A Yeast TATA-Binding Protein Mutant That Selectively Enhances Gene Expression from Weak RNA Polymerase II Promoters

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We describe a unique gain-of-function mutant of the TATA-binding protein (TBP) subunit of Saccharomyces cerevisiae TFII D that, at least in part, renders transcriptional transactivators dispensable for efficient mRNA expression. The yTBPN69S mutant enhances transcription from weaker yeast promoter elements by up to 50-fold yet does not significantly increase gene expression directed by highly active promoters. Therefore, this TBP mutant and transcriptional transactivators appear to affect a common rate-limiting step in transcription initiation. Consistent with the hypothesis that this step is TFII D recruitment, tethering of TBP to a target promoter via a heterologous DNA binding domain, which is known to bypass the need for transcriptional transactivators, also nullifies the enhancing effect exerted by the N69S mutation. These data provide genetic support for the hypothesis that TFII D recruitment represents a rate-limiting step in the initiation of mRNA transcription that is specifically enhanced by transcriptional transactivators.

Eukaryotic mRNA expression requires the functional interaction of a complex set of cellular transcription factors with promoter DNA, leading ultimately to the recruitment of RNA polymerase II (Pol II). Many cellular factors involved in initiation of transcription by Pol II have been identified through biochemical and/or genetic approaches, and these have been typically classified as either general transcription factors (GTFs) or transcriptional transactivators. GTFs can be defined as those factors required for low levels of accurate transcription initiation from Pol II-dependent promoters in vitro, including, but not limited to, TFII A, TFII B, TFII D, TFII E, TFII F, and TFII H (13, 34, 51). Transcriptional transactivators, on the other hand, act via specific promoter-distal DNA or RNA targets to activate transcription by, most probably, facilitating GTF recruitment.

Many, but not all, eukaryotic Pol II promoters contain a TATA box that is required for efficient transcription initiation and that directly binds the TATA-binding protein (TBP) component of TFII D (16). Precise DNA sequence requirements for TATA box function have been identified by mutational analysis (8, 47) or by random selection of functional TATA elements (37), leading to identification of the consensus sequence 5′-TATAA-3′. In higher eukaryotes, some Pol II promoters contain an initiator (Inr) element in addition to or instead of a consensus TATA box, that overlaps the transcription start site and that can recruit TFII D to the promoter element via a direct interaction with a TBP-associated factor (TAF) component of TFII D (13, 46). Pol II promoters in the yeast Saccharomyces cerevisiae that lack a consensus TATA box have been less well characterized. However, several of these, including the HIS3 Try promoter element, are known to contain essential promoter-proximal A/T-rich sequences (28). Currently, therefore, it remains unclear whether a direct interaction between TBP and DNA is invariably required for transcription from yeast TATA-less promoters or whether such promoters instead contain alternative TFII D binding sites functionally equivalent to Inr elements.

Recruitment of TFII D to promoter elements is essential for transcription initiation in eukaryotes and appears to represent a rate-limiting step in vivo that can be specifically enhanced by at least some transcriptional transactivators (24). TFII D may nucleate the assembly of the basal transcription machinery on Pol II promoters, analogous functionally to prokaryotic σ factors (13). The TBP component of TFII D consists of a nonconserved amino-terminal domain and a highly conserved carboxy-terminal or core domain, which contains two imperfect repeats of ~60 amino acids in length. The solved cocrystal structure of TBP bound to TATA DNA reveals that the TBP core domain folds into a symmetrical structure that resembles a molecular saddle (20, 22, 31). TBP residues located in the concave underside of the molecular saddle directly contact TATA DNA, resulting in a large distortion of the DNA helix. Conversely, TBP residues predicted to be involved in protein-protein interactions map to the convex side of the saddle.

Although TBP has been extensively mutationally characterized, point mutations in TBP that significantly enhance Pol II transcription have not been described. The identification and characterization of mutations that enhance TBP function should provide valuable insights into the role of TBP and TFII D in activated and/or basal transcription from Pol II promoters. In an attempt to achieve this, we developed a genetic screen designed to identify yeast TBP (yTBP) mutants that facilitate the activity of weak transcriptional activators. We have identified one such TBP mutant (yTBP N69S) that increases the level of gene expression directed by several weak or basal yeast promoter elements. In contrast, transcription from highly active yeast promoters was not found to increase significantly. A single amino acid substitution (Asn to Ser) at residue 69 of yTBP, which is predicted to contact DNA, was sufficient to confer the mutant phenotype. Further analysis showed that yTBP N69S mediated high levels of gene expression from a yeast promoter containing a mutant TATA sequence that was not efficiently utilized by wild-type yTBP and demonstrated

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that direct DNA binding is not required for yTPB69S function. Nevertheless, the competition of the activities of yTPB69S and wild-type yTPB targeted to promoter DNA by heterologous DNA binding domains indicates that the observed increase in yTPB69S-mediated gene expression results from an increase in the efficiency of yTPB69S promoter recruitment.

