed by PCR (9) using TBPm3 as a template. As before, TBP libraries were carried on TRP1-marked centromeric plasmids. Separation of the double mutant (S118L, S121T) was achieved by subcloning the AgeI-HindIII fragment of the double mutant into the parental TBPm3 context (to give S121T) and subcloning the same fragment from parental TBPm3 into the double mutant (to give S118L). Generation of the TBP mutants L114K, L189K, and K211L was accomplished by PCR mutagenesis as described in subsection 3. The CUP1 coding sequence. The libraries were introduced into strain BY2Δ (within the TBP coding sequence) and BamHI sites of TBP. TBPm3 genes were purified as instructed by the manufacturer (Novagen). Proteins thus purified were estimated to be greater than 50% pure by Coomassie blue staining. Conclusions were determined by the Bradford assay and calculated for wild-type TBP. Protein concentrations for the TBP mutants were estimated by Coomassie blue staining, using serial dilutions of wild-type TBP as a standard. Gel mobility shift experiments were performed by incubating a 45-bp fragment (0.5 ng) containing the adenovirus E1B TATA box with the indicated amounts of the TBP derivative in the presence of 200 ng of poly(GC)-dC, 100 mM KCl, 40 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES; pH 7.9), 20 mM Tris (pH 7.5), 5 mM MgCl2, 10% glycerol, 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride. TBP complexes were electrophoretically separated on a 5% polyacrylamide gel in a Tris-glycine buffer system containing 4 mM MgCl2. Column association experiments involved incubating 20 µl of glutathione-agarose beads containing glutathione S-transferase (GST), GST-VP16, and GST-TFIIB with TBP derivatives for 1 h in 100 µl of buffer containing 100 mM KCl, 20 mM HEPES (pH 7.9), 5 mM MgCl2, 20 µg bovine serum albumin, 0.2 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 10% glycerol, and 0.03% Nonidet P-40. The beads were washed five times with 200 µl of buffer, and bound proteins were eluted in 40 µl of sodium dodecyl sulfate-polyacrylamide gel electrophoresis loading buffer and separated by electrophoresis. TBP bound to the various columns tested was visualized by Western blotting (immunoblotting) with an anti-TBP antibody.

RESULTS

A genetic screen for isolating activation-deficient derivatives of TBP. To obtain TBP mutants with defects in activated transcription, we exploited the unique property of an altered-specificity TBP derivative (TBPm3) to bind and function at promoters bearing the mutant TATA element, TGTTAAA (43). TBPm3 versions of yeast and human derivatives have been examined for activation in yeast and human cells (22, 43, 46). A yeast genetic screen performed in the context of TBPm3 has several advantages. First, because the screen is carried out in the presence of wild-type TBP, mutations that adversely affect both activation and viability are not lost. Second, TBPm3 is required for transcription from TGTTAAA-containing promoters but not from native yeast promoters; this reduces the likelihood of isolating activation-deficient TBP mutants that indirectly generate their phenotypes by altering expression of genes other than the reporter. Third, the availability of genetic screens makes it possible to examine many TBP derivatives for the desired properties.

The directed genetic screen for activation-defective TBP derivatives was diagrammed in Fig. 1. Six highly complex and compact libraries generated by regional codon randomization (9) were reconstructed in the TBPm3 background. These libraries are strongly biased to the convex surface of TBP that mediates protein-protein interactions; of the 95 residues mutated, only 14 are located on the concave, DNA-binding surface. The libraries were introduced into strain yML1, which contains his3 promoter derivatives containing the TGTTAAA TATA element fused to selectable structural genes. The non-

RNA analysis. For analysis of Ac1 activation, yML6 derivatives supported by wild-type or mutant TBPs were grown in synthetic minimal glucose medium to mid-log phase and split into two series of parallel cultures in fresh medium. When these cultures reached mid-log phase, one series of cultures was induced with 200 µM CuSO4 for 1 h, at which point both series of cultures were harvested for RNA analysis. For the genetic complementation assay, strains supported by the wild-type and mutant TBP derivatives were transformed with TBPm3 and grown in a Casamino Acids medium containing raffinose (nonrepressing) as the sole carbon source. Cultures were grown to mid-log phase and split into Casamino Acids medium containing either glucose (repressing) or galactose (inducing) as the sole carbon source and harvested at mid-log phase. In all cases, RNA levels were measured quantitatively by S1 nuclease analysis using oligonucleotides probes (8, 28).

