Interdependent Interactions between TFIIB, TATA Binding Protein, and DNA

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Temperature-sensitive mutants of TFIIB that are defective for essential interactions were isolated. One mutation (G204D) results in disruption of a protein-protein contact between TFIIB and TATA binding protein (TBP), while the other (K272I) disrupts an interaction between TFIIB and DNA. The TBP gene was mutagenized, and alleles that suppress the slow-growth phenotypes of the TFIIB mutants were isolated. TFIIB with the G204D mutation [TFIIB(G204D)] was suppressed by hydrophobic substitutions at lysine 239 of TBP. These changes led to increased affinity between TBP and TFIIB. TFIIB(K272I) was weakly suppressed by TBP mutants in which K239 was changed to hydrophobic residues. However, this mutant TFIIB was strongly suppressed by conservative substitutions in the DNA binding surface of TBP. Biochemical characterization showed that these TBP mutants had increased affinity for a TATA element. The TBPs with increased affinity could not suppress TFIIB(G204D), leading us to propose a two-step model for the interaction between TFIIB and the TBP-DNA complex.

Transcription by RNA polymerase II requires a remarkably large number of accessory factors (33). Basal transcription factors are necessary to correctly position the polymerase at the promoter DNA. The assembly of the transcription complex has been studied predominantly in vitro, but the models derived from these experiments need to be validated by in vivo experiments.

A primary event in transcription is the binding of TATA binding protein (TBP) to the TATA element of the promoter (33). It is generally believed that much transcription regulation occurs at this step. Despite years of study, many questions remain about the TBP binding event. TBP is a saddle-shaped molecule, and DNA binds in a severely distorted manner to the concave face of the protein (27, 32) (Fig. 1). Kinetic and molecular studies indicate that TBP binding occurs in several steps, with bending of the DNA being a critical event (22, 23, 34, 36, 37, 42, 45). TBP may arrive at promoters as part of the larger TFIID complex, although it is not clear how the TBP-associated factors (TAFs) affect binding. In vivo, TBP binding is further complicated by the presence of histones, which can affect DNA accessibility and topology.

The basal transcription factor TFIIB interacts directly with TBP (33). TFIIB has three domains: an N-terminal zinc ribbon and two cyclin-fold domains (4, 6, 8, 19, 21, 46). The zinc ribbon interacts with RNA polymerase II, while the cyclin-fold domains interact with the TBP-DNA complex. A cocrystal structure of TBP, TFIIB, and DNA (31) (Fig. 1) shows that TFIIB makes protein-protein contacts with one of the two TBP "stirrups." In addition, each cyclin fold of TFIIB contacts the

DNA directly. One contacts DNA upstream of TBP, and the other contacts DNA downstream. The TFIIB-DNA contacts are made possible by the TBP-induced bending of DNA.

We generated mutants in TFIIB that were defective in either the TFIIB-TBP or TFIIB-DNA interaction. All of these mutants caused severe growth defects in vivo and were unable to form stable TBP-TFIIB-DNA complexes in vitro. We then selected for altered TBP alleles that could suppress the TFIIB defects. Interestingly, the TFIIB mutants were suppressed by distinct classes of TBP mutations. The most interesting TBP suppressor mutants were found on the DNA binding surface. Surprisingly, these mutants had increased affinity for a TATA element. Our results suggest that the TFIIB-TBP interaction occurs in two steps, one primarily mediated by the protein-protein interaction and the second involving the TFIIB-DNA interactions.

MATERIALS AND METHODS

DNA cloning. The 3.2-kb *Eco*RI-*Cla*I fragment from plasmid pDW5462 (a gift of Mike Hampsey) (38) was cloned into pRS313 (41) to create pRS313-*SUA7*. The 3.2-kb *Xba*I-*Xho*I fragment from pRS313-*SUA7* was cloned into pRS413 (41) to create pRS413-*SUA7*. pRS313-*sua7*(G204D) was isolated as a temperature-sensitive mutant following hydroxylamine mutagenesis of pRS313-*SUA7* and screening for the inability to complement an *sua7* deletion in YSB141. pRS313-*sua7*(K272I) was created by oligonucleotide-directed phagemid mutagenesis of pRS313-*SUA7* by using the oligonucleotide *SUA7*-F (5′GATCAAA GAAACTGCAGGTANATCCCCTATTAC3′) followed by subcloning of the 1.6-kb *Bst*BI fragment into pRS313. Other *sua7* mutant alleles were generated by oligonucleotide-directed phagemid mutagenesis of pRS413-*SUA7* by using oligonucleotides *SUA7*-B (5′GTGCTGACGTNTCCGGAGTNCAAAGTC TACCC3′) for the C24F and C27Y mutations, *SUA7*-C2 (5′CTTTGAAG GNGABATCAATGGAG3′) for K166I/T/R (a change from K to I, T, or R at position 166), *SUA7*-D (5′GATGTTGTATHTGCTCTATNTGGTCTAGTA3′) for C45Y/F, *SUA7*-E (5′ACAAGGATCCGAMNGGGTGAAACCACGGAT A3′) for K98M/R, *SUA7*-F for K272T/I/R and I269T, and *SUA7*-G (5′TTT TAAGAGGCABGAGCGAAGATGGTTTC3′) for K217R/M. For protein expression in bacteria, the open reading frames of wild-type, G204D mutant, and K272I mutant Sua7 genes were amplified by PCR and cloned into pET-11d (11).

