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# A new regulatory domain on the TATA-binding protein

**Yong Cang, David T.Auble[1] and Gregory Prelich[2]**

Department of Molecular Genetics, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461 and [1]Department of Biochemistry and Molecular Genetics, University of Virginia Health Sciences Center, Charlottesville, VA 22908, USA

[2]Corresponding author
e-mail: prelich@aecom.yu.edu

**Recognition of the TATA box by the TATA-binding protein (TBP) is a highly regulated step in RNA polymerase II-dependent transcription. Several proteins have been proposed to regulate TBP activity, yet the TBP domains responsive to all these regulators have not been defined. Here we describe a new class of TBP mutants that increase transcription from core promoters *in vivo*. The majority of these mutations alter amino acids that cluster tightly on the TBP surface, defining a new TBP regulatory domain. The mutant TBP proteins are defective for binding the transcriptional regulator yNC2, are resistant to inhibition by yNC2 *in vitro* and exhibit allele-specific genetic interactions with yNC2. These results provide strong biochemical and genetic evidence that TBP is directly repressed *in vivo*, and define a new TBP domain important for transcriptional regulation.**

*Keywords*: BUR6/NC2/repression/TBP/transcription

## Introduction

The TATA-binding protein (TBP) is a frequent target for transcriptional regulators. TBP performs essential roles in transcription by all three nuclear RNA polymerases (Cormack and Struhl, 1992); as a component of TFIID, the binding of TBP to the TATA box is the first step in the assembly of the RNA polymerase II (Pol II) pre-initiation complex (PIC) (Matsui *et al*., 1980). Formation of the TBP–TATA complex triggers recruitment of the remaining general transcription factors (GTFs) and Pol II, possibly as an intact Pol II holoenzyme (Koleske and Young, 1994; Ranish *et al*., 1999). Because TBP binding is the initial, and often rate-limiting step in transcription (Kuras and Struhl, 1999; Li *et al*., 1999), it is highly subject to both positive and negative regulation.

TBP has been reported to contact directly a wide variety of general and promoter-specific transcription factors, including TFIIA, TFIIB, the C-terminal domain of the largest subunit of Pol II, TBP-associated factors (TAFs), many gene-specific activators, including GAL4-VP16 and p53, the Spt3 subunit of the SAGA complex and the negative regulators Mot1 and negative cofactor 2 (NC2) (Hampsey, 1998). Because TBP performs such a central role in transcription, it is important to identify the precise contacts between TBP and these TBP-interacting proteins. Structural data have identified TBP residues that interact directly with DNA (J.L.Kim *et al*., 1993; Y.Kim *et al*., 1993), TFIIA (Geiger *et al*., 1996; Tan *et al*., 1996), TFIIB (Bagby *et al*., 1995; Nikolov *et al*., 1995) and $dTAF_{II}230$ (D.Liu *et al*., 1998). TBP resembles a molecular saddle, with DNA binding to the concave surface. The DNA-binding surface is also contacted by $dTAF_{II}230$, which interacts with almost identical TBP residues as the TATA box, thus inhibiting TATA-binding activity by direct competition. By contrast, TFIIA and TFIIB recognize residues on the outer convex surface of TBP; TFIIA primarily contacts a basic region located between the two ~90-amino-acid repeats of TBP, whereas TFIIB contacts a small domain near the TBP C-terminus. The surfaces responsible for interactions with the other TBP-interacting proteins are not yet understood at the structural level; however, genetic analysis indicates that Spt3 interacts with region H1′, near the TBP C-terminus (Eisenmann *et al*., 1992), whereas a double mutant within the basic region is defective for binding Mot1 *in vitro* (Auble and Hahn, 1993).