In vitro analysis of TBPs. TBP derivatives were cloned into the NdeI and BamHI sites of vector PET-15b (Novagen) as follows. An NdeI-BamHI fragment containing the N terminus of yeast TBP (7) was ligated to the BglII-BamHI fragment of the TBP derivatives containing the rest of the TBP coding sequence, with destruction of the BamHI site. All mutations analyzed were present between the BglII (within the TBP coding sequence) and BamHI sites of TBP. TBPm3 genes were purified as instructed by the manufacturer (Novagen). Proteins thus purified were estimated to be greater than 50% pure by Coomassie blue staining. Concentrations were determined by the Bradford assay and calculated for wild-type TBP. Protein concentrations for the TBP mutants were estimated by Coomassie blue staining, using serial dilutions of wild-type TBP as a standard. Gel mobility shift experiments were performed by incubating a 45-bp fragment (0.5 ng) containing the adenovirus E1B TATA box with the indicated amounts of the TBP derivative in the presence of 200 ng of poly(GC)-dC, 100 mM KCl, 40 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES; pH 7.9), 20 mM Tris (pH 7.5), 5 mM MgCl2, 10% glycerol, 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride. TBP complexes were electrophoretically separated on a 5% polyacrylamide gel in a Tris-glycine buffer system containing 4 mM MgCl2. Column association experiments involved incubating 20 µl of glutathione-agarose beads containing glutathione S-transferase (GST), GST-VP16, and GST-TFIIB with TBP derivatives for 1 h in 100 µl of buffer containing 100 mM KCl, 20 mM HEPES (pH 7.9), 5 mM MgCl2, 20 µg bovine serum albumin, 0.2 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 10% glycerol, and 0.03% Nonidet P-40. The beads were washed five times with 200 µl of buffer, and bound proteins were eluted in 40 µl of sodium dodecyl sulfate-polyacrylamide gel electrophoresis loading buffer and separated by electrophoresis. TBP bound to the various columns tested was visualized by Western blotting (immunoblotting) with an anti-TBP antibody.
activated promoter drives expression of HIS3, which can be monitored by resistance to AT, a competitive inhibitor of the HIS3 gene product. The Gcn4-activated promoter directs expression of CUP1, which can be monitored by resistance to exogenous copper sulfate in the growth medium (31). The promoters for the two reporters are identical in all respects except that one contains a Gcn4-binding site while the other does not. In comparison with the parental TBPm3, an activation-deficient mutant should show high activity from the nonactivated reporter but low activity from the activated reporter.

Isolation of a rare class of TBP mutants that is impaired in transcriptional activation. Approximately 5,000 transformants from each TBPm3 library were screened on medium containing either 0.5 mM AT or 160 μM CuSO4. Colonies that grew fairly well on AT but poorly on CuSO4 were selected, and the plasmids encoding the TBPm3 derivatives were retransformed into an isogenic yeast strain carrying the nonactivated and Gcn4-activated promoters fused to the HIS3 structural gene. Figure 2A shows a graphical representation of the range of mutant phenotypes on the nonactivated promoter. Four TBPm3 derivatives were isolated at the top of the panel. As expected, defects in activated transcription are strongly correlated with defects in transcription from the nonactivated promoter. However, within one class of mutants having a modestly decreased level of nonactivated transcription (Fig. 2A, boxed), we observed a wide distribution of phenotypes on the activated promoter.