The plasmid carrying *spt15-206*(K239E) was a gift of Fred Winston (16). Dominant suppressors of the temperature-sensitive phenotype of *sua7*(G204D)

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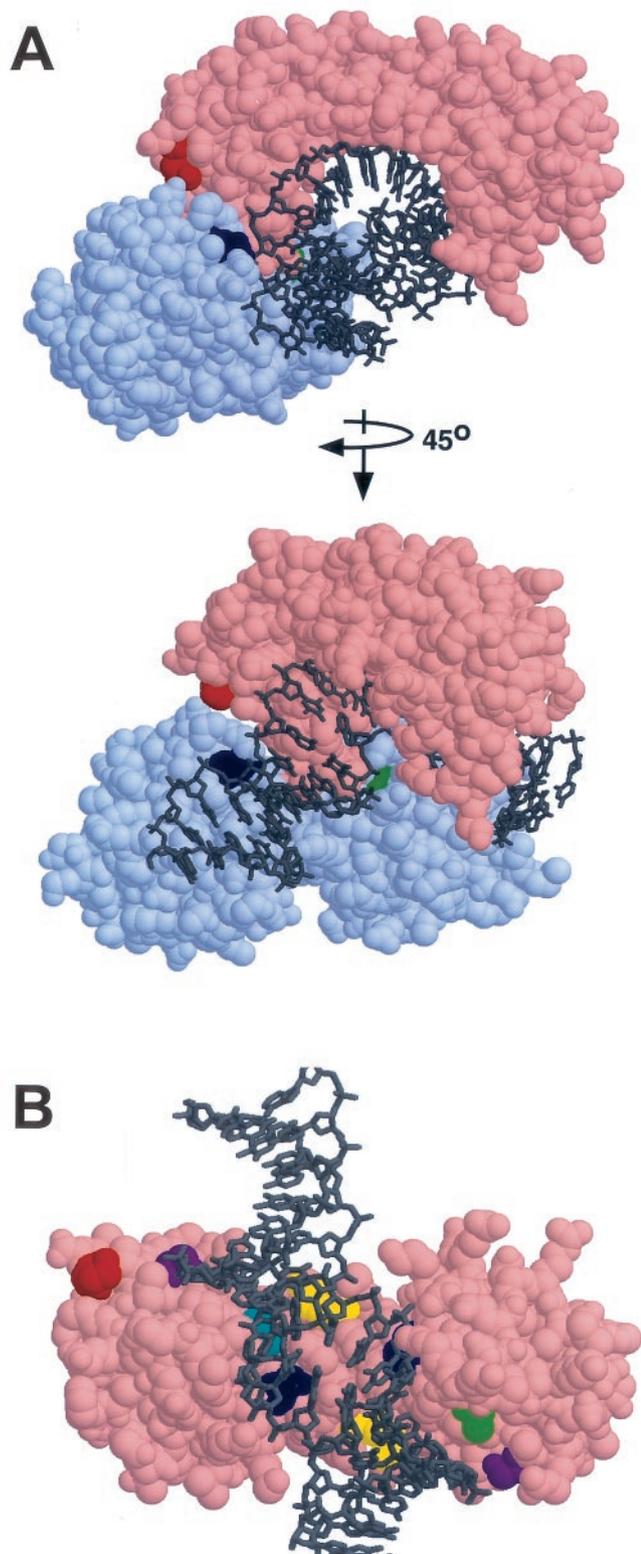† Present address: Department of Biochemistry, Cambridge University, Cambridge, United Kingdom.

or of the slow-growth phenotype of *sua7*(K272I) were isolated as described below from mutant TBP libraries N1 to N6 in vector YCplac22 (a gift of Kevin Struhl) (14) and from hydroxylamine-mutagenized plasmid pUN45-IID (9). The T215 and S209 changes in Spt15 were generated by oligonucleotide-directed phagemid mutagenesis of pUN45-IID by using oligonucleotide TBP-S209 (5′GTTGTTA ATTTTTGTTAMCGGAAAGATTGTTC3′) or TBP-T215 (5′AAGATTGT TCTTKTGGGTGCAAAGCAA3′). For protein expression in bacteria, the open reading frames of wild-type and mutant Spt15 genes were amplified by PCR and cloned into pET-11d or pET-24a.

Yeast methods. The *Saccharomyces cerevisiae* strains used in this study are listed in Table 1. Standard methods were used for medium preparation, yeast transformation, and plasmid recovery (18). YDE11, YDE13, and L662 were gifts of Fred Winston (16). YSB141 was used for plasmid shuffling and complementation testing of *SUA7* alleles. YDE11 and YDE13 were used for plasmid shuffling and complementation testing of *SPT15* alleles. YSB170 and YSB287 were generated by plasmid shuffling of pRS313-*sua7*(K272I) and pRS313-*sua7*(G204D), respectively, into YSB141. YSB170 and YSB287 were transformed with pNKY1009 (3) to create the Trp⁻ strains YSB288 and YSB299. Isolation of dominant suppressors of *sua7*(G204D) and *sua7*(K272I) was carried out by transformation of YSB289 or YSB288 with mutant TBP libraries or with hydroxylamine-treated pUN45-IID. Transformants were plated at 37°C (YSB289) or 30°C (YSB288), and colonies showing improved growth were selected. Plasmids carrying *SPT15* were recovered and sequenced by standard methods.

Protein methods. Recombinant TBP and TFIIB proteins were produced in bacteria as previously described (8, 9, 11). Native gel electrophoresis was also carried out as previously described (7, 8). Gels containing 3 mM MgCl₂ were used to assay the TBP-DNA complex, while the TBP-TFIIB-DNA complex was assayed with gels containing no magnesium. Equilibrium dissociation constants were calculated by assaying increasing concentrations of TBP (0.5 to 8 nM) by use of native gel electrophoresis. The fractional occupancy of the probe was measured by a phosphorimager, and data were plotted using the Langmuir isotherm (37). The K_d was derived from the slope of this line. Titrations were done in triplicate to allow determination of error.

RESULTS

Isolation of TFIIB mutant alleles. In order to explore the functions of TFIIB, mutants with interesting phenotypes were isolated in two ways. First, randomly mutagenized TFIIB plasmids were screened for conditional growth phenotypes. Approximately 50,000 hydroxylamine-mutagenized clones were tested for the ability to grow at 30°C but not at 37 or 15°C. Four clones that were sensitive to cold were isolated. Sequencing of the genes revealed that all of the cold-sensitive alleles corresponded to a change of amino acid 62 from glutamate to lysine. This change is identical to that for the original *sua7-1* allele that causes a shift in transcription start sites (38, 39). Biochemical characterization of this mutant has been presented elsewhere (11, 40).

