MOLECULAR AND CELLULAR BIOLOGY, May 1997, p. 2888–2896 Vol. 17, No. 5
0270-7306/97/$04.00+0

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

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Received 7 November 1996/Returned for modification 31 December 1996/Accepted 14 February 1997

We describe a unique gain-of-function mutant of the TATA-binding protein (TBP) subunit of *Saccharomyces cerevisiae* TFIID 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 TFIID 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 TFIID 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, TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH (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 TFIID (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′-TATAAA-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 TFIID to the promoter element via a direct interaction with a TBP-associated factor (TAF) component of TFIID (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* T_C 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 TFIID binding sites functionally equivalent to Inr elements.

Recruitment of TFIID 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). TFIID may nucleate the assembly of the basal transcription machinery on Pol II promoters, analogous functionally to prokaryotic σ factors (13). The TBP component of TFIID 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 TFIID 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 (yTBPN69S) 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 yTBPN69S 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 yTBPN69S function. Nevertheless, comparison of the activities of yTBPN69S and wild-type yTBP targeted to promoter DNA by heterologous DNA binding domains indicates that the observed increase in yTBPN69S-mediated gene expression results from an increase in the efficiency of yTBPN69S promoter recruitment.

MATERIALS AND METHODS

Plasmids. The GAL4 fusion protein expression plasmids pGAL4, pGAL4-VP16, and pGAL4-RelA have been previously described (3). pGAL4-(AAM) and pGAL4-(AAM)-(ΔAAM), which encode GAL4-activation domain fusions expressed from a truncated *ADH1* promoter, were constructed by the digestion of the mammalian expression vectors pGAL4-(AAM) and pGAL4-(AAM)-(ΔAAM) (4) with the restriction endonucleases *Xho*I and *Eco*RI. The resulting *Xho*I/*Eco*RI fragment, containing both GAL4 sequences and (AAM) or (AAM)-(ΔAAM) activation domain sequences, was introduced into the yeast GAL4 expression vector pGBT9 (Clontech) digested with *Xho*I and *Eco*RI. Alternatively, these same DNA fragments were introduced into a derivative of pGBT9 containing the *PGK* promoter in place of the *ADH1* promoter to construct pGAL4K-(AAM) or pGAL4K-(AAM)-(ΔAAM). pGAL4-yTBPwt and pGAL4-yTBPN69S were constructed by amplification of a wild-type TBP cDNA, or a TBP cDNA encoding the N69S mutation, by PCR with primers that introduced flanking *Eco*RI restriction sites. Amplified products were then digested with *Eco*RI and introduced into the pGBT9 expression plasmid.

yTBP expression plasmids (e.g., pyTBPwt) were generated by PCR amplification of yTBP sequences by using primers that contained unique *Xho*I or *Eco*RI restriction sites. Digested products were then inserted into a Ycplac111 (12) ARS-CEN-based plasmid derivative, which contains the yeast *ADH1* promoter, at unique *Xho*I and *Eco*RI sites. The library of TBP mutations was generated in the same manner, except that PCR was carried out under conditions that favored the random incorporation of missense mutations. A yTBP expression plasmid encoding a lysine substitution for leucine 205 (L205K) was generated by recombinant PCR with oligonucleotides spanning yTBP-coding sequences encoding residues 199 to 208 and 5′ and 3′ flanking oligonucleotide primers containing unique *Xho*I or *Eco*RI restriction sites, respectively. A yTBP expression plasmid encoding a Lys 138-to-Thr and Tyr 139-to-Ala amino acid substitution mutation (N2-1) was similarly generated by PCR amplification of yTBP sequences with a primer that spanned yTBP sequences encoding residues 131 to 141 and a 3′ flanking oligonucleotide primer that contained a unique *Eco*RI restriction site. Amplified products were digested with *Msc*I and *Eco*RI and ligated to yTBP expression plasmids digested with the same restriction endonucleases. *Escherichia coli* expression plasmids expressing yTBP were constructed by PCR amplification of TBP sequences with primers that contained *Xho*I or *Pst*I sites and ligation of the amplified products into pQE-31 (Qiagen) digested with *Sal*I and *Pst*I. TBP-coding regions were sequenced in their entireties from plasmids generated by PCR to ensure their identities.