From this class of TBPm3 mutants having equivalent phenotypes on the nonactivated promoter, four TBPm3 derivatives exhibiting the lowest level of Gcn4-activated transcription were selected for further analysis (Fig. 2A, circled). Three of these mutants contain single amino acid substitutions (N159L, V161A, and F148L); the remaining mutant contained two changes (S118L and S128T). Compared with the parental TBPm3, all four mutants exhibit only slightly reduced levels of activity on the nonactivated promoter, whereas they show dramatic effects on the Gcn4-activated promoter (Fig. 2B). In contrast, TBPm3 derivatives containing K145R, R137S, A140C, plus Q144L, and S136M, which display similar functions on the nonactivated promoter, give levels of activation more characteristic of the general distribution of mutants. Separation of the S118LS128T double mutant indicates that S118L is responsible for the activation-defective phenotype; S128T behaves indistinguishably from the parental TBPm3. Double mutants representing the six pairwise combinations of the activation mutations were equally or more impaired in the ability to support Gcn4-dependent activation compared with the corresponding single mutants (Fig. 2B).

We tested the four TBPm3 derivatives for G4-dependent activation by introducing them into a strain carrying a TATAAA-containing promoter with four binding sites for the Gal4 acidic activator (Fig. 2C). In comparison with parental TBPm3, the four TBPm3 derivatives are impaired in the ability to support Gal4-dependent activation, although to different extents. The S118L, F148L, and V161A derivatives show very little activity on this Gal4-dependent promoter, whereas N159L is only partially defective.

Activation defects are retained in the context of wild-type TBP. To verify that the activation-deficient phenotypes were not an artifact of the TBPm3 context, we introduced the mutations into the context of wild-type TBP. Strains containing the four derivatives as the sole source of TBP grow well at 30°C, with little or no effect on doubling time in YPD medium. At 37°C, strains containing S118L, N159L, and V161A exhibit slightly decreased growth compared with strains containing F148L or wild-type TBP. Growth rates of double mutant strains were equal to or slightly slower than those of the corresponding single-mutant strains. As determined by Western blotting, TBP levels in the mutant strains were indistinguishable from that of the wild-type strain (see Fig. 4B).

To test whether these mutant strains were deficient for activation by Gal4 and Ace1, we generated strains containing appropriate reporter constructs with a wild-type (TATAAA) element. In all four mutant strains, β-galactosidase activity from the Gal4-dependent reporter under activating conditions (in galactose) varied between 6 and 24% of the activity of the wild-type strain (Fig. 3A); in the double-mutant strains, activities ranged from 6 to 12% (Fig. 3B). As expected, the behaviors of the activation-deficient TBPs in this assay paralleled their activities in the TBPm3 context (compare Fig. 2C with Fig. 3A). With the exception of N159L, which was least affected for Gal4 activation, the mutant TBP derivatives were also impaired in transcriptional activation by Ace1 (Fig. 3C). V161A was very deficient (about 5% of wild-type activity), whereas S118L and F148L showed about 25% of wild-type activity. The apparent defects observed in the absence of copper are Ace1 dependent (10) and do not represent defects in nonactivated transcription.

TBP derivatives do not affect Pol I, Pol III, and constitutive Pol II transcription. In the TBPm3 context used in their isolation, the activation-deficient derivatives appear to be slightly defective for nonactivated transcription (Fig. 2A and B). However, this apparent defect may be due not to the S118L, F148L, N159L, and V161A mutations per se but rather to the fact that they are analyzed in the context of TBPm3, which contains three mutations on the DNA-binding surface that mediate the altered TATA element specificity. We therefore analyzed the
mutations in the context of wild-type TBP (same strains as used for Fig. 3) for transcription from representative Pol I (rRNA), Pol III (tRNA-I), TATA-less Pol II (TRP3), and constitutive Pol II (DED1 and RPS4) promoters. For all five genes tested, transcription in the mutant strains was similar to that observed in the wild-type strain (Fig. 4). Although basal transcription as defined in vitro cannot be measured in vivo (see the introduction), these results indicate that the TBP mutations do not generally impair Pol II transcription. Thus, in the context of wild-type TBP, the mutant proteins are specifically defective in the response to acidic activators in vivo.