A single temperature-sensitive allele was isolated in the screen which grew slowly even at 30°C (Fig. 2A and data not shown). The protein contained a mutation from glycine to aspartate at residue 204. Based on the cocrystal structure of *Arabidopsis* TBP, human TFIIB, and DNA, it was determined that TFIIB residue 204 (residue 192 in the human protein) is

FIG. 1. Positions of TBP and TFIIB mutants discussed in this paper. (A) Cocrystal structure of TBP, TFIIB, and DNA (31), but note that the amino acid numbers in reference 31 have been changed to those of yeast TBP and TFIIB. TBP is shown in pink, TFIIB is shown in light blue, and DNA is shown in gray. The top view is a perspective from upstream of the transcription complex. The bottom view is the same, except rotated 45° as indicated. TFIIB residue K272 (contacting

the DNA phosphate backbone) is shown in dark blue and G204 (contacting the TBP stirrup) is shown in green. TBP residue K239, which suppresses both TFIIB mutants, is shown in red. (B) TBP suppressor mutations on the DNA binding surface. Starting with the upper view in panel A, TFIIB was removed and the structure was rotated to show a view from the DNA perspective. For orientation, K239 is shown again in dark orange. T124 and T215 are shown in dark blue, V213 is shown in cyan, Q68 and Q158 are shown in yellow, S118 and S209 are shown in purple, and A100 and A101 are shown in green.
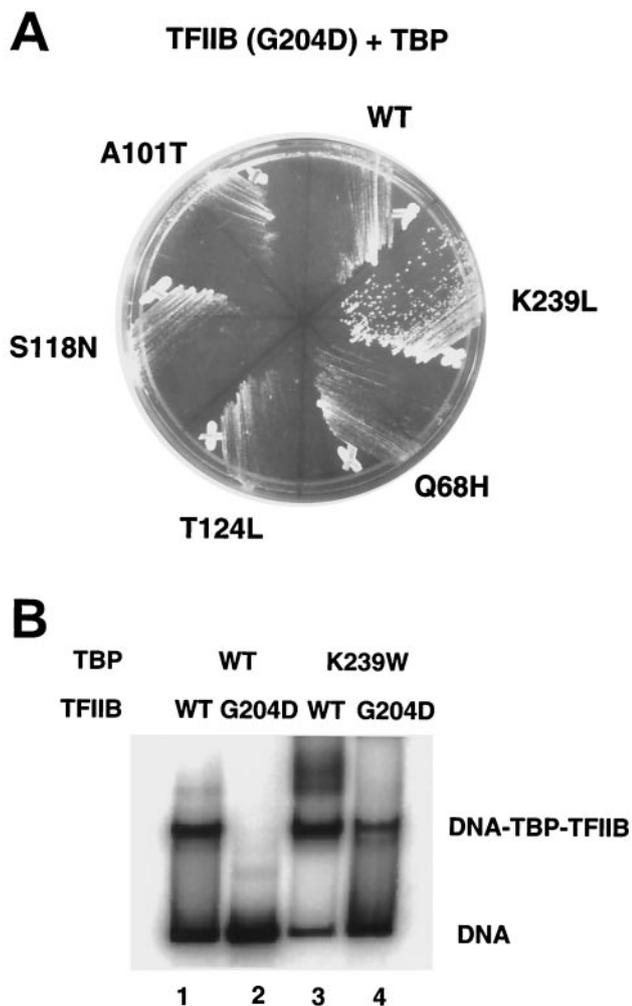
FIG. 2. Suppression of TFIIB(G204D) by TBP(K239L). (A) Suppression in vivo. Low-copy plasmids carrying the indicated alleles of the TBP gene (*SPT15*) were transformed into yeast strain YSB289 that carries the TFIIB(G204D) allele. Transformants were streaked for single colonies on selective media and incubated for 3 days at 30°C. (B) Suppression in vitro. Native gel electrophoresis was carried out on binding reactions containing different combinations of TBP [wild type (WT), lanes 1 and 2, or TBP(K239W) mutant, lanes 3 and 4] and TFIIB [wild type (WT), lanes 1 and 3, or TFIIB(G204D) mutant, lanes 2 and 4].

located near the TBP stirrup. In fact, the crystal structure predicts that leucine 189 of the yeast TBP stirrup makes a van der Waals contact with glycine 204 in TFIIB (31) (Fig. 1A). These amino acids are adjacent to TBP glutamate 188, which makes an essential hydrogen bond between the TBP stirrup and TFIIB (31).

In addition to the random mutagenesis, site-directed mutagenesis of conserved TFIIB residues was performed. We found that changes in the conserved cysteines of the TFIIB zinc finger (C24F, C27Y, C45Y, or C45F) resulted in proteins that could not support viability. Changes in several other conserved residues (G97R, K98 M/R, I269T, or K217R/M) did not result in any mutant phenotypes (data not shown).

Interestingly, each repeat of TFIIB contains a positively charged residue (arginine or lysine) that is absolutely conserved in all TFIIB genes characterized to date. These two residues are predicted to participate in ionic interactions with the phosphate backbone of the DNA (31) (Fig. 1A). Yeast TFIIB mutants with a change at residue K166 or K272 were shuffled into yeast. Interestingly, mutations in the second repeat that removed the positive charge (K272I or K272T) caused a dramatically slow-growth phenotype at 22 and 30°C and resulted in the inability to grow at 37°C (Fig. 3A and data not shown). A conservative substitution mutant (K272R) supported viability similarly to wild-type TFIIB at all temperatures, indicating that a positive charge at this residue is essential for function (data not shown). In contrast to a mutation in the second repeat, that in the first repeat (K166I or K166T) resulted in wild-type phenotypes at all temperatures (data not shown). In the TBP-TFIIB-TATA cocrystal structure (31), the lysine in the first repeat (arginine 154 in human TFIIB) contacts DNA downstream of the TATA box while the residue in the second repeat (arginine 248 in human TFIIB) contacts residues near the 5′ end of the TATA box. Our genetic results agree well with earlier biochemical results from studies using human TFIIB mutants (8) and indicate that these positive residues do not contribute equally to TFIIB function.