MATERIALS AND METHODS

Plasmids. The GAL4 fusion protein expression plasmids pGALA, pGAL4-VP16, and pGAL4-RelA have been previously described (3). pGAL4-(AAM) and pGAL4-(AAM)-Δ(AAAM), which encode GAL4-activation domain fusion proteins from a truncated ADH1 promoter, were constructed by amplification of the mammalian expression vectors pGAL4-(AAM) and pGAL4-(AAM)-Δ(AAAM) (4) with the restriction endonucleases Xhol and EcoRI. The resulting XhoI/EcoRI fragment, containing both GAL4 sequences and (AAM) or (AAAM)-Δ(AAAM) activation domain sequences, was introduced into the yeast GAL4 expression vector pGBT9 (Clontech) digested with Xhol and EcoRI. Alternatively, these same DNA fragments were introduced into a derivative of pGAL4-(AAM) containing the PGK-ADH promoter in place of the ADH1 promoter to construct pGAL4K-(AAM) or pGAL4K-(AAM)-Δ(AAAM). pGAL4-yTPBwt and pGAL4-yTPBmut were constructed by amplification of a wild-type TBD cDNA, or a TBD CDNA encoding the N69S mutation, with PCR primers that introduced flanking EcoRI restriction sites. Amplified products were then digested with EcoRI and introduced into the pGBT9 expression plasmid.

yTPB expression plasmids (e.g., pYTPBwt) were generated by PCR amplification of yTPB encoding sequences by using primers that contained unique Xhol and EcoRI restriction sites. Digested products were then inserted into a Yeplac112 (12) ADH1 promoter plasmid, digested with Xhol and EcoRI, which contains the yeast ADH1 promoter at unique Xhol and EcoRI sites. The library of TBD mutants was generated in the same manner, except that PCR was carried out under conditions that favored the random incorporation of mis sense mutations. A yTPB expression plasmid encoding a lysine substitution for leucine 205 (L205K) was generated by recombinant PCR in which oligonucleotides spanning yTPB-coding sequences encoding residues 199 to 208 and 5′ and 3′ flanking oligonucleotide primers containing unique Xhol or EcoRI restriction sites, respectively. A yTPB expression plasmid encoding a Lys138-to-Thr and Tyr139-to-Ala amino acid substitution mutant (N2-1) was similarly generated by PCR amplification of yTPB sequences with a primer that spanned yTPB sequences encoding residues 131 to 141 and a 3′ flanking oligonucleotide primer that contained a unique EcoRI restriction site.

Amplified products were digested with Xhol and EcoRI and ligated to yTPB expression plasmids digested with the same restriction endonucleases. Escherichia coli expression plasmids expressing yTPB were constructed by PCR amplification of TBD expression sequences with primers that contained Xhol or PstI sites and ligation of the amplified products into pQE-31 (Qiagen) digested with Sall and PstI. TBD-coding regions were sequenced in their entires from plasmids generated by PCR to ensure their identities.

The pADH1lacZ or pPGKlacZ reporter plasmids were constructed by PCR amplification of ADH1 (2) or PGK (32) promoter sequences extending ~412 nucleotides (nt) or ~575 nt 5′ of their Intromechinon codons, respectively, with oligonucleotide primers containing unique Xhol or BamHI sites and insertion of the amplified promoter products into pLGS5-D5 digested with Xhol and BamHI. pUASGCYC1/lacZ and pCYC1/lacZ are derivatives of pLGS5D5 and pLB (10), respectively. The pCycGAL1A reporter plasmid was constructed by amplification of CYC1 promoter sequences from pLGS5D5 with primers corresponding to CYC1 sequences extending ~140 nt 5′ of the Intromechinon codon, which contain Xhol restriction sites, and primers that correspond to 5′ promoter-proximal lacZ sequences. Amplified products were digested with Xhol and BamHI and ligated into pLGS5-D5 digested with Xhol and BamHI. The pCycGALB mutant promoter-lacZ reporter construct was generated by the same method, except that the 5′ CYC1 promoter primers encoded the (Ato-G) nucleotide substitution in CYC1 TATA sequences. In vitro transcription plasmid pBSβ-GAL was generated by digestion of pCMV/β-GAL (11) with EcoRI and BamHI, purification of DNA fragments containing the 3′ 450 bp of lacZ sequences, and ligation of the purified DNA fragments into BlueScript II KS digested with EcoRI and BamHI.