The pADH1/lacZ or pPGK/lacZ reporter plasmids were constructed by PCR amplification of *ADH1* (2) or *PGK* (32) promoter sequences extending −412 nucleotides (nt) or −572 nt 5′ of their Inr methionine codons, respectively, with oligonucleotide primers containing unique *Xho*I or *Bam*HI sites and insertion of the amplified products into pLGSD5 (15) digested with *Xho*I and *Bam*HI. $pUAS_GCYC1/lacZ$ and pCYC1/lacZ are derivatives of pLGSD5 and pJLB (10), respectively. The pCYCd1A reporter plasmid was constructed by amplification of *CYC1* promoter sequences from pLGSD5 with primers corresponding to *CYC1* sequences extending −140 nt 5′ of the *CYC1* Inr methionine codon, which contain *Xho*I restriction sites, and primers that correspond to 3′ promoter-proximal *lacZ* sequences. Amplified products were digested with *Xho*I and *Bam*HI and ligated into pLGSD5 digested with *Xho*I and *Bam*HI. The pCYCd1B mutant promoter-*lacZ* reporter construct was generated by the same method, except that the 5′ *CYC1* promoter primers encoded the (A-to-G) nucleotide substitution in *CYC1* TATA sequences. In vitro transcription plasmid pBS/β-GAL was generated by digestion of pCMV/β-GAL (11) with *Eco*RI and *Bam*HI, 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 *Eco*RI and *Bam*HI.

Yeast transformation and analysis. To identify yTBP gain-of-function mutations, yeast strain Y190, which contains an integrated *HIS3* growth-selectable marker and an integrated *lacZ* reporter, both under the control of GAL4 upstream activating sequences, was cotransformed with pGAL4-(AAM)-(ΔAAM) and a yTBP expression plasmid encoding a library of yTBP mutations. Yeast colony growth was selected for on synthetic dextrose-supplemented medium 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 (β-GAL) activity, as previously described (5), to ensure that yeast colony growth correlated with increased transcriptional activity. yTBP mutants were then isolated from yeast colonies exhibiting increased levels of induced β-GAL activity compared to that observed from yeast transformed with the GAL4-(AAM)-(ΔAAM) effector and a wild-type yTBP (pyTBPwt) expression plasmid. Mutant yTBP expression plasmids were sequenced, reconstructed, and cotransformed along with the GAL4 effector plasmids to ensure that the observed increase in transcription activation was yTBP sequence dependent.

To characterize yTBP mutants, yeast strain Y190, which contains an integrated *lacZ* reporter, was cotransformed with GAL4 expression vectors and yTBP expression vectors. Alternatively, yeast strain LDY434 was cotransformed with GAL4 expression vectors, yTBP expression vectors, and the pLGSD5 reporter plasmid. Yeast growth was selected for on synthetic dextrose-supplemented media either lacking both tryptophan and leucine or lacking tryptophan, leucine, and uridine. For the analysis of different yeast promoters, yeast strain PSY316 was cotransformed with yTBP expression plasmids and the pADH1/lacZ, pPGK/lacZ, or pCYC1/lacZ 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, pGAL4K-(AAM), or pGAL4K-(AAM)-(ΔAAM), and either yTBPwt or yTBPN69S, 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 PSY316) cotransformed with yTBP expression plasmids and pADH1/lacZ. RNase T_1 protection analysis was performed as follows. To generate the riboprobe, pBS/β-GAL was digested with *Eco*RI and used as a template for bacteriophage T7 RNA polymerase in the presence of [α-^{32}P]CTP. Ten micrograms of total RNA was hybridized to 5×10^5 cpm of RNA probe at 42°C before RNase T_1 digestion. RNase protection was quantitated with a PhosphorImager (Molecular Dynamics, Inc.). As a control for quantities of RNA analyzed, 0.5 μg of total cellular RNA was immobilized on nitrocellulose filters and hybridized to oligonucleotide probes, corresponding to rRNA sequences, labeled by incubation with [γ-^{32}P]ATP and bacteriophage T4 polynucleotide kinase.