A genetic complementation experiment verifies the primary nature of activation deficiency. There are two mechanisms by which a TBP mutant might generate an activation-deficient phenotype. In the desired case, TBP mutants specifically and directly affect the process of activation. Alternatively, as TBP plays an essential role in all transcriptional events in vivo, an activation-deficient phenotype on a particular reporter might represent an indirect effect due to the altered expression of a gene(s) important for activation. For example, TBP derivatives defective in the synthesis of specific activator proteins, TAFs, or other coactivators might lead to the appearance of an activation-defective phenotype.

We have designed a genetic complementation experiment to distinguish between these possibilities (Fig. 5A). Strain yML7 contains a Gal4-dependent, TGTAAA-containing promoter that drives HIS3 expression and is supported by wild-type TBP on a URA3-marked plasmid. The four TBP mutants (in the wild-type context) were introduced into strain yML7 by plasmid shuffling, and activation deficiency was confirmed by analysis with the Gal4-dependent lacZ reporter (data not shown). The resulting strains (and the wild-type TBP control) were transformed by a plasmid expressing the parental (i.e., otherwise wild-type) TBPm3. If a TBP mutant indirectly generates an activation-deficient phenotype by affecting expression of gene(s) important for the activation process, then TBPm3 should also show an activation-defective phenotype on its cognate promoter. On the other hand, in a cell supported by a TBP mutant with a primary activation defect, TBPm3 should function normally in activated transcription from a TGTAAA promoter. Thus, in this genetic complementation experiment, TBPm3 serves as a probe for the activation competency of the cell.

In the control strain supported by wild-type TBP, TBPm3 drives HIS3 transcription when the cells are grown in galactose (Fig. 5B). In strains supported by the S118L, N159L, V161A, and F148L derivatives, TBPm3 function varied between 80 and 100% of wild-type function. Thus, the activation-deficient phenotypes of the four TBP mutant strains are directly due to inherent functional defects in transcriptional activation.

Activation-deficient TBP alleles are defective for DNA binding. Mapping of the TBP mutations onto the X-ray structure of a TBP-TATA element complex (23, 25) reveals that three of

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**FIG. 2.** Isolation of activation-deficient TBP mutants. (A) Phenotypes obtained from the screen. Mutants were scored for activity from each reporter, using an arbitrary scale (phenotypes conferred by wild-type TBP and the parental TBPm3 were set at the bottom and top of the scale, respectively). Each point on the graph denotes a phenotypic class and represents one or more mutant TBP derivatives. Boxed, series of phenotypic classes with equivalent function on the nonactivated promoter; circled, class of mutants defined to be activation deficient. (B) Phenotypic assays for nonactivated and Gen4-activated HIS3 expression conferred by mutant TBPm3 derivatives. The HIS3 promoter derivatives are identical to those depicted in Fig. 1 except that the activated reporter directs expression of HIS3. Mutants were introduced into strain yML2 (nonactivated reporter) or yML3 (activated reporter), and the resulting cells were spotted on plates containing either 0.5 mM AT (nonactivated) or 5.0 mM AT (activated). Mutants K145G, R137S, A140C, Q144L, and S136M are activation-competent mutants whose activities on the nonactivated promoter are similar to those of the activation-deficient mutants S118L, S128T, N159L, V161A, and F148L. Individual amino acid changes in S118L, S128T were tested separately to determine the basis for the activation-deficient phenotype. Analysis of the six possible double mutants between S118L, N159L, V161A, and F148L constructed in TBPm3 is shown at the right. (C) Responses of TBP mutants to the Gal4 activator. TBPm3 derivatives were introduced into strain yML4 (Gal4-dependent TGTAAA-containing HIS3, with four Gal4-binding sites denoted by black boxes), and the resulting strains were tested for growth in medium containing either glucose or galactose as the carbon source and 5 mM AT.
the four TBP derivatives isolated here (S118L, N159L, and V161A) affect residues that directly contact DNA. Given that only 14 of the 95 residues represented in the screened libraries map to the DNA-binding surface, this result was very surprising (P < 0.01). This result was also surprising given that nearly all models of transcriptional activation involving TBP have emphasized protein-protein interactions mediated by the convex (i.e., non-DNA-binding) surface.