Several proteins that inhibit TBP have been identified. These inhibitors can affect either the initial binding of TBP to the TATA box or the subsequent TBP-dependent recruitment of the GTFs and the Pol II holoenzyme. TBP DNA-binding activity is inhibited by at least three distinct activities *in vitro*: histones, $dTAF_{II}230$ and Mot1. Histones (Workman and Roeder, 1987) and $dTAF_{II}230$ (D.Liu *et al*., 1998) inhibit TBP by direct competition, whereas Mot1 actively disrupts pre-formed TBP–TATA complexes in an ATP-dependent manner (Auble and Hahn, 1993). TBP can also be inhibited subsequent to formation of the TBP–TATA complex by NC2. NC2 binds to TBP, blocking interactions between TBP and the GTFs TFIIA and/or TFIIB *in vitro* (Meisterernst and Roeder, 1991; Goppelt *et al*., 1996; Mermelstein *et al*., 1996). NC2 consists of two evolutionarily conserved subunits, designated NC2α and NC2β (Goppelt *et al*., 1996; Mermelstein *et al*., 1996); in *Saccharomyces cerevisiae* the NC2 subunits are encoded by *BUR6/NCB1* (yNC2α) (Goppelt and Meisterernst, 1996; Gadbois *et al*., 1997; Kim *et al*., 1997; Prelich, 1997) and *YDR1/NCB2* (yNC2β) (Goppelt and Meisterernst, 1996; Gadbois *et al*., 1997; Kim *et al*., 1997). Genetic analysis is consistent with a role for yNC2 as a transcriptional repressor; *bur6* mutations increase transcription from a core (UAS-less) promoter in yeast (Prelich and Winston, 1993; Prelich, 1997), whereas *bur6* (*ncb1*) and *ydr1* (*ncb2*) mutations suppress temperature-sensitive defects in the *SRB4* subunit of the Pol II holoenzyme (Gadbois *et al*., 1997; Lee *et al*., 1998). *BUR6* and *YDR1* are required for normal cell growth; deletion of either gene causes lethality (Gadbois *et al*., 1997; Kim *et al*., 1997) or extreme sickness (Prelich, 1997).

Despite the wealth of data demonstrating direct TBP–repressor interactions *in vitro*, there is little evidence that TBP is repressed directly *in vivo*; indeed, several results question the simplistic view of the repressors described above. First, the transcriptional effects of these proposed repressors could be independent of direct effects on TBP. The identification of *BUR6* and *YDR1* as suppressors of *srb4* and *srb6* mutations (Gadbois *et al*., 1997; Lee *et al*., 1998), for example, connects yNC2 most directly with the Pol II holoenzyme, not with TBP. Secondly, nearly all biochemical characterization of Mot1 and NC2 has been performed with TBP, not with the intact TFIID complex, so the effects of Mot1 and NC2 on TBP under physiological conditions remain unknown. Thirdly, although *mot1* and *bur6* mutations increase transcription of weakened promoters *in vivo* (Davis *et al*., 1992; Prelich and Winston, 1993), consistent with their role as repressors, they also decrease transcription from other promoters (Collart, 1996; Madison and Winston, 1997; Prelich, 1997), implying a positive role at some transcription units. The contrasting promoter-specific effects may be due to secondary effects or to multiple functional roles for these proteins *in vivo*. Identifying the direct targets of these proteins *in vivo* would help to clarify their specific roles.

We previously used a genetic selection in *S.cerevisiae* to identify proteins that have general roles in transcriptional regulation. By selecting for mutations that increase the activity of a core promoter (a promoter that has been deleted of its upstream activating sequence and is therefore incapable of activated transcription), we identified several genes previously implicated in repression, including the histone loci, several *SPT* genes, and six other genes designated *BUR1–BUR6* (Prelich and Winston, 1993). The *BUR* genes have properties consistent with roles as general repressors of transcription, since loss-of-function mutations in all these genes increase transcription from core promoters and some alleles suppress *snf/swi* defects. Cloning of the *BUR* genes supports this model; *BUR3* is identical to *MOT1*, *BUR5* encodes histone H3 and *BUR6* encodes the α-subunit of yeast NC2 (Prelich and Winston, 1993; Prelich, 1997). The Bur$^-$ selection has thus been informative for identifying general transcriptional regulators. Here we utilize the Bur$^-$ phenotype to investigate the regulation of TBP. We reasoned that if TBP was inhibited *in vivo*, we should be able to obtain TBP mutants that are resistant to inhibition, resulting in increased transcriptional activity. Here we describe the identification of hyperactive TBP mutants, their biochemical characterization and allele-specific interactions with *BUR6* and *MOT1*. These TBP Bur$^-$ mutants provide strong evidence that TBP is directly repressed *in vivo*, and define a new surface domain of TBP required to bind yNC2.