In vitro analysis of TBP DNA binding. His-tagged TBPs were expressed from pQE-31 plasmids in *E. coli* and purified to greater than 50% homogeneity as described by the manufacturer. TBP concentrations were quantitated by serial dilution of purified TBPs, electrophoresis on sodium dodecyl sulfate-polyacrylamide gels, Coomassie blue staining, and comparison to standards of known concentrations. Ten, 25, or 50 ng of purified TBPs 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-^{32}P]ATP and T4 polynucleotide kinase. Alternatively, 50 ng of TBP was incubated with a ^{32}P-labeled DNA probe corresponding to the *CYC1* promoter (spanning sequences 50 to 140 nt 5′ of the *CYC1* Inr methionine codon) containing either the wild-type TATA sequences or the TGTA mutant. TBP-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 $MgCl_2$.

RESULTS

Identification of a yTBP mutant that facilitates transcription. We and others have previously demonstrated that natural acidic activation domains, such as those found in the VP16 and RelA transcriptional transactivators, consist of multiple, highly synergistic acidic activation modules (AAMs) of ~11 amino acids in length (4, 35, 36). To identify TBP mutations that facilitated transcription initiation, we used a weak synthetic transcriptional transactivator, termed GAL4-(AAM)-(ΔAAM), 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 *GAL1:lacZ* 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 TBP gain-of-function mutants, yeast strain Y190 was cotransformed with the GAL4-(AAM)-(ΔAAM) effector plasmid and a library of mutant yTBP expression plasmids generated by amplification of yTBP sequences by PCR under conditions that favor random missense mutations. Growth on selective media led to the identification of a single yeast colony that expressed a mutant yTBP (yTBPN69S) that

TABLE 1. Induction of β-GAL activity by GAL4-activation domain fusions in the presence of yTBPwt or yTBPN69S[a]

Promoter	Effector	β-GAL activity with[a]:		Fold activation[b]
		yTBPwt	yTBPN69S	
ADH1[c]	GAL4	<1 (20)	<1 (98)	4.9
	GAL4-(AAM)	8	243	31
	GAL4-(AAM)-(ΔAAM)	136	428	3.1
	GAL4-RelA	4,960	5,760	1.2
	GAL4-VP16	4,900	6,490	1.3
PGK[d]	GAL4-(AAM)	60	309	5.2
	GAL4-(AAM)-(ΔAAM)	538	938	1.7

[a] 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.
[b] Ratio of β-GAL activity induced in the presence of yTBPN69S to that seen in the presence of yTBPwt.
[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 pLGSD5 reporter plasmid.

could significantly facilitate Pol II-dependent gene expression. As shown in Table 1, β-GAL activities induced by GAL4-(AAM)-(ΔAAM), or by the even less active GAL4-(AAM) fusion protein, were 3- and 31-fold greater, respectively, in the presence of yTBPN69S than in the presence of yTBPwt. yTBPN69S-mediated effects on gene expression were not activation domain dependent in that β-GAL levels observed in cells expressing the GAL4 DNA binding domain alone (measured at a later time point) were also ~5-fold higher in the presence of yTBPN69S than in the presence of yTBPwt. In contrast, GAL4 fusion proteins containing the potent activation domains of RelA or VP16, expressed in amounts nontoxic to yeast cell growth, induced similar levels of β-GAL activity in the presence of either yTBPwt or yTBPN69S (Table 1).