Yeast transformation and analysis. To identify yTPB gain-of-function mutations in yeast strain Y190, which contains an integrated HIS3 growth-suppressed marker and an integrated lacZ reporter, both under the control of GAL4 upon upstream activating sequences, was cotransformed with pGAL4-(AAM)-Δ(AAAM) and a yTPB expression plasmid encoding a library of yTPB mutants. Yeast colonies were collected for an additional streptomycin-supplement resistant growth on lacking tryptophan, leucine, and histidine and containing 40 mM 3-amino-1,2,4-triazole. After ~5 days of growth on selective medium, large colonies were harvested and subjected to a secondary screen that measured induced β-galactosidase activity. For yTPB expression vectors, yeast strain LLYV (5) was cotransformed with pGAL4-(AAM)-Δ(AAAM) effector plasmid and a library of mutant yTPB expression plasmids generated by amplification of yTPB sequences by PCR under conditions that favor random mis sense mutations. Growth on selective media led to the identification of a single yeast colony that expressed a mutated yTPB (yTPB69S) that

formulated along with the GAL4 effector plasmids to ensure that the observed increase in transcription activation was yTPB sequence dependent.

To characterize yTPB mutants, yeast strain Y190, which contains an integrated lacZ reporter, was cotransformed with GAL4 expression vectors and yTPB expression vectors. Alternatively, yeast strain LDD34A, which carries GAL4 expression vectors, yTPB expression vectors, and the pLGS5D5 reporter plasmid. Yeast growth was selected for on synthetic dextrose-supplemented media either lacking both tryptophan and leucine or lacking tryptophan, leucine, and histidine. For the analysis of different yeast mutants, yeast strain PS316 was cotransformed with yTPB expression plasmids and the pADH1lacZ, pPGKlacZ, or pCYC1lacZ reporter construct, and growth was selected for on synthetic medium lacking leucine and uridine. After growth selection, yeast cells were harvested and β-GAL activity from yeast cell extracts was measured as described previously (5).

Expression levels of GAL4 fusion proteins were determined by Western blot analysis. Briefly, yeast cells cotransformed with pGAL4-(AAM), pGAL4-VP16, pGAL4-K-(AAM), or pGAL4-K-(AAM)-Δ(AAAM), and either yTPBwt or yTPB69S, were harvested, and yeast cell proteins were concentrated by trichloroacetic acid precipitation. Concentrated proteins were resuspended in Laemmli sample buffer, subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred to nitrocellulose prior to Western analysis with polyclonal antiserum directed against the GAL4 DNA binding domain. Immune complexes were visualized by autoradiography with enhanced chemiluminescence.

RNA isolation and analysis. Total cellular RNA was isolated by the hot-phenol method from yeast cells (strain PS316) cotransformed with yTPB expression plasmids and pADH1lacZ. RNase T1 protection analysis was performed as follows. To generate the riboprobe, pBSS-GAL was digested with EcoRI and used as a template for bacteriophage T7 RNA polymerase in the presence of [α-32P]CTP. Ten micrograms of total RNA was hybridized to 5 × 106 cpm of RNA probe at 42°C before RNase T1 digestion. RNase protection was quantitated with a PhosphorImager (Molecular Dynamics, Inc.). As a control for quantification of RNA analysis, reactions were run on nitrocellulose filters and hybridized to oligonucleotide probes, corresponding to RNA sequences, labeled by incubation with [γ-32P]ATP and bacteriophage T4 polynucleotide kinase.