## Results

Most of the TBP mutants identified by previous selections caused a reduction in TBP activity (Eisenmann *et al*., 1989; Cormack and Struhl, 1992, 1993; Zhou and Buratowski, 1992; Arndt *et al*., 1995; Bryant *et al*., 1996; Stargell and Struhl, 1996; Tang *et al*., 1996). We reasoned that TBP alleles with increased activity would provide unique insights into the regulation of TBP not revealed by existing mutations. To identify potential hyperactive TBP alleles, we screened for mutants that increase transcription from a core (UAS-less) promoter in *S.cerevisiae*. The starting strain, YY1 (Figure 1A), contains a genomic *suc2Δuas*(*-1900/-390*) allele that removes 1.5 kb of *SUC2* upstream DNA, including the entire *SUC2* UAS, yet leaves the core promoter intact (Sarokin and Carlson, 1984). This deletion abolishes *SUC2* transcription, causing an inability to grow on media containing sucrose as the carbon source. Although *suc2Δuas* is transcriptionally inactive, previous studies have shown that it can be partially activated by mutations in histones, certain *SPT* genes (Malone *et al*., 1991; Swanson and Winston, 1992), *MOT1*, *BUR6* and other *BUR* genes (Prelich and Winston, 1993); increased transcription from *suc2Δuas* defines the Bur$^-$ (bypass UAS requirement) phenotype. YY1 also contained a genomic deletion of *SPT15*, which encodes TBP in yeast (Eisenmann *et al*., 1989), and an *SPT15*$^+$ *CEN* plasmid to maintain viability. A plasmid shuffle strategy (Sikorski and Boeke, 1991) utilizing a PCR-mutagenized *spt15* library (Arndt *et al*., 1995) was used to introduce random *spt15* mutations into YY1; following loss of the wild-type *SPT15*$^+$ plasmid, Bur$^-$ TBP mutants were identified by screening for the ability to grow on sucrose plates.

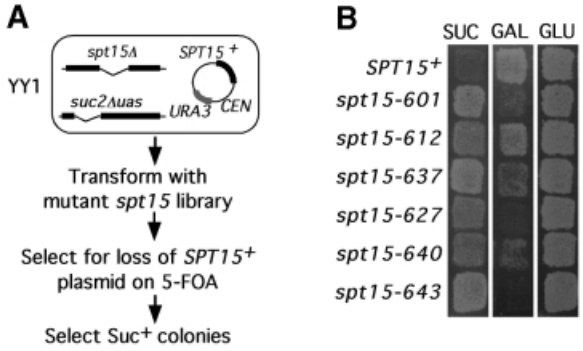


**Fig. 1.** Isolation of TBP Bur$^-$ mutants. (**A**) Plasmid shuffle strategy to identify TBP Bur$^-$ mutants. Strain YY1 contains a genomic *spt15* deletion, with the wild-type *SPT15* gene on a *URA3* plasmid. A PCR-mutagenized *spt15 LEU2*$^+$ library was introduced into YY1 and colonies that had lost the *SPT15*$^+$ plasmid were identified by growth on 5-FOA plates. Mutants that increase transcription from *suc2Δuas* were identified by growth on sucrose-containing media. (**B**) Growth of representative *spt15* mutants on media containing sucrose (SUC), galactose (GAL) or glucose (GLU) as the carbon source. All strains contain the *suc2Δuas*(*-1900/-390*) allele, in combination with the *spt15* mutations shown on the left. The *SPT15*$^+$ strain shown on top is a control to show the phenotypes of the starting strain.