The data presented in Table 1 demonstrate that the modest level of gene expression derived from the integrated *GAL1:lacZ* indicator gene in the presence of weak activator proteins is significantly enhanced by coexpression of the yTBPN69S mutant. This effect could reflect not only a direct effect of the yTBPN69S mutant on the integrated *GAL1* promoter element but also an indirect effect resulting from the enhanced expression of activator proteins, which in this experiment were expressed under the control of a truncated *ADH1* promoter element. To examine whether yTBPN69S affected activator protein expression, steady-state levels of GAL4-(AAM) and GAL4-VP16 protein expression were measured by Western blot analysis. As shown in Fig. 1A, both GAL4-(AAM) and GAL4-VP16 were indeed expressed at markedly higher levels from the *ADH1* promoter in the presence of yTBPN69S (compare lanes 3 and 5 with lanes 2 and 4). Therefore, the yTBPN69S mutant can affect the activity of promoters both directly, as in the case of the *ADH1* promoter (Fig. 1A), and indirectly, as seen with the *GAL1* reporter element in the presence of weak activators (Table 1).

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 yTBPwt 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 yTBPwt 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 yTBPwt (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

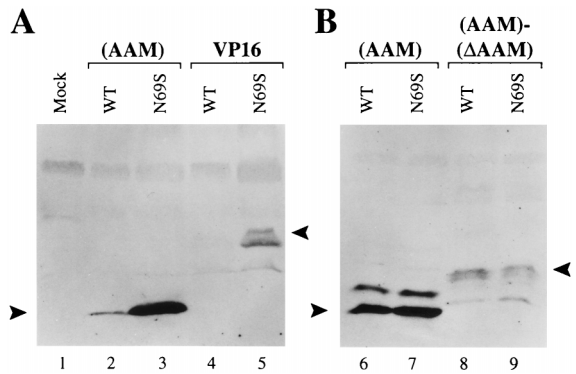

FIG. 1. Western blot analysis of activator expression levels in the presence of yTBPwt or yTBPN69S. Yeast strain Y190 (A) was cotransformed with pGAL4-(AAM) (lanes 2 and 3) or pGAL4-VP16 (lanes 4 and 5), which express GAL4-(AAM) and GAL4-VP16, respectively, under the control of the *ADH1* promoter and with plasmids expressing yTBPwt (WT) or yTBPN69S. Alternatively, yeast strain LDY434 (B) was cotransformed with plasmids expressing GAL4-(AAM) (lanes 6 and 7) or GAL4-(AAM)-(ΔAAM) (lanes 8 and 9) under the control of the *PGK* promoter and with plasmids expressing either yTBPwt or yTBPN69S. After selection, yeast cell proteins were concentrated by precipitation and subjected to polyacrylamide gel electrophoresis. Resolved proteins were transferred to nitrocellulose, and GAL4 fusion proteins were detected by Western blot analysis with a GAL4 DNA binding domain-specific antiserum and enhanced chemiluminescence. The resultant autoradiogram in panel B was exposed for a shorter period of time than that in panel A to ensure comparable signals. Arrowheads denote the positions of the GAL4 fusion proteins.

TABLE 2. β-GAL expression from yeast promoter-*lacZ* fusions in the presence of yTBPwt or yTBPN69S[a]

Promoter fusion	β-GAL activity with:		Fold activation
	yTBPwt	yTBPN69S	
CYC1-lacZ	7	116	17
ADH1-lacZ	48	1,057	22
PGK-lacZ	22,300	34,580	1.5

[a] β-GAL activities from promoter-*lacZ* fusions were measured as described in the footnotes to Table 1.

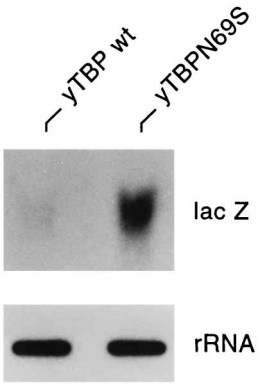

FIG. 2. mRNA expression from the yeast *ADH1* promoter in the presence of yTBPwt or yTBPN69S. Yeast strain PSY316 was cotransformed with the pADH1/lacZ reporter plasmid and with plasmids expressing yTBPwt or yTBPN69S. Transformed yeast cells were harvested, and total yeast cell RNA was isolated and subjected to RNase T_1 protection analysis with a ^{32}P-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 ^{32}P-labeled probe complementary to rRNA.