TFIIB mutants are defective for formation of the TFIIB-TBP-DNA complex in vitro. The TFIIB proteins with the G204D and K272I mutations [TFIIB(G204D) and TFIIB(K272I)] were expressed in bacteria, purified, and tested for the ability to interact with the TBP-DNA complex by use of native gel electrophoresis. Both mutants were severely compromised in their ability to produce a stable complex in a gel shift assay (Fig. 2B and 3B). Under these conditions, the TBP-DNA complex is not seen, but the TBP-TFIIB-DNA complex

TABLE 1. *S. cerevisiae* strains used in this study

| Strain	Genotype
YSB141	*MAT**a** sua7Δ::LEU2 ura3-52 leu2-3,112 his3Δ200* [pDW5462]
YSB170	*MAT**a** sua7Δ::LEU2 ura3-52 leu2-3,112 his3Δ200* [pRS313-sua7(G204D)]
YSB287	*MAT**a** sua7Δ::LEU2 ura3-52 leu2-3,112 his3Δ200* [pRS313-sua7(K272I)]
YSB288	*MAT**a** sua7Δ::LEU2 ura3-52 leu2-3,112 trp1::hisG-URA3-hisG his3Δ200* [pRS313-sua7(K272I)]
YSB289	*MAT**a** sua7Δ::LEU2 ura3-52 leu2-3,112 trp1::hisG-URA3-hisG his3Δ200* [pRS313-sua7 (G204D)]
YSB299	*MAT**a** sua7Δ::LEU2 ura3-52 leu2-3,112 his3Δ200* [pRS413-sua7(K272T)]
YDE11	*MATα spt15Δ::LEU2 ura3-52 leu2-3,112 trp1Δ1 his4-912δ lys2-128δ* [pDE38-9]
YDE13	*MAT**a**/α spt15Δ::LEU2/spt15Δ::LEU2 ura3-52/ura3-52 leu2-3,112/leu2-3,112 trp1Δ1/trp1Δ1 his4-912δ/his4-912δ lys2-128δ/lys2-128δ* [pDE38-9]
L662	*MATα spt15Δ101::LEU2 ura3-52 leu2Δ1 trp1Δ1 his4-917δ spt3-401 lys2-173r2* [pDE38-9]
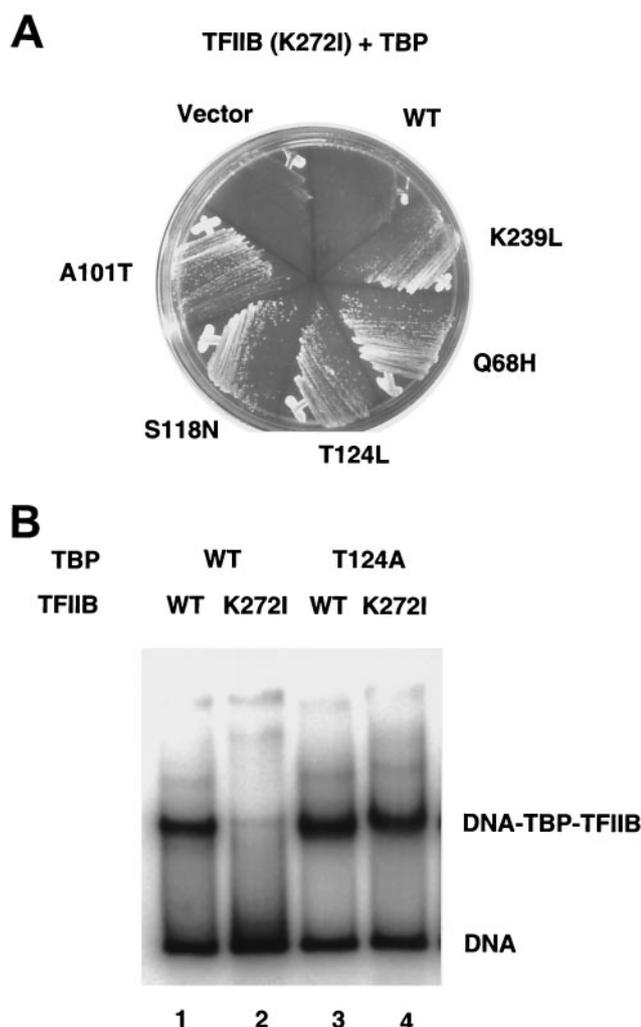
FIG. 3. Suppression of TFIIB(K272I) by TBP mutant alleles. (A) Suppression in vivo. Low-copy plasmids carrying the indicated alleles of the TBP gene (*SPT15*) were transformed into yeast strain YSB288 that carries the TFIIB(K272I) allele. Transformants were streaked for single colonies on selective media and incubated for 3 days at 30°C. (B) Suppression in vitro. Native gel electrophoresis was carried out on binding reactions containing different combinations of TBP [wild type (WT), lanes 1 and 2, or TBP(T124A) mutant, lanes 3 and 4] and TFIIB [wild type (WT), lanes 1 and 3, or TFIIB(K272I) mutant, lanes 2 and 4]. Similar results were obtained with the S209N, Q68W, and S118N mutations.

is stable. Based on the cocrystal structure of the TBP-TFIIB-DNA complex (31) (Fig. 1A), the G204D defect is due to defective protein interaction between TFIIB and TBP. In contrast, the unstable binding of K272I is the result of a loss of interaction between TFIIB and the DNA phosphate backbone. These two mutants with distinct defects were used for further genetic experiments.

TBP suppressors of TFIIB G204D mutation. Because the conditional phenotypes of the TFIIB mutants were due to defects in the ability to form a stable TFIIB-TBP-DNA complex, we decided to screen for mutant TBP alleles that would suppress the temperature sensitivity of TFIIB. We predicted that these would increase the interaction between TBP and

TFIIB. Because such TBP alleles would involve a gain of function and should be dominant, the screen was performed by transforming mutant TBP libraries into yeast strains that contained a wild-type copy of the TBP gene. The TBP libraries were mutated on a regional codon basis, and mutations were targeted primarily to non-DNA binding regions of TBP (14).