Of 27 000 total transformants, 36 colonies contained plasmids that conferred Suc$^+$ growth (Figure 1B; Table I). Many of these mutations also conferred other mutant phenotypes, including Gal$^-$ and Ino$^-$, suggesting that they affect the transcription of genes besides *SUC2* and that they affect the transcription of wild-type genes with an intact UAS. All 36 alleles were sequenced, revealing 17 different amino acid changes localized within the conserved 180-amino-acid C-terminal core domain of TBP (Table I; Figure 2A). The 17 substitutions alter amino acids at nine different positions, only two of which (V71 and K145) had been identified by previous genetic screens (Zhou and Buratowski, 1992; Arndt *et al*., 1995); the *spt15-627* (V71M) mutation is on the DNA-binding surface (Figure 2B), whereas the *spt15-640* (K145E) mutation is in the basic region that is implicated in binding TFIIA

**Table I.** TBP mutations that increase transcription from *suc2Δuas*

| Amino acid replacement | Structural domains[a] | Conserved residue[b] | *spt15* alleles (times isolated) |
|---|---|---|---|
| V71M | S1 | yes | *spt15-627* (1) |
| K145E | H2 | yes | *spt15-640* (1) |
| H179Q | H1′–S2′ loop | yes | *spt15-602* (1) |
| H179P | H1′–S2′ loop | | *spt15-613* (1) |
| H179Y | H1′–S2′ loop | | *spt15-612* (1) |
| H179R, F155L | H1′–S2′ loop, S1′ | | *spt15-611* (10) |
| F182V | H1′–S2′ loop | yes | *spt15-601* (1) |
| F182S, G162S | H1′–S2′ loop, S1′ | | *spt15-614* (4) |
| S183P | S2′ | yes | *spt15-628* (1) |
| Y195C | S3′ | yes | *spt15-637* (3) |
| Y195H | S3′ | | *spt15-623* (4) |
| V198G | S3′–S4′ loop | no | *spt15-605* (2) |
| V198A | S3′–S4′ loop | | *spt15-632* (1) |
| V198A, S58P | S3′–S4′ loop, N-terminus | | *spt15-625* (1) |
| P232L | H2′ | yes | *spt15-631* (1) |
| V233A | H2′ | no | *spt15-607* (2) |
| V233L | H2′ | | *spt15-608* (1) |
| K145E, F182V | H2, H1′–S2′ loop | yes | *spt15-643*[c] |

[a]Structural domain nomenclature as reported in Nikolov *et al.* (1992) and Y.Kim *et al.* (1993). H, α-helix; S, β-strand.
[b]Residues conserved in all TBP sequences compiled in Nikolov *et al.* (1992).
[c]A double mutant constructed *in vitro* by combining the mutations present in *spt15-601* and *spt15-640*.