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 yTBP 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 plasmids expressing either yTBPwt or yTBPN69S. After growth on selective media, cells were harvested and total cellular RNA was isolated and subjected to RNase T_1 protection analysis with a ^{32}P-labeled RNA probe complementary to *lacZ* sequences (Fig. 2). Consistent with the data presented above, levels of *lacZ* mRNA induced from the *ADH1* promoter, quantitated by phosphorimager scanning of the RNase protection analysis, were ~10-fold higher in the presence of yTBPN69S than in the presence of yTBPwt. In addition, DNA slot blot analysis showed that yTBPN69S had no effect on reporter plasmid copy number (data not shown). Therefore, these data demonstrate that yTBPN69S affects gene expression exclusively at the mRNA level.

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 grove 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 ^{32}P-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-

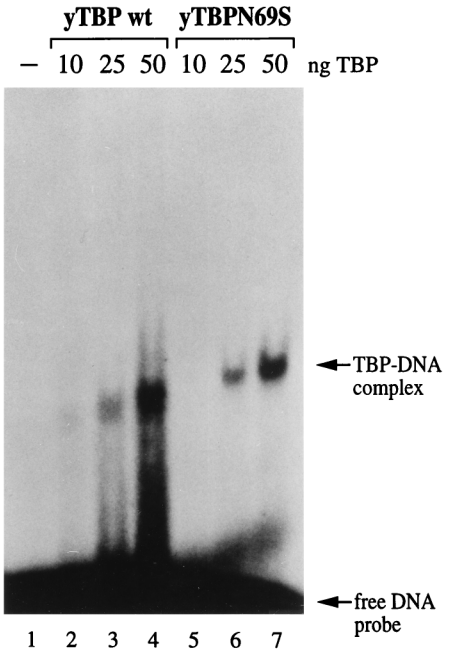

FIG. 3. Electrophoretic mobility shift analysis of yTBP-TATA DNA interactions. Increasing concentrations of recombinant yTBP or yTBPN69S protein were incubated with a ^{32}P-labeled DNA probe containing the ADMLP TATA box. TBP-DNA complexes were separated from free probe by native polyacrylamide gel electrophoresis. –, no TBP added.

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 either the *CYC1* promoter (pCYC1/lacZ) only or an identical *CYC1* promoter flanked by 5′ promoter-proximal GAL4 DNA binding sites (pUAS$_G$CYC1/lacZ). As shown in Table 3, both GAL4-yTBPwt and GAL4-yTBPN69S induced similar levels of β-GAL activity from the pUAS$_G$CYC1/lacZ reporter, which were >25-fold higher than those induced by the GAL4 DNA binding domain alone. In contrast, GAL4-yTBPN69S induced an ~4-fold-higher level of gene expression from the pCYC1/lacZ reporter compared to GAL4-yTBPwt (Table 3), thus demonstrating that fusion to the GAL4 DNA binding domain does not inhibit the enhanced activity described above for yTBPN69S on promoters lacking GAL4 DNA binding sites. These data demonstrate that yTBPwt mediates transcription at levels closely comparable to those of yTBPN69S when targeted to a promoter by a heterologous DNA binding domain and, therefore, indicate that yTBPN69S acts at the step of promoter recruitment in vivo.

TABLE 3. Induction of β-GAL activity by GAL4-yTBP fusions[a]

Reporter	Effector	β-GAL activity[b]
(UAS$_G$)*CYC1-lacZ*	GAL4	24
	GAL4-yTBPwt	629
	GAL4-yTBPN69S	661
CYC1-lacZ	GAL4-yTBPwt	17
	GAL4-yTBPN69S	70

[a] β-GAL activities induced from a *CYC1-lacZ* reporter containing [(UAS$_G$)*CYC1-lacZ*] or lacking (*CYC1-lacZ*) 5′ promoter-proximal GAL4 DNA binding sites were measured 5 min after addition of the substrate.
[b] Activities are in milli-optical density units.