In vitro analysis of TBD DNA binding. His-tagged TBDs were expressed from pQE-31 plasmids in E. coli and purified to greater than 50% homogeneity as described by the manufacturer. TBD concentrations were quantitated by serial dilution of purified TBDs, electrophoresis on sodium dodecyl sulfate-polyacrylamide gels, Coomassie blue staining, and comparison to standards of known concentrations. Ten, 25, or 50 ng of purified TBDs were incubated in gel shift buffer (25) with an oligonucleotide probe containing a 20-nt sequence spanning the adenovirus major late promoter TATA box, which was labeled by incubation with [γ-32P]ATP and T4 polynucleotide kinase. Alternatively, 50 ng of TBD was incubated with a 32P-labeled DNA probe corresponding to the CYC1 promoter (spanning sequences 30 to 140 nt of the CYC1 Intromechinon codon) containing either the wild-type TATA sequences or the TGT4 mutation. TBD-DNA complexes were separated from free probe by electrophoresis on 4.5% native polyacrylamide gels run in a Tris-glycine buffer system supplemented with 5 mM MgCl2.

RESULTS

Identification of a yTPB mutant that facilitates transcription. We and others have previously demonstrated that natural activation domains, such as those found in the VP16 and RelA transcriptional activators, consist of multiple, highly synergistic acidic activation modules (AAMs) of ~11 amino acids in length (4, 35, 36). To identify TBD mutations that facilitated transcription initiation, we used a weak synthetic transcriptional activator, termed GAL4-(AAM)-Δ(AAAM), consisting of the GAL4 DNA binding domain linked to one functional and one mutant 11-amino-acid AAM (4). In the context of the yeast Y190 indicator strain, this weak activator induces low but readily detectable levels of an integrated GAL1lacZ indicator gene but is not able to induce levels of expression of an integrated GAL1-HIS3 selectable marker adequate to sustain yeast cell growth on histidine-minus media containing 40 mM 3-amino-1,2,4-triazole.

To screen for TBD gain-of-function mutants, yeast strain Y190 was cotransformed with pGAL4-(AAM)-Δ(AAAM) effector plasmid and a library of mutant yTPB expression plasmids generated by amplification of yTPB sequences by PCR under conditions that favor random mis sense mutations. Growth on selective media led to the identification of a single yeast colony that expressed a mutated yTPB (yTPB69S) that
TABLE 1. Induction of β-GAL activity by GAL4-activation domain fusions in the presence of yTBPwt or yTBPN69S

| Promoter | Effector | yTBPwt | yTBPN69S | Fold activation
<table>
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<tbody>
<tr>
<td>ADH1a</td>
<td>GAL4</td>
<td>&lt;1 (20)</td>
<td>&lt;1 (98)</td>
<td>4.9</td>
</tr>
<tr>
<td></td>
<td>GAL4-(AAM)</td>
<td>8</td>
<td>243</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>GAL4-(AAM)-(ΔAAM)</td>
<td>136</td>
<td>428</td>
<td>3.1</td>
</tr>
<tr>
<td></td>
<td>GAL4-RelA</td>
<td>4,960</td>
<td>5,760</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>GAL4-VP16</td>
<td>4,900</td>
<td>6,490</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>GAL4-(AAM)</td>
<td>60</td>
<td>309</td>
<td>5.2</td>
</tr>
<tr>
<td></td>
<td>GAL4-(AAM)-(ΔAAM)</td>
<td>538</td>
<td>938</td>
<td>1.7</td>
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Induced β-GAL activities were measured 5 min after addition of the substrate and are presented in milli-optical density units, which are corrected for any dilution required to maintain samples in the measurable range. Numbers in parentheses represent β-GAL activities measured 5 h after addition of the substrate.

a Ratio of β-GAL activity induced in the presence of yTBPN69S to that seen in the presence of yTBPw.
b GAL4 fusion proteins differed by less than 5 and 40%, respectively, in the presence of yTBPw versus yTBPN69S. c GAL4 fusions were expressed from the ADH1 promoter and induced β-GAL activities were measured from an integrated GAL1::lacZ reporter. d GAL4 fusions were expressed from the PGK promoter, and induced β-GAL activities were measured from the pLG5DS reporter plasmid.