The strain with the TFIIB(G204D) mutant grows very slowly at 30°C and not at all at 37°C (Fig. 2A). We screened 10,000 to 20,000 TBP mutants from each of six localized mutant libraries for the ability to restore growth at 37°C to the strain containing TFIIB(G204D). Each library represents a highly enriched collection of mutants in a limited region of TBP (14). Strikingly, no suppressing plasmids were obtained from five of the libraries, while over 100 were obtained from a library containing mutations in TBP residues 226 to 240. Forty-eight of the suppressor TBP genes were sequenced. Remarkably, every single suppressing allele involved an amino acid change at lysine 239 (Table 2) (Fig. 2B). Furthermore, all of the changes at K239 were to hydrophobic residues. Most were changes to leucine, isoleucine, or methionine, although phenylalanine and tryptophan substitutions were also isolated. Although some suppressor alleles contained amino acid substitutions in addition to that of lysine 239, these changes followed no pattern and were not required for suppression.

TBP suppressors of TFIIB(K272I). The TFIIB(K272I) mutant reduces the stability of the TFIIB-TBP-DNA complex due to presumed loss of ionic interactions between the lysine and the DNA phosphate backbone. However, our characterization preceded the development of the crystal structure and the discovery of TFIIB-DNA contacts. Under the assumption that K272 was involved in contacts with TBP, a screen for TBP suppressors of the TFIIB(K272I) mutant was carried out as described above for the TFIIB(G204D) mutant, except that the selection was done at the semipermissive temperature of 30°C. Surprisingly, several dominant suppressing TBP alleles associated with various parts of the protein were isolated (Table 2 and Fig. 3B).

Sequencing of the TBP suppressors of TFIIB(K272I) revealed that they fell into two classes. The first class involved changes at lysine 239 of TBP. As previously observed for suppressors of TFIIB(G204D), changes of the lysine to the hydrophobic residue leucine, methionine, or phenylalanine improved growth in the TFIIB(K272I) strain (Fig. 3B). However, this class of TBP alleles contained the weakest suppressing alleles isolated.

Surprisingly, the second class of suppressors all mapped to the DNA binding face of TBP. Specific changes were isolated at Q68, T124, S118, and F152/Q158 (Table 2). Each of these mutated residues is predicted to make protein-DNA contacts in the cocrystal structure (17, 27, 31, 35, 44) (Fig. 1B). These results were particularly astonishing for two reasons. First, the vast majority of published amino acid substitutions in the DNA binding surface of TBP abolish binding to TATA elements. Second, the mutagenized TBP libraries used in this screen were designed to primarily target the non-DNA binding surfaces of TBP.

Since TBP is a pseudosymmetric protein, we asked whether suppressor amino acid changes in one repeat would also suppress TFIIB(K272I) when constructed in the corresponding residue of the other repeat. In each case, suppression could be

TABLE 2. TBP suppressors of TFIIB mutants

TBP allele	Source[a]	Suppression of *sua7* (K272I) at 30°C	Suppression of *sua7* (G204D) at 37°C	Phenotype[b]
K239M	Cormack N5	Very weak	Moderate	Weak TS
K239L	Cormack N5	Very weak	Strong	Weak TS
K239F	Cormack N5	Very weak	Strong	Weak TS
K239I	Cormack N5	None	Strong	Very Weak TS
K239W	Cormack N5	None	Weak	Weak TS
Q68H	Cormack N6	Strong	None	WT
Q68W	Cormack N6	Strong	None	Weak TS, weak Gal⁻
T124V	Cormack N1	Strong	None	Very weak TS
T124L	Cormack N1	Strong	None	Strong TS, Gal⁻
T124A	Cormack N1	Strong	Not tested	Strong TS, Gal⁻
T215V	Site directed	Strong	None	Weak TS
T215L	Site directed	Weak	None	Weak TS
S118T	Cormack N1	Weak	None	Very weak TS
S118N	Cormack N1	Strong	None	Weak TS
S209T	Site directed	Strong	None	Wild type
S209N	Site directed	Strong	None	Very weak TS
S209K	Site directed	None	None	Lethal
A101T	Hydroxylamine	Strong	None	Weak TS
A101V	Hydroxylamine	Weak	None	Strong TS, Gal⁻
A100P	Hydroxylamine	Strong	None	Strong TS
V2131	Hydroxylamine	Very weak	None	Weak TS
F152W, Q158W	Cormack N3	Weak	None	TS, weak Gal⁻

[a] See reference 14 for details of Cormack libraries. Site directed, site-directed mutagenesis.
[b] TS, temperature sensitive; WT, wild type; Gal⁻, unable to grow on 2% galactose as a carbon source.

mediated by the same amino acid substitution in either repeat (Table 2). For example, both Q68W and Q158W were isolated from the codon-mutagenized libraries. T124 in the first TBP repeat corresponds to T215 in the second repeat. Changing either of these residues to either valine or leucine resulted in suppression of TFIIB(K272I). Mutation of either S118 or S209 to asparagine or threonine had a similar effect. Therefore, the two TBP repeats appear to make roughly equivalent contributions to the suppressor phenotype.

Since the libraries used in the original screen were not mutagenized in large regions of the TBP DNA binding surface, the entire TBP gene was mutagenized using hydroxylamine and the screen for suppressors of TFIIB(K272I) was repeated. Again, suppressors were found in the DNA binding face of TBP. The mutations V213I, A100P, A101T, and A101V were isolated in this screen. These residues were not mutagenized in the libraries of the original screen. Interestingly, these mutations would increase the size of the hydrophobic side chains facing the TATA element minor groove.

Although the TBPs with mutations at lysine 239 suppressed both the TFIIB allele that affects protein-protein interactions and the allele that disrupts the TFIIB-DNA contact, this was not true of the TBP binding face mutants. When transformed into the TFIIB(G204D) strain, these TBP alleles did not cause any improvement in cell growth. Therefore, there is allele specificity in the suppression pattern.