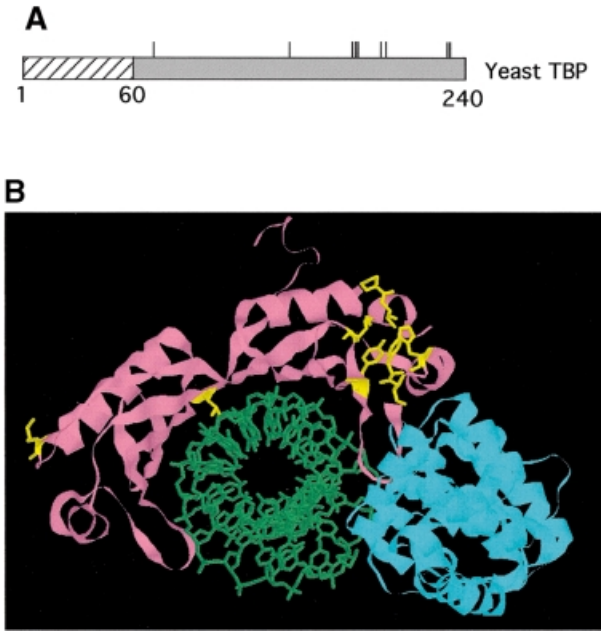


**Fig. 2.** TBP Bur⁻ mutations. (**A**) Linear representation of yeast TBP. The conserved 180-amino-acid C-terminal core domain of TBP is shaded, whereas the divergent N-terminal domain is depicted by diagonal lines. The nine amino acid residues affected by the 36 TBP Bur⁻ mutations are represented by vertical bars. (**B**) Location of the TBP mutations on the DNA–TBP–TFIIB crystal structure (Nikolov *et al.*, 1995). The DNA is green, TBP is purple and TFIIB is blue. The residues affected in TBP Bur⁻ mutants are shown in yellow. K145E is on the left, V71M is on the DNA-binding surface and the clustered mutants are located on the right, adjacent to the TFIIB-binding domain. This picture was generated using RasMol.

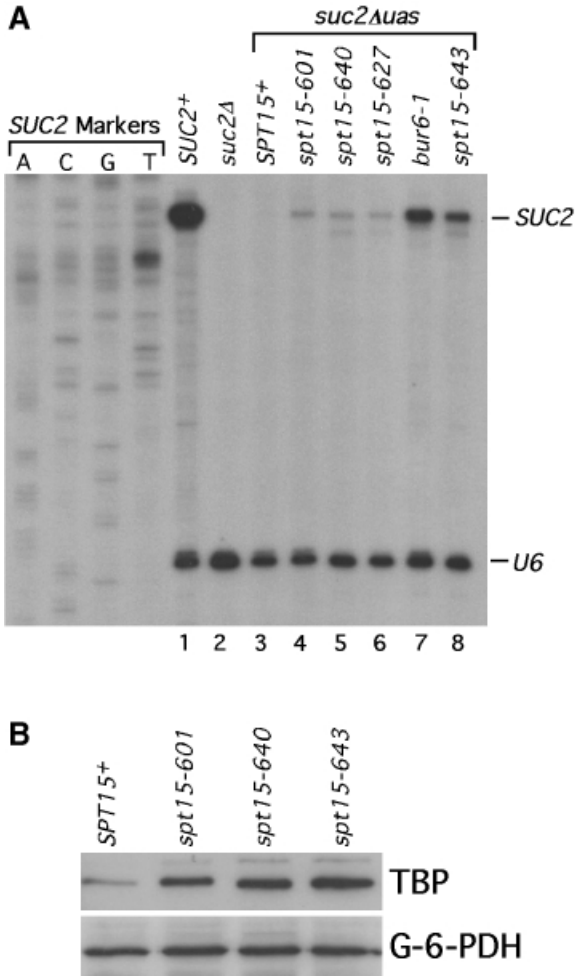


**Fig. 3.** The *spt15* mutations increase transcription from *suc2Δuas* (*-1900/-390*). (**A**) Primer extension analysis. RNA was prepared from strains with the indicated genotypes grown under derepressing conditions, and primer extension analysis was performed using primers specific for *SUC2* and *U6*. The positions of the *SUC2* and *U6* transcripts (loading control) are indicated and *SUC2* size markers are shown on the left. (**B**) Western blot analysis of TBP levels in the *spt15* mutants. Whole-cell protein extracts were prepared from *SPT15*⁺, *spt15-601*, *spt15-640* and *spt15-643* strains and probed with antibody against TBP or glucose-6-phosphate dehydrogenase (G-6-PDH).

and Mot1. Although the seven novel positions are scattered within the final 61 amino acids on the linear sequence of TBP and fall within four structural sub-domains, they define a remarkably tight cluster on the exposed surface of TBP in the three-dimensional structure, adjacent to the TFIIB-binding site (Figure 2B). The tight clustering and surface localization suggest that these mutations identify an interaction site between TBP and a regulator of basal transcription.