yTBPN69S enhances gene expression from a promoter lacking a functional TATA box. To determine if a functional TATA box was required for yTBPN69S-mediated gene expression, a single nucleotide substitution was introduced into the consensus TATA sequences (TATA→TGTA) of the *CYC1* promoter to give the pCYC1B indicator plasmid. This mutation is known to essentially block TATA element function both in vitro and in vivo (8, 47). In addition, a negative control plasmid (pdCYC) that lacked all *CYC1* promoter sequences and instead retained only the *CYC1* translation initiation codon fused in frame with the *lacZ* gene was constructed. Each reporter construct was cotransformed into yeast cells, along with plasmids expressing either yTBPwt or yTBPN69S. After growth on selective media, yeast cells were harvested and β-GAL activity from cell extracts was measured.

Consistent with the data in Table 2, a significant increase (~10-fold) in β-GAL activity was measured from a *CYC1* reporter containing wild-type TATA sequences (pCYCd1A) in the presence of yTBPN69S compared to yTBPwt (Fig. 4). As expected, very low levels of β-GAL activity were induced by the pdCYC reporter, which lacks *CYC1* promoter sequences, in the presence of either yTBPwt or yTBPN69S. Introduction of an A-to-G nucleotide substitution into the *CYC1* TATA box (TATA→TGTA) in the pCYCd1B mutant led to a marked, >10-fold drop in the level of β-GAL activity observed in the presence of yTBPwt (Fig. 4). In contrast, this mutation had a far less pronounced effect in the presence of yTBPN69S, leading to only an ~2-fold drop in β-GAL activity compared to that with the wild-type pCYCd1A reporter plasmid (Fig. 4). Therefore, yTBPN69S enhanced gene expression from the effectively TATA-less promoter present in pCYCd1B by ~50-fold, an effect that clearly markedly exceeds the ~10-fold effect seen with the wild-type *CYC1* promoter element. These data there-

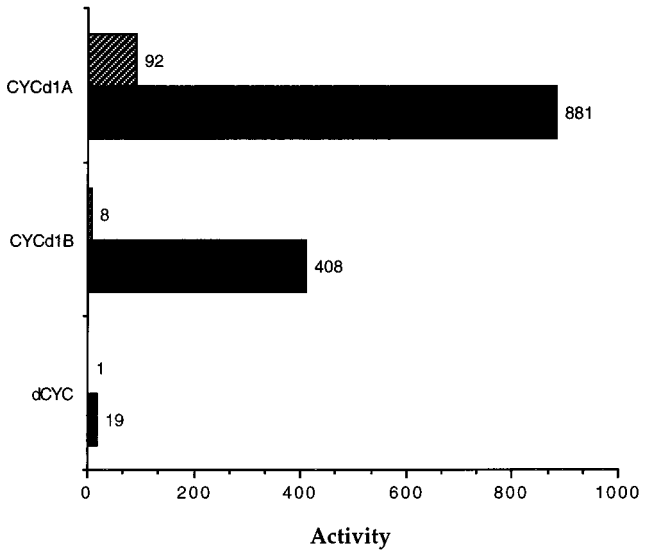

FIG. 4. Activities of *CYC1* promoter derivatives in the presence of yTBPwt or yTBPN69S. Yeast strain PSY316 was cotransformed with either pCYCd1A, which contains a wild-type *CYC1* TATA element; pCYCd1B, which contains a mutant (TATA→TGTA) *CYC1* TATA element; or pdCYC, which contains only the *CYC1* initiation codon fused in frame with the *lacZ* gene, and with plasmids expressing either yTBPwt (hatched bars) or yTBPN69S (solid bars). Transformed yeast cells were harvested and β-GAL activity (given in milli-optical density units) was measured.