Properties of the TBP(K239) suppressors of TFIIB mutants. In order to characterize the mechanism of the TBP suppressors, the proteins were produced in *Escherichia coli* and tested by gel shift assay. The TBP(K239) mutants were tested for the ability to bind the adenovirus major late promoter (AdMLP) TATA element. The DNA binding of the mutants was indistinguishable from that of wild-type TBP (data not shown). The ability to form the DNA-TBP-TFIIB complex was also tested

by gel shift analysis (Fig. 2B). Whereas wild-type TBP could bind wild-type TFIIB, no stable complex was observed with TFIIB(G204D). The TBP(K239W) protein bound wild-type TFIIB to form a complex with mobility that was identity to that of the wild type. However, the suppressor TBP protein could also stably bind the TFIIB(G204D) protein. Therefore, the in vivo suppression is apparently a direct result of improved interactions between the suppressor TBP and mutant TFIIB.

Suppression of TFIIB mutants by TBP(K239) mutants was predicted to occur by an increase in the affinity of the protein-protein interactions between TBP and TFIIB. Since TBP residue 239 is not predicted to be close to either TFIIB residue 204 or K272 (31) (Fig. 1), it seemed likely that the change of lysine 239 to a hydrophobic residue either created a new favorable contact or removed an unfavorable contact between the proteins. Therefore, the TBP(K239) mutant should also show increased affinity for wild-type TFIIB. In support of this hypothesis, gel shifts using equivalent amounts of TBP (as determined by Coomassie staining and the ability to form a DNA-TBP complex) consistently detected more DNA-TBP-TFIIB complex with the TBP(K239) mutant than with the wild type (Fig. 2B and data not shown).

Properties of the TBP-DNA binding face suppressors of TFIIB mutants. Although the TBP(K239) mutants were expressed in *E. coli* at levels comparable to that of wild-type TBP, the DNA binding face mutants were apparently more toxic to the bacteria. When ampicillin was used as the selectable marker for the expression plasmid, the mutant plasmids were lost at high frequency early in log phase growth. The TBP binding face mutants were recloned into an expression vector conferring kanamycin resistance, and this resulted in low but satisfactory levels of protein expression (data not shown).

Because all previously reported TBP mutations in the DNA binding surface have been found to drastically reduce binding
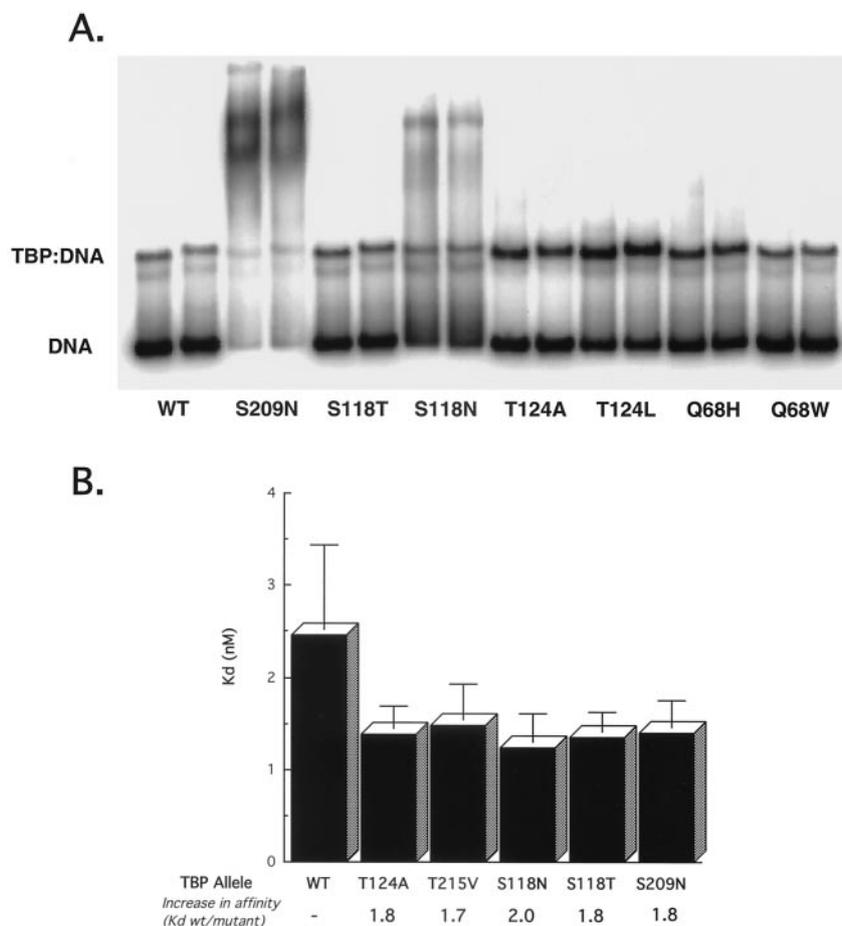
FIG. 4. TBP suppressors of TFIIB(K272I) have increased DNA binding affinity. (A) Native gel electrophoresis of wild-type and mutant TBP. Thirty nanograms of each protein was incubated with an AdMLP probe. Duplicate lanes for each protein are shown. (B) K_d values for wild-type and mutant TBPs. Titrations of TBP binding to the AdMLP were assayed using native gel electrophoresis, and the K_ds were calculated as described in Materials and Methods. K_ds for Q68H and Q68W were not significantly different from that for wild-type TBP.

to the TATA element, the suppressor TBP proteins were first tested in a gel shift assay. Remarkably, the suppressor proteins did not show any reduction in affinity for the AdMLP TATA element (Fig. 4A). This was particularly surprising because some of the changed amino acids are predicted to directly contact DNA bases (31) (Fig. 1B). For example, T124 and T215 are predicted to make two of the rare hydrogen bonds in the TBP-DNA interface, yet changing either of these residues to alanine does not reduce binding.

Three possible mechanisms of TFIIB suppression by the TBP binding face mutants were considered when the in vitro tests were designed. First, it was possible that the mutations actually affected TBP dimerization rather than DNA binding. TBP dimers have been demonstrated both in vitro and in vivo, and it has been proposed that the transition to monomer form could be limiting for transcription (12, 13, 24, 25, 43). The ability of the suppressor TBPs to dimerize was tested by glutaraldehyde cross-linking (12, 43). Although this is not a quantitative assay, the TBP mutants all appeared to form dimers in vitro (data not shown). Also, the suppressor mutations on the crystal structure of the yeast TBP dimer are not located at the

interface of the TBP molecules (10). Therefore, it is very unlikely that suppression is due to effects on TBP dimerization.