### *The TBP mutations increase transcription from suc2Δuas*

To determine whether the Suc⁺ phenotype of the TBP Bur⁻ mutants was due to increased transcription from *suc2Δuas*, primer extension analysis was performed using RNA prepared from strains grown under derepressing conditions for *SUC2* expression. The *spt15* mutations increase *SUC2* transcript levels in the *suc2Δuas* background, whereas wild-type TBP is inactive (Figure 3A, lanes 3–6). Furthermore, the transcripts initiate at the normal *SUC2* 1.9 kb mRNA start site, indicating that the increased transcription is not due to aberrant initiation

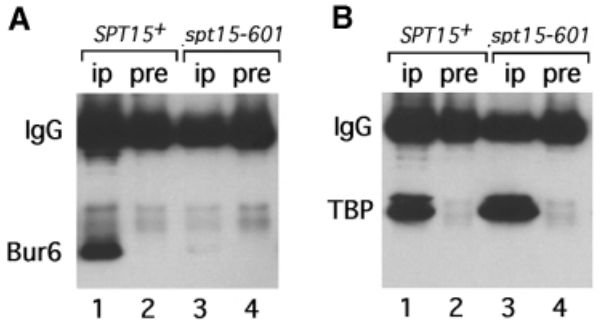


**Fig. 4.** F182V (*spt15-601*) is defective for interacting with Bur6. Extracts were prepared from wild-type and *spt15-601* strains overexpressing FLAG-Bur6, and 2 mg of each extract were incubated with antibody against TBP (ip) or pre-immune serum (pre). Immune complexes were precipitated with protein A–Sepharose beads, washed, and loaded onto 15% SDS–polyacrylamide gels. Proteins were transferred to filters, which were then probed with (**A**) anti-Bur6 antibody or (**B**) anti-TBP antibody.

events. To determine whether increased *suc2*Δ*uas* transcription is due simply to altered levels of TBP, we examined TBP levels in the mutant strains. Although TBP levels were increased slightly in three mutants tested (Figure 3B), the increased *suc2*Δ*uas* transcription is not caused simply by TBP overproduction, since overexpression of wild-type TBP from a high copy number plasmid does not result in Suc$^+$ growth, and overexpression of the TBP mutants from a high copy number plasmid does not enhance the Suc$^+$ growth (Y.Cang and G.Prelich, unpublished data). Finally, the experiments described below demonstrate specific biochemical defects consistent with increased or unregulated TBP activity. We conclude that the altered TBP proteins are more active at the *SUC2* core promoter *in vivo*, initiating transcription under conditions that are normally inhibitory for wild-type TBP.

### *The clustered mutants are defective for binding yNC2*

Based on their surface localization and the resulting increased transcription at *suc2*Δ*uas*, we suspected that the clustered mutants were defective for binding a general transcriptional repressor. The most likely candidates were yNC2 (a heterodimer of Bur6 and Ydr1) and Mot1, since *bur6* and *mot1* mutations also cause a Bur$^-$ phenotype (Prelich, 1997). Preliminary genetic characterization (see Figure 8) suggested that mutations in the cluster were all defective for the same function; to characterize the cluster biochemically we chose to analyze in detail *spt15-601* (F182V), the mutant that caused the strongest Suc$^+$ growth. We first tested whether F182V (*spt15-601*) associates with yNC2 using a co-immunoprecipitation assay. Extracts were prepared from *SPT15*$^+$ and *spt15-601* strains that overexpress Ydr1 and Flag-tagged Bur6. Equivalent amounts of TBP were immunoprecipitated from both extracts (Figure 4B, lanes 1 and 3); Bur6 co-immunoprecipitated with wild-type TBP, whereas barely detectable levels of Bur6 co-immunoprecipitated with F182V (Figure 4A, lanes 1 and 3). F182V is thus defective for binding to Bur6 in whole-cell extracts.

To determine whether this was a direct effect, gel mobility shift analysis was performed using recombinant yNC2 in combination with wild-type and mutant TBPs that were expressed and purified from *Escherichia coli*.