A recent study reported an inverse correlation between activation domain potency and the levels of activator required for site saturation on promoter DNA (42). Therefore, increases in the levels of weak activator expression may result in disproportionate increases in reporter gene activity. Such a phenomenon was not inconsistent with the profile observed with GAL4 fusions expressed from the ADH1 promoter (Table 1), and it remained possible that the yTBPN69S-mediated effects on reporter gene expression in the presence of weak activators might be entirely indirect. To examine such a possibility, the GAL4-(AAM) and GAL4-(AAM)-(ΔAAM) fusion proteins were expressed from the full-length PGK promoter (Table 1). The activity of the highly potent PGK promoter is largely unaffected by yTBPN69S (see below); therefore, steady-state levels of activator protein expressed from the PGK promoter should be similar in the presence of yTBPw or yTBPN69S. This expectation was confirmed by the Western blot analysis shown in Fig. 1B. Pixel quantitation of the Western blot analysis in Fig. 1B with the NIH Image 1.60 program demonstrated that expression levels of the GAL4-(AAM) and GAL4-(AAM)-(ΔAAM) proteins differed by less than 5 and 40%, respectively, in the presence of yTBPw versus yTBPN69S. Despite very similar activator expression levels, a significant increase (fivefold) in GAL4-(AAM)-induced β-GAL activity was observed in the presence of yTBPN69S compared to that observed in the presence of yTBPw (Table 1). In contrast, the yTBPN69S-induced increase in β-GAL expression observed in the presence of the more active GAL4-(AAM)-(ΔAAM) fusion protein was a more modest ~2-fold (Table 1). Therefore, these data demonstrate that yTBPN69S can increase activator-induced gene expression in the absence of any change in the...
level of the activator protein. In addition, these data further confirm the hypothesis that the level of enhancement of activator function by yTBPN69S is inversely correlated with activator strength.

It is important to note that the yTBPN69S mutant fails to support yeast viability when expressed in the absence of wild-type yeast TBP (data not shown), and the effect of yTBPN69S on gene expression in vivo could therefore be measured only in the presence of endogenously expressed wild-type yTBP. However, although yTBPN69S is presumably expressed at higher levels than yTBPwt due to yTBPN69S-mediated up-regulation of its cognate promoter, it is unlikely that such an increase contributed significantly to the mutant phenotype. Previous studies have demonstrated that overexpression of yTBPwt at levels up to 20-fold greater than those of endogenous TBP had no significant effect on basal or activated transcription in yeast cells (33, 52). Consistent with these earlier studies, we observed that overexpression of yTBPwt from ARS-CEN-based yeast plasmids or high-copy-number 2µm yeast plasmids also had no effect on reporter gene expression (data not shown).

**yTBPN69S selectively enhances RNA expression from weak promoters.** The data presented in Table 1 and Fig. 1 demonstrate that yTBPN69S can enhance the level of gene expression directed by relatively weak promoter elements such as the ADH1 promoter or basal or weakly activated forms of the GAL1 promoter element. However, yTBPN69S failed to enhance the level of gene expression directed by the GAL1 promoter when this was also acted upon by the potent GAL4-VP16 or GAL4-RelA transcriptional transactivators. These observations raised the possibility that yTBPN69S might selectively act on only weak promoter elements.

To test this hypothesis directly, reporter constructs containing the lacZ gene under the control of either a truncated ADH1 promoter, a truncated CYC1 promoter, or the PGK promoter were cotransformed into yeast cells along with plasmids expressing either yTBPwt or yTBPN69S. As shown in Table 2, the level of β-GAL activity observed with these ADH1 or CYC1 promoter derivatives increased by 22- and 17-fold, respectively, in the presence of yTBPN69S when compared to yTBPwt. In contrast, and as also shown in Fig. 1B, the level of β-GAL activity induced by the stronger PGK promoter was essentially unaffected by yTBPN69S. Taken together with the results presented in Table 1 and Fig. 1, these data demonstrate that yTBPN69S induces levels of gene expression from three different weak or basal yeast promoter elements that are from 4- to 31-fold higher than those seen with yTBPwt alone. In contrast, gene expression directed by highly active promoters, such as the PGK promoter, is not significantly affected by the presence of yTBPN69S. As no DNA sequence recognition elements for upstream transcriptional activators are known to be present in the ADH1 or CYC1 yeast promoter sequences used for Table 2, yTBPN69S most likely enhances gene expression levels via a direct, activator-independent mechanism in these cases.

Next, the effect of yTBPN69S on mRNA expression was directly measured by using yeast cells cotransformed with the pADH1/lacZ reporter plasmid and with plasmids expressing yTBPwt or yTBPN69S. After growth on selective media, cells were harvested and total cellular RNA was isolated and subjected to RNase T1 protection analysis with a 32P-labeled RNA probe complementary to the 3′-450 nt of the lacZ gene. To ensure that comparable amounts of RNA were analyzed, a portion of the yeast cell RNA was bound to nitrocellulose filters and hybridized to a 32P-labeled probe complementary to rRNA.