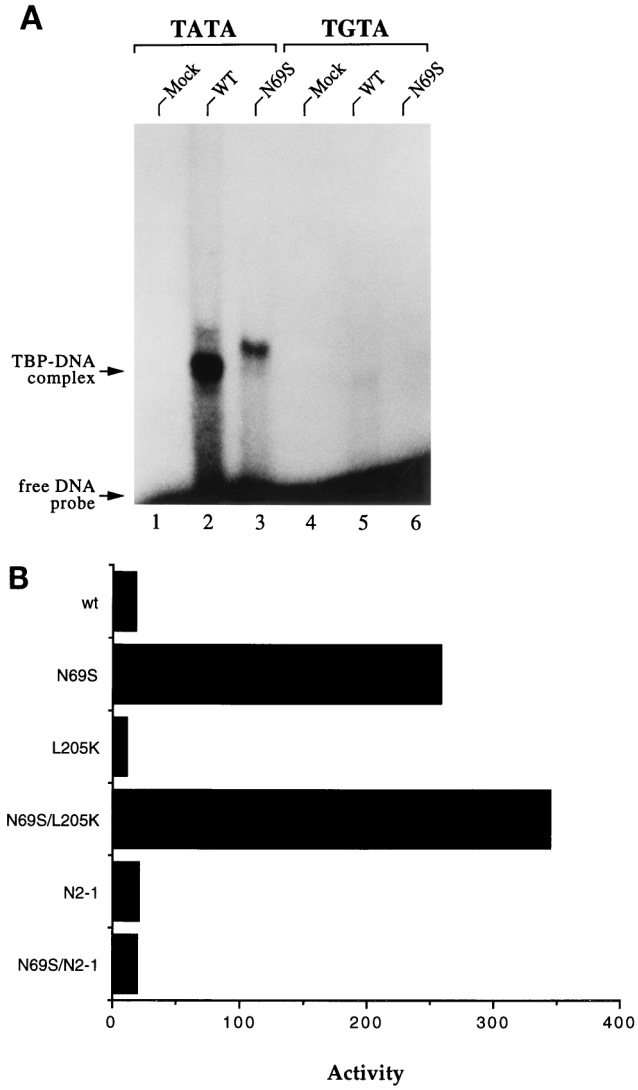

FIG. 5. Functional analysis of yTBPN69S-DNA interactions in vitro and in vivo. (A) Recombinant yTBPwt (WT) or yTBPN69S protein (50 ng) was incubated with ^{32}P-labeled probes corresponding to the *CYC1* promoter containing either a wild-type or TGTA mutant TATA element. TBP-DNA complexes were separated from free probe by native polyacrylamide gel electrophoresis. Mock, DNA probes incubated in gel shift buffer alone. (B) Yeast strain PSY316 was cotransformed with the pCYCd1A reporter plasmid and with plasmids expressing yTBPwt, yTBPN69S, yTBPL205K, yTBPN69S/L205K, yTBPN2-1, or yTBPN69S/N2-1. Transformed yeast cells were harvested, and β-GAL activity from cell extracts (given as milli-optical density units) was measured.

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 yTBPwt or yTBPN69S proteins were incubated with ^{32}P-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 yTBPwt 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-mediated 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 yTBPwt and yTBPN69S. A *lacZ* reporter construct containing wild-type *CYC1* promoter sequences (pCYCd1A) was then cotransformed into yeast along with plasmids expressing either yTBPwt, 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 in the presence of yTBPN69S compared to that in the presence of yTBPwt 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 TFIID 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 TFIID (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 TFIID recruitment is a rate-limiting step in transcription initiation that is specifically enhanced by transcriptional transactivators leads to the prediction that TFIID mutants with interesting phenotypes should be obtainable. While no gain-of-function mutations of TFIID 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 TFIID recruitment in activated transcription would be provided by a gain-of-function mutation within a TFIID 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 TFIID 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 TFIID 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 TFIID recruitment.

Alternative explanations for the mechanism of yTBPN69S enhancement of gene expression could be proposed. Thus, the N69S mutation could interfere with TFIID dimerization (41), thus giving rise to increased pools of endogenous TFIID 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.

ACKNOWLEDGMENTS

This work was supported by the Howard Hughes Medical Institute.

We thank S. Goodwin for secretarial assistance and R. Roeder and K. Struhl for reagents used in this work.

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