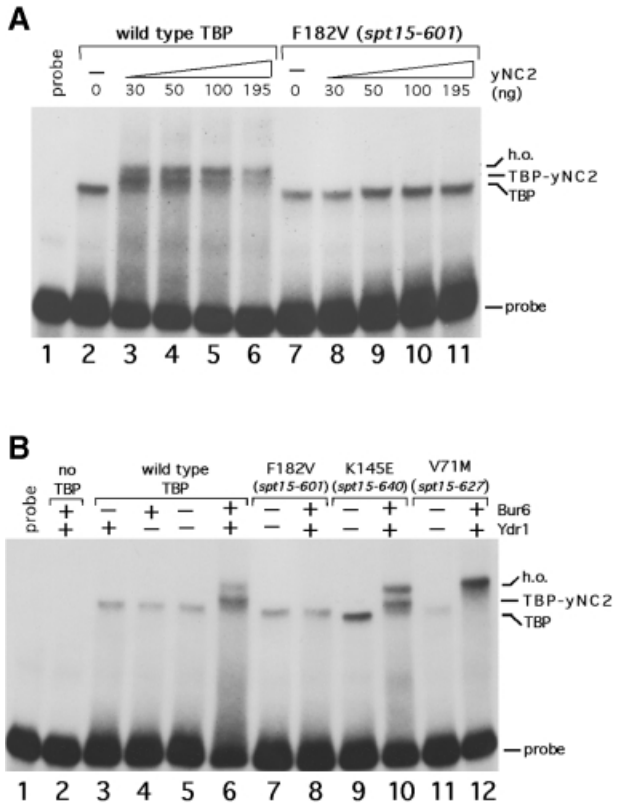


**Fig. 5.** F182V (*spt15-601*) fails to bind to yNC2 *in vitro*. Gel shift reactions were performed using the adenovirus major late promoter with either wild-type TBP or recombinant mutant TBP as indicated. TBP and DNA were incubated with recombinant Bur6, Ydr1 or yNC2 (Bur6 + Ydr1 in a 1:1 molar ratio) at 30°C for 30 min and the products were resolved in 6% non-denaturing polyacrylamide gels at 4°C. (**A**) The TBP F182V mutant is defective for yNC2 interaction. For each 10 µl reaction, the amounts of yNC2 used were 30 ng (lanes 3 and 8), 50 ng (lanes 4 and 9), 100 ng (lanes 5 and 10) or 195 ng (lanes 6 and 11). The positions of the monomeric (TBP–yNC2) and higher order (h.o.) yNC2–TBP–DNA complexes are indicated. (**B**) Other TBP non-cluster mutants bind to yNC2. Reactions were assembled as in (A) except that 30 ng of yNC2 were used for each binding assay where indicated.

As described previously (Goppelt *et al*., 1996), Bur6 and Ydr1 individually do not shift a TBP–DNA complex (Figure 5B, lanes 3 and 4), whereas Bur6 and Ydr1, expressed and purified from *E.coli* and combined in a 1:1 molar ratio (hereafter referred as as yNC2), form stable complexes with wild-type TBP (Figure 5B, lane 6; Figure 5A, lanes 3–6). The yNC2–TBP–DNA complexes are visualized as two bands in non-denaturing gels; these bands represent monomeric and higher order (h.o.) yNC2–TBP–DNA complexes (Goppelt *et al*., 1996). yNC2, however, failed to bind the F182V cluster mutant stably (Figure 5A, lanes 7–11; Figure 5B, lanes 7 and 8), even using >6-fold more yNC2 (Figure 5A, lanes 3 and 11). Identical results were obtained with another cluster mutant, *spt15-637* (Y195C; data not shown). This defect was specific for the cluster mutants as the two non-cluster mutants tested, K145E and V71M, bound yNC2 as well as wild-type TBP (Figure 5B, lanes 6, 10 and 12). Interestingly, V71M (*spt15-627*) has diminished DNA-binding ability by itself, yet forms a stable complex with yNC2 (Figure 5B, lanes 11 and 12). The cluster mutants are, therefore, specifically defective for binding yNC2,