**yTBPN69S and yTBPwt exhibit similar affinities for TATA DNA in vitro.** Analysis of the sequence of the yTBPN69S mutant demonstrated that a single amino acid substitution (Asn to Ser) at residue 69 of yTBP was sufficient to confer the mutant phenotype. The cocrystal structure of yeast TBP with the CYC1 promoter TATA box predicts that yTBP residue 69 interacts through hydrogen bonding with two thymidine acceptor residues in the minor groove of TATA DNA (22). These observations suggested that the yTBPN69S mutant might exhibit an increased affinity for promoter DNA compared to wild-type TBP, resulting in increased recruitment and, hence, increased transcription initiation. To test this hypothesis directly, yTBPwt and yTBPN69S proteins were expressed in E. coli and then purified by column chromatography (see Materials and Methods). Recombinant yTBPwt or yTBPN69S proteins were incubated at increasing concentrations with a 32P-labeled DNA probe that includes the ADMLP TATA box, and TBP-DNA complexes were separated from unbound probe by nondenaturing polyacrylamide gel electrophoresis. As shown in Fig. 3, yTBPwt and yTBPN69S exhibit similar affinities for TATA-containing DNA probes, with yTBPN69S, if anything, showing a slightly lower affinity for TATA DNA. Of interest, yTBPN69S-DNA complexes exhibited a slightly slower electro-
phoretic mobility than yTBPwt-DNA complexes in nondenaturing polyacrylamide gels (Fig. 3). Competition experiments with wild-type or mutant TATA sequences demonstrated that the observed complexes were specific for wild-type TATA sequences, while kinetic analyses of yTBPwt- or yTBPN69S-DNA complex formation failed to reveal significant differences in association or dissociation rates in vitro (data not shown). These findings therefore suggest that the effect of yTBPN69S on Pol II-driven gene expression does not result from an increased affinity for TATA DNA. Further comparative analyses of TBP–wild-type TATA DNA interactions in vitro (e.g., by DNA footprinting) were not performed based on the demonstration that a direct TBP-DNA interaction was not required for yTBPN69S-mediated enhancement of gene expression (see Fig. 5).

yTBPN69S increases gene expression by increasing TBP promoter recruitment in vivo. If TBP/TFIID recruitment to Pol II promoter elements represents a rate-limiting step in transcription initiation, it should then be possible to at least partly bypass the need for transcriptional activators by efficiently targeting TBP to promoters by using a heterologous DNA binding domain. As shown by several groups, this is indeed the case (6, 23, 49). With this approach, it has recently proven possible to subdivide inactive mutants of TBP into a class that is inefficiently recruited to promoter elements, and that can therefore be rescued by fusion to a heterologous DNA binding domain, and into a second class that is defective for a step subsequent to promoter recruitment, and that is therefore not rescued (39).

We have therefore used this in vivo TBP recruitment assay to determine if yTBPN69S acts at the level of TBP promoter recruitment or at a step following recruitment. Plasmids expressing GAL4-yTBPwt or GAL4-yTBPN69S fusions were cotransformed with lacZ reporter constructs that contained ei-
...fore demonstrate that activation of weak promoter elements by the yTBPN69S mutant is not dependent upon the integrity of the TATA element.

yTBPN69S function does not require direct interaction with DNA. The observation that the yTBPN69S mutant can activate transcription from a promoter containing a mutant TATA element (Fig. 4) raises the possibility that this mutant might exhibit a higher affinity for such a TATA element. To directly test this hypothesis, recombinant yTBPTwt or yTBPN69S proteins were incubated with 32P-labeled probes corresponding to CYC1 promoter DNA containing either the TGTA or wild-type TATA sequences. TBP-DNA complexes were separated from free probe by electrophoresis on nondenaturing polyacrylamide gels. Consistent with the data shown in Fig. 3, yTBPWT and yTBPN69S exhibited similar affinities for CYC1 promoter DNA containing a wild-type TATA box (Fig. 5A, lanes 2 and 3). Also consistent with the data in Fig. 3, yTBPN69S-DNA complexes again exhibited a slower electrophoretic mobility than yTBPWT-DNA complexes. However, neither yTBPTwt nor yTBPN69S bound efficiently to CYC1 promoter sequences containing the TGTA mutant TATA box (Fig. 5A, lanes 5 and 6). These results were surprising given the observation that yTBPN69S mediated readily detectable levels of gene expression from a CYC1 promoter containing a TGTA mutant TATA box (Fig. 4) and suggested that yTBPN69S-mediated gene expression might not depend on a direct TBP-DNA interaction.