To test whether DNA binding was indeed affected, we expressed histidine-tagged TBP mutant proteins (in a wild-type context) in E. coli and purified them by nickel affinity column chromatography. As assayed by gel retardation, wild-type TBP bound specifically to a TATA-containing probe, whereas none of the mutants could bind detectably at similar TBP concentrations (Fig. 6A). Indeed, none of the mutants could bind detectably at 10-fold-higher protein concentrations, with the exception of F148L, which gave a very faint signal. Thus, the four activation-defective mutants possess DNA-binding capacities at least 100-fold below wild-type levels. Addition of TFIIA or TFIIIB to the reaction does not rescue the DNA-binding defect (data not shown). The inability to bind TATA sequences is not due to a gross unfolding or inactivation of the E. coli-generated proteins. When tested for interactions with TFIIA or VP16, two factors with well-characterized associations with TBP, all four mutants behaved indistinguishably from wild-type TBP (Fig. 6B).

A subset of other mutations on the TBP DNA-binding surface confer activation defects in vivo. Three TBP mutants that support basal transcription but do not respond to the VP16 acidic activation domain have been previously identified by assays in vitro (24). While the mutations involved (L114K, L189K, and K211L) cause a variety of biochemical defects, they all affect residues that directly contact DNA. As most of the TBP derivatives described in the current study affect residues on the DNA-binding surface, we therefore examined whether these previously identified TBP mutants are defective for transcriptional activation in vivo.

We first tested whether the L114K, L189K, and K211L de-
derivatives (in an otherwise wild-type context) could support cell growth as the sole source of TBP. Of the three mutants, only L114K gave any detectable complementation of the TBP deletion; growth was extremely feeble when L114K was present on a centromeric plasmid and slow when it was introduced on a multicopy vector. When tested for activation by Gal4 and Ace1, the L114K strain was nearly as defective as the strain containing V161A (Fig. 3A and C). As a second test, all three mutants were constructed in the TBP m3 context and analyzed for the ability to mediate nonactivated and Gcn4-activated transcription from appropriate TGTAAA-containing promoters (Fig. 7). L114K, L189K, and K211L behaved very similarly to V161A, which was isolated in the present study.

To test whether any DNA-binding mutant could confer this phenotype, we assayed two TBP mutants (K110L and K120L) with known DNA-binding defects in vitro (55). Both of these mutants are not activation deficient by our assay; the K110L derivative behaves similarly to the parental TBP m3, whereas the K120L derivative shows equivalent decreases in nonactivated and Gcn4-activated transcription from appropriate TGTAAA-containing promoters (Fig. 7). L114K, L189K, and K211L behaved very similarly to V161A, which was isolated in the present study.

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Our results strongly suggest that the four TBP derivatives are specifically impaired in the response to acidic activators. Strains supported by these TBP derivatives are defective (to various extents) for transcriptional activation by Gcn4, Gal4, and Ace1. These three activator proteins have structurally unrelated DNA-binding domains and are physiologically regu-
lated in very distinct manners, but they all stimulate transcription through acidic activation domains (10, 16, 35). In contrast, transcription from Pol I, Pol III, and TATA-less Pol II promoters (rRNA, tRNA, and TRP3, respectively) is unaffected in the mutant TBP strains (Fig. 4), even though functional TBP is required in all cases in vivo (8). Furthermore, transcription from constitutive, TATA-containing Pol II promoters (RPS4 and DED1) is normal in our mutant TBP strains. Although basal transcription as defined in vitro cannot be measured in vivo (see the introduction), the selective impairment of transcription mediated by three acidic activators excludes the possibility that the TBP mutations have a general effect on Pol II function.