A second possibility was that the suppressor mutations could cause differences in the DNA bending induced by TBP. Since TFIIB recognizes both TBP and the bent DNA, mutations in TBP that change the angle or stability of the bend might increase the affinity of TFIIB for the TBP-DNA complex. To test the bend angle of the TBP-DNA complexes, gel shifts were performed using circularly permuted probes that placed the center of the bend (i.e., the TATA element) at different positions within the DNA fragment (42). TBP-DNA complexes with the mutant proteins migrated in the same way as wild-type complexes, suggesting that the overall shape of the TBP-DNA complex was not affected by the DNA binding face mutations (data not shown).

A third model for suppression postulates that binding energy lost due to the missing TFIIB-DNA contact can be compensated for by stronger contacts between TBP and the DNA (1, 2). The TBP suppressors would therefore be predicted to have increased affinity for TATA elements. To test this, quantitative binding experiments were performed. Equivalent amounts of

protein were tested for binding to a consensus TATA element. Binding was plotted using the Langmuir isotherm formula (37), and equilibrium dissociation constants were calculated from the slope of the curves (Fig. 4B). Wild-type TBP had a K_d of approximately 2.5 nM, in good agreement with other published estimates. Most of the mutants did exhibit increased affinity (about twice that of the wild-type protein).

Phenotypes of TBP suppressor mutants. The TBP suppressors of TFIIB mutants were all dominant, since they were isolated in the presence of the wild-type TBP. In order to determine whether these mutants had their own recessive phenotypes, plasmid shuffling was used to introduce them into cells in the absence of any other TBP alleles (Table 2). Several of the mutants with increased affinity (those with the A100P, A101V, A101T, T124A, T124L, and F152W/Q158W mutations) were temperature sensitive and grew poorly on galactose as a carbon source. Many of the others grew more slowly than the wild type at 37°C. This suggests that increased affinity for the TATA element may actually be detrimental to proper gene regulation and growth. Alternatively, these mutations may adversely affect transcription by RNA polymerase I or polymerase III.

DISCUSSION

This study began with the characterization of several TFIIB mutants. Two interesting mutants that caused a severe growth defect in vivo and failed to form a stable TBP-TFIIB-DNA complex in vitro were found. However, the two TFIIB mutants were defective for distinct TFIIB interactions. One disrupts the interaction with TBP, while the other disrupts the TFIIB-DNA interaction.

The TFIIB(G204D) protein is mutated in a residue that makes a van der Waals contact with TBP. The change of glycine to aspartate is predicted to disturb the protein-protein interface between the two factors. When we selected for mutations in TBP that could compensate for this reduction in affinity, we isolated multiple amino acid substitutions at lysine 239 of TBP. Interestingly, all of the changes were to hydrophobic amino acids.

In the TBP-TFIIB-TATA cocrystal structure, the residue corresponding to K239 (K197 in *Arabidopsis* TBP) hangs down from the C-terminal H1′ helix of TBP and makes a salt bridge with residue D243 of human TFIIB (31) (Fig. 1A). Interestingly, the corresponding TFIIB residue in the yeast protein is lysine 267. This raises the possibility that the TBP suppressors work by removing some charge repulsion between yeast TBP K239 and TFIIB K267, thereby strengthening the TBP-TFIIB interface. One might ask why no suppressors were isolated that change K239 to an acidic residue. It should be noted that TFIIB K267 is surrounded by several other acidic residues that may clash with a negatively charged residue at TBP K239.

Interestingly, a mutation at lysine 239 of TBP has previously been identified in a screen of TBP alleles that can suppress the Spt⁻ phenotype of the *spt3-401* allele (15). The TBP(K239E) allele does not have an Spt⁻ phenotype by itself, and the TBP(K239E) mutant could not suppress the temperature-sensitive phenotypes of the TFIIB mutations described in this report (data not shown). Furthermore, the TBP(K239L) and TBP(K239W) alleles, which do suppress the TFIIB mutations, are unable to suppress the Spt⁻ phenotype of an *spt3-401* strain. In fact, double-mutant cells [*spt3-401*, *spt15*(K239I or K239W)] had a stronger Spt⁻ phenotype (assayed with the *his4-912δ* and *lys2-128δ* markers) than cells containing only the *spt3* mutation (data not shown). This raises the possibility that the effects of the interacting Spt15 and Spt3 mutations are actually mediated by influencing the TBP-TFIIB interaction. Perhaps TFIIB and Spt3 compete for binding to TBP.

The second interesting TFIIB mutant characterized here was that with the K272I substitution. TFIIB contains two cyclin-fold domains that have significant sequence similarity to each other. Each TFIIB repeat contains a highly conserved pair of residues: a glycine followed by a positively charged residue. The TBP-TFIIB-DNA cocrystal structure shows that the positively charged residues make hydrogen bonds with the phosphate backbone of the DNA (31) (Fig. 1A). The N-terminal repeat contacts DNA downstream of the TATA box, while the C-terminal repeat contacts DNA upstream. Human TFIIB mutants carrying changes in the glycine-arginine pair have been analyzed previously (8). Mutations in the first-repeat pair had little effect in vitro, while mutations in the second-repeat pair led to defects in the formation of the TBP-TFIIB-DNA complex and a reduced ability to support transcription in vitro. In the present study, we provide further evidence that the DNA contact made by the C-terminal TFIIB is important in vivo (leading to the slow growth phenotype of the yeast TFIIB mutants with the K272I and K272T substitutions), while similar changes in the first-repeat residues have little effect.

When TBP suppressors of TFIIB(K272I) were isolated, two classes of mutants were recovered. The first comprised the same TBP mutants that suppressed TFIIB(G204D), i.e., TBPs with lysine 239 changed to a hydrophobic residue. The suppressing phenotype of these TBP alleles can be rationalized by the same mechanism proposed for the suppression of TFIIB(G204D). The TFIIB(K272I) mutant has lost stability because the contact between K272 and DNA is no longer made. Since stability of the interaction between TFIIB and the TBP-DNA complex is dependent upon both protein-protein and protein-DNA interactions, loss of binding energy in one can be compensated for by an increase in the other (1, 2). Therefore, the improved TFIIB contact made by the TBP mutants with changes at K272 compensates for the lost TFIIB-DNA contact in TFIIB(K272I).