To directly address this hypothesis in vivo, the effect of a second amino acid substitution on yTBPN69S-directed gene expression was examined. A Leu-to-Lys amino acid substitution at yTBP residue 205, which totally disrupts TBP DNA binding activity in vitro and in vivo (6, 50), was introduced into the contexts of both yTBPTwt and yTBPN69S. A lacZ reporter construct containing wild-type CYC1 promoter sequences (pCYCd1A) was then cotransformed into yeast along with plasmids expressing either yTBPTwt, yTBPN69S, yTBPL205K, or the yTBPN69S/L205K double mutant. After growth on selective media, β-GAL activity from yeast cell extracts was measured. Consistent with the data presented above, an ~10-fold increase in β-GAL activity from the pCYCd1A reporter plasmid was observed in the presence of yTBPN69S compared to that in the presence of yTBPTwt was measured (Fig. 5B). Remarkably, a similar increase in β-GAL activity was also observed for the yTBPN69S/L205K double mutant compared to yTBPL205K (Fig. 5B). These data demonstrate that the L205K yeast TBP mutation is unable to block the effect of the N69S substitution on Pol II-directed gene expression in vivo. Consistent with a previous report (50), electrophoretic mobility shift analysis confirmed that yTBPN69S/L205K exhibited no detectable DNA binding activity (data not shown). Therefore, taken together...
with the findings presented above, these data demonstrate that yTBPN69S-mediated gene expression does not depend on a direct TBP-DNA interaction.

Although a direct TBP-DNA interaction is not required, a functional TBP-TFIIA interaction is essential for yTBPN69S enhancement of gene expression. As shown in Fig. 5B, the introduction of the amino acid substitutions Lys to Thr and Tyr to Ala at yTBP residues 138 and 139, respectively, abolished the yTBPN69S mutant phenotype. This double substitution mutation, which is termed N2-1, has been shown to specifically impair the response of yTBP to acidic activators in vivo by disrupting yTBP-TFIIA interactions (38). Therefore, these data are again consistent with the hypothesis that yTBPN69S and transcriptional transactivators affect the same step in the enhancement of gene expression.

**DISCUSSION**

Transcription of a eukaryotic Pol II-dependent gene in vivo requires the recruitment of a complete set of GTFs to the relevant promoter element. Considerable evidence now indicates that transcriptional transactivators increase the rate of transcription by increasing the efficiency with which GTFs are recruited to specific promoter elements. While evidence suggesting direct contacts between activation domains and several GTFs has been presented, evidence for a critical role for TFIIID recruitment in activated transcription is particularly strong. Relevant observations include the in vitro demonstration of specific contacts between activation domains and both the TBP and TAF components of TFIIID (4, 9, 14, 17–19, 26, 27, 40, 45, 48, 50), the finding that specific TAFs are required to mediate transcriptional activation in vitro by specific classes of activation domains (7, 30, 44), the in vivo demonstration that acidic activation domains enhance the kinetics of TBP recruitment (24), and, finally, the in vivo demonstration that tethering TBP to a promoter via a heterologous DNA binding domain results in efficient transcription initiation even in the absence of DNA-bound transcriptional transactivators (6, 23, 49).

The hypothesis that TFIIID recruitment is a rate-limiting step in transcription initiation that is specifically enhanced by transcriptional transactivators leads to the prediction that TFIIID mutants with interesting phenotypes should be obtainable. While no gain-of-function mutations of TFIIID or TBP have been reported previously, Kim et al. (21) have presented data suggesting that mutations that disrupt the in vitro interaction between yeast TBP and the VP16 activation domain can specifically block in vivo transcription activation by VP16 without affecting basal transcription. However, the subsequent report by Tansey and Herr (43) that a mutation in human TBP that blocks a presumably similar reported in vitro interaction with the VP16 activation domain fails to inhibit transcription activation by VP16 in human cells has rendered the interpretation of this earlier result more difficult. Clearly, strong support for a critical role for TFIIID recruitment in activated transcription would be provided by a gain-of-function mutation within a TFIIID subunit that, at least in part, rendered transcriptional transactivators dispensable for efficient transcription initiation in vivo. The N69S missense mutation described in this report represents a mutation in the TBP component of yeast TFIIH that conforms precisely to this predicted phenotype.