Although these TBP mutations confer slightly reduced function on the nonactivated promoter in the TBPm3 context, it should be emphasized that the mutants were initially selected because of their markedly reduced ability to support activated transcription as compared with other mutants with similar competency on the nonactivated promoter. Moreover, interpretation of this weak effect on nonactivated transcription is complicated by the facts that TBPm3 already contains three substitutions on the DNA-binding surface and that it binds less strongly to TATAAA than wild-type TBP binds to TATAAA (43). Thus, we conclude that the S118L, N159L, and V161A substitutions specifically affect activated transcription and that the observed effects on nonactivated transcription in the TBPm3 context reflect perturbations of the DNA-binding surface and TATA element interaction enhanced by the three mutations that confer altered specificity.

Role of DNA binding in activation. Given that most models for transcriptional activation invoke protein-protein interactions at the promoter, we screened libraries biased against mutations on the concave, DNA-bindingsurface of TBP. Thus, it was quite unexpected (P = 0.01) that three of the four derivatives altered residues that directly interact with the TATA element. These three TBP mutants (S118L, N159L, and V161A) do not bind detectably to the TATA element in vitro, and the remaining mutant (F148L) is severely defective in DNA binding (<1% of the wild-type level). The common defect in DNA binding does not reflect gross unfolding or inactivation because all four TBP derivatives interact normally with VP16 and TFIIB in vitro.

While it is possible that these mutants are compromised in their interactions with factors that we have not tested, the localization of these mutations to the DNA-binding surface is very suggestive of the primary significance of the TBP-TATA element interaction. In support of this view, previously characterized TBP mutants that map to the DNA-binding surface and are activation defective in vitro (24) behave similarly in vivo to the mutants isolated in this paper (Fig. 7). After this report was submitted for publication, TBP mutants defective for Gal4-activated transcription in vivo were isolated by a very different genetic screen (1). These mutants also map on the DNA-binding surface of TBP and in two cases occur at the same position (although not the same substitution) as the mutants described here; however, they do not appear to be defective in activation by Gen4 (1). Taken together, the evidence argues that mutations at the TBP-DNA surface can preferentially affect activated transcription.

Although these mutants fail to bind TATA elements in vitro, their ability to support viability indicates that they interact efficiently with these elements in vivo. This finding strongly suggests that the TBP mutants are recruited or stabilized to the TATA element in vivo, possibly by proteins (TFIIA, TFIIB,  

**FIG. 6.** In vitro analysis of TBP mutants. (A) Gel mobility shift assay for DNA binding. The indicated amount of histidine-tagged TBP protein was incubated with a TATAAA-containing probe and electrophoretically separated on a Tris-glycine 4% native polyacrylamide gel supplemented with 4 mM MgCl2. WT, wild type. (B) Affinity chromatography assay for protein-protein interactions with TFIIB and VP16. Fifty nanograms of each TBP derivative was incubated with an excess of glutathione-agarose beads containing GST, GST-VP16, or GST-TFIIB. Approximately 30% of the input TBP’s was retained on the columns.

**FIG. 7.** Phenotypic analysis of previously characterized TBP derivatives with mutations on the DNA-binding surface of TBP. The L114K, L189K, K211L, K110L, and K120L substitutions (17, 55) were introduced into the TBPm3 context and examined for nonactivated and Gen4-activated transcription (strains yML2 and yML3, respectively) as described in the legend to Fig. 2. The K120L derivative shows equivalent decreases in nonactivated and Gen4-activated transcription (in comparison with V161A, a representative of the activation-deficient mutants isolated here, K120L has a much weaker signal on the nonactivated promoter and a slightly greater activated signal); hence, it is not considered an activation-deficient mutant.
and TAF150) that can extend the footprint of TBP on DNA in vitro (2, 49). We do not believe, however, that the activation-deficient phenotypes are due simply to a general reduction in TBP affinity for DNA. The L189K protein binds TATA sequences with wild-type affinity (55) but is activation deficient. Conversely, K110L does not bind DNA in vitro (55) but appears fully competent for activated transcription in vivo (Fig. 7) or in vitro (24). Moreover, we recently tested the four mutants isolated here in a highly sensitive assay for DNA binding, using a prebound TATA sequence (37). In this assay, V161A, the mutant most severely affected for activation, binds DNA detectably, while N159L, the least severely affected mutant, fails to bind at all (29). Taken together, the data indicate that activation deficiency cannot be explained as a simple consequence of a reduced TBP-TATA interaction.

Possible molecular mechanisms. In the simplest models, the central role of TBP in preinitiation complex formation predicts that defects in DNA binding will affect both nonactivated and activated transcription. Indeed, analysis of numerous derivatives of the TATA element suggests that the level of basal TBP-dependent transcription in vitro is very strongly (but not completely) correlated with the level of transcriptional activation in vivo (12, 52). Thus, it is very surprising to find that mutations on the DNA-binding surface of TBP can specifically affect the response to acidic activators.

Why are the TBP mutants isolated in this study specifically defective for activation? We consider four explanations that are not mutually exclusive. First, these mutants (aided by other factors such as TAFs, TFIIA, or TFIIIB) might bind DNA in a conformation that differs from that of wild-type TBP such that the resulting TBP-DNA complex cannot be recognized by activators and/or coactivators. Although TBP conformation (unlike that of the TATA element) does not appear dramatically altered upon binding (23, 25), conformational differences might not need to be large to be significant and might reside in the DNA rather than in TBP. Second, activators might require that a given TBP molecule be present at the promoter for a certain length of time before they can mediate their stimulatory action. The mutant TBPs might have a slower rate or an increased off rate from the TATA element such that an activator would not be effective. In this view, the effective occupancy time required for nonactivated transcription would be less (which might account for the lower level of transcription). Third, transcriptional activation in vivo might involve the remodelling of chromatin by factors such as the Swi/Snf complex that can enhance the accessibility of TBP for the TATA element in vitro (18). If a TBP mutant cannot exploit the remodelled chromatin generated by Swi/Snf, then activation might be greatly reduced. Fourth, the concave surface of TBP might be bifunctional such that it can interact with both DNA and activators. All of these models are consistent with the observation that only a subset of mutations on the DNA-binding surface specifically affect transcriptional activation.

Potential physiological relevance. Although the molecular mechanisms are unknown, our results indicate that interactions at the TBP-TATA interface can specifically affect the response to acidic activators. While the activation-deficient TBPs described here are artificial, our results are likely to be physiologically relevant because the affinity of TBP for natural TATA elements varies over a wide range. Indeed, there are several examples in which specific TATA sequences display differential responses to activator proteins (12, 15, 40, 44, 51). In S. cerevisiae, there are numerous examples of closely packed genes that are differentially regulated. In such situations, functionally distinct TATA elements might permit an activator protein that functions bidirectionally to stimulate only one of a pair of diverently transcribed genes. For example, a Gcn4-binding site is located at a comparable distance upstream of the HIS3 and PET56 TATA elements, but Gcn4 is able to stimulate only HIS3 transcription (44). Thus, the subtleties involved in interactions at the TBP-TATA interface that specifically affect transcriptional activation might contribute to the extraordinary diversity of eukaryotic gene regulatory patterns.

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REFERENCES