The second class of TBP mutants that suppress TFIIB(K272I) mutant phenotypes mapped to the DNA binding surface of TBP (Fig. 1B). The suppression observed with this group was notably stronger than that observed with the TBP(K239) class. It was surprising to isolate mutants on the DNA binding face of TBP, since most previously reported mutations on this highly conserved surface have detrimental effects on TBP binding to DNA. Remarkably, we found that many of these mutant TBP proteins actually had an increased affinity for a consensus TATA element. The proteins with the T124A, T215V, S118N/T, and S209N mutations all bound to the AdMLP with approximately twofold greater affinity than did wild-type TBP. We did not observe increased binding of the proteins with Q68H/W mutations, although it is certainly possible that these mutants would show increased affinity had we tested a variety of TATA elements. We did not assay the

proteins with mutations at A100 or A101. However, J. V. Spencer and K. M. Arndt (personal communication) also isolated the A100P mutant in experiments designed to identify TBPs that increased transcription from a "reverse" TATA element (26). They found that this mutant also increased affinity for the AdMLP by two- to threefold.

As discussed above, the mechanism of TFIIB suppression by the TBP mutants is easily understood by considering that the stability of the TBP-TFIIB-DNA complex derives from the sum of all of its protein-protein and protein-DNA contacts (1, 2). The TFIIB(K272I) mutant loses one TFIIB-DNA interaction, but this can be compensated for by an increase in the affinity of the TBP-TFIIB interaction (via the mutations at K272 of TBP) or the TBP-DNA interaction (via the DNA binding face mutations). It is not clear why the particular amino acid substitutions that were isolated lead to the increased affinity of TBP for DNA. However, it is striking that all of the affected residues make contacts with the DNA.

In some cases (T124L/A, T215V/L, V213I, and Q68H/W), there is an increase in the hydrophobicity of the DNA binding surface. Therefore, it is possible that affinity is increased by making solvation of free TBP more costly energetically, i.e., shifting equilibrium towards bound TBP by making free TBP less favorable. In vivo, it is possible that changing the TBP binding face disrupts a protein-protein interaction that occurs when TBP is not bound to DNA. Both TBP dimerization (12, 13, 24, 25, 43) and an inhibitory TBP-TAF1 interaction (5, 28, 29) occur via the TBP DNA binding face. However, we found that TBP dimerization was not strongly affected in our mutants (data not shown), and our in vitro results showing increased affinity were obtained with purified recombinant TBPs lacking any TAFs.

The S118T/N and S209T/N mutations are not easily explained. These serines make hydrogen bonds to the phosphate backbone. The substituted amino acids can still form hydrogen bonds, and perhaps the difference in side-chain conformation strengthens the bonds. Similarly, it is not clear how the A100P and A101T/V mutations affect DNA binding. The position corresponding to A100 in the second repeat is a proline (P191), and it has been suggested that this asymmetry contributes to the directionality of TBP binding (26). These residues immediately follow the phenylalanines that intercalate between the DNA bases to distort and bend the TATA element. It seems likely that the A100 and A101 positions may influence this intercalation and bending process.

Interestingly, several of the DNA binding face mutants that we isolated occur naturally in the TBP protein of the malaria-causing parasite *Plasmodium falciparum*. The Q68H, A101T, S118N, and V213I substitutions all appear in the wild-type TBP of *P. falciparum* (30). Although this TBP is highly diverged from TBPs of other eukaryotes, it is unlikely that these particular substitutions would arise by chance. The *P. falciparum* genome is extraordinarily A-T rich (90%), suggesting that its TBP must have evolved extremely selective binding for discrimination of true TATA elements from related sequences.

Based on our model of suppression by energetic compensation, one is forced to ask why the TBP mutants with increased affinity for DNA suppress TFIIB(K272I) but not TFIIB(G204D). In contrast, the TBP(K239) mutants suppress both classes of TFIIB mutants. We believe that this allele

specificity provides genetic evidence for multiple steps in the formation of the TBP-TFIIB-DNA complex. In one step, a TBP-TFIIB interaction may occur that is not sensitive to the DNA binding state of TBP. The TFIIB(G204D) mutant disrupts this protein-protein interaction and is suppressed only by the TBP(K239) mutants that increase the TBP-TFIIB affinity. Another step may involve DNA contact by both TBP and TFIIB. TFIIB(K272I) decreases the interaction between DNA and the complex formed in the first step, but this can be compensated for by the additional binding affinity provided by the TBP DNA binding face mutants. This step can also be suppressed (at least partially) by the TBP(K239) mutants because the intermediate complex formed in the first step is necessarily a precursor to the second step.

At least three molecular models are consistent with this proposal. In the first, TBP and TFIIB form a complex before TBP completes binding to the DNA. This could mean that TBP and TFIIB bind first as a heterodimer off the DNA. Alternatively, since TBP binding to DNA appears to occur in multiple steps, TFIIB may first recognize an intermediate form of the TBP-DNA complex. Most models of TBP binding to DNA propose one or more intermediates, perhaps with varying degrees of DNA bending. The interaction of K272 of TFIIB with DNA may occur in only one of the later TBP-DNA intermediates.

A third model that might explain our data is one in which TFIIB binding to the preformed TBP-DNA complex occurs in two steps. Some support for this idea comes from comparison of the nuclear magnetic resonance structure of the free TFIIB core domain (4, 20) with TFIIB within the TBP-TFIIB-DNA cocrystal structure (31). The two structures are quite similar when the individual TFIIB cyclin domains are compared, but the relative orientation of the domains is quite different. This suggests that TFIIB might undergo a significant conformation change upon binding to the TBP-DNA complex. TFIIB binding could occur in two steps, with the first mediated primarily by the protein-protein contact with TBP. The second step could then involve a conformation change that allows the TFIIB-DNA contact to form. Future biophysical experiments will help to test these models.

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