An important property of the yTBPN69S mutant is that it specifically and potently enhances transcription from several weak or basal yeast promoter elements but does not increase gene expression directed by highly active natural or synthetic yeast promoters (Tables 1 and 2 and Fig. 1). This is precisely the pattern that one would expect if this mutation is affecting the same rate-limiting step that is also targeted by transcriptional transactivators. Consistent with this, amino acid substitution mutations in yTBP sequences, which specifically inhibit activated, rather than basal, transcription in vivo, also block yTBPN69S-mediated enhancement of gene expression. If the N69S mutation indeed acts exclusively at the step of promoter recruitment, which is also thought to be modulated by transcriptional transactivators, then this mutant should lack a phenotype when recruitment is no longer rate limiting, i.e., if TBP is efficiently recruited to a promoter element by tethering to a heterologous DNA binding domain. As shown in Table 3, this prediction is fully borne out, thus strongly suggesting that N69S indeed acts by enhancing promoter recruitment efficiency.

Because the asparagine residue mutated in yTBPN69S is predicted, in the reported crystal structure of TBP-DNA complexes, to directly contact DNA, it appeared possible that this TBP mutation acted by directly affecting DNA affinity. Several lines of evidence argue against this simple hypothesis: (i) the mutant yTBPN69S protein binds TATA DNA with an affinity similar to that of the wild type when measured in vitro (Fig. 3 and 5A); (ii) the yTBPN69S mutant protein strongly enhances gene expression directed by a promoter containing a mutant TATA box that is not effectively utilized efficiently by wild-type TBP (Fig. 4) and that does not bind recombinant wild-type or N69S mutant TBP detectably (Fig. 5A); and (iii) a mutation in TBP that totally blocks specific DNA binding fails to block the increased level of promoter expression induced by the N69S mutation (Fig. 5B). Taken together, these data clearly argue that the N69S mutation in yTBP does not act by directly promoting DNA binding by TBP. We therefore conclude that the N69S mutation is acting indirectly, most probably by promoting the interaction of other TFIIID components with promoter DNA and/or other DNA-bound GTFs (13). The observation of numerous TATA-less Pol II promoters both in higher eukaryotes and in yeast clearly indicates that at least one such TATA-independent mechanism exists. Indeed, Martinez et al. (29) have demonstrated that a human TBP mutant, which is defective for TATA binding activity, can direct efficient transcription from TATA-less promoters in vitro. We therefore hypothesize that yTBPN69S is acting to increase the efficiency of this alternate, TATA box-independent path of TFIIID recruitment.

Alternative explanations for the mechanism of yTBPN69S enhancement of gene expression could be proposed. Thus, the N69S mutation could interfere with TFIIID dimerization (41), thus giving rise to increased pools of endogenous TFIIID competent for DNA binding and transcription initiation. Or, similarly, the N69S mutation could interfere with the ability of global repressors of Pol II transcription, such as Mot1 (1), to inhibit TBP binding to promoter DNA. However, both of these proposed mechanisms would clearly predict a requirement for a direct interaction between the yTBPN69S mutant and responsive promoter elements, which was not found to be the case (Fig. 4 and 5). A final potential explanation for the N69S phenotype could be that yTBPN69S effectively sequesters negative regulators of transcription, thus resulting in increased levels of reporter gene expression mediated by the endogenous wild-type TBP. However, this explanation is also inconsistent with the observation that yTBPN69S expression resulted in high levels of gene expression directed by a promoter containing a mutant TATA element that is not utilized effectively by wild-type TBP (Fig. 4). Additionally, yTBPN69S enhancement of gene expression was blocked by the N2-1 amino acid substitution mutation of yTBP, which has been shown previously.
to specifically disrupt the interaction of yTBP with TFIIA (38). Because this mutation has been shown to selectively block activated, rather than basal, transcription (38), it appears very unlikely that it would also exert a pleiotropic effect on the interaction of yTBP with other protein targets, such as potential negative regulators. Therefore, while the precise mechanism by which N69S increases promoter recruitment of yeast TFIID remains unclear, the phenotype of this TBP mutation does provide important genetic support for the hypothesis that TFIID recruitment is both rate limiting for transcription initiation in vivo and a specific target for transcriptional transactivator function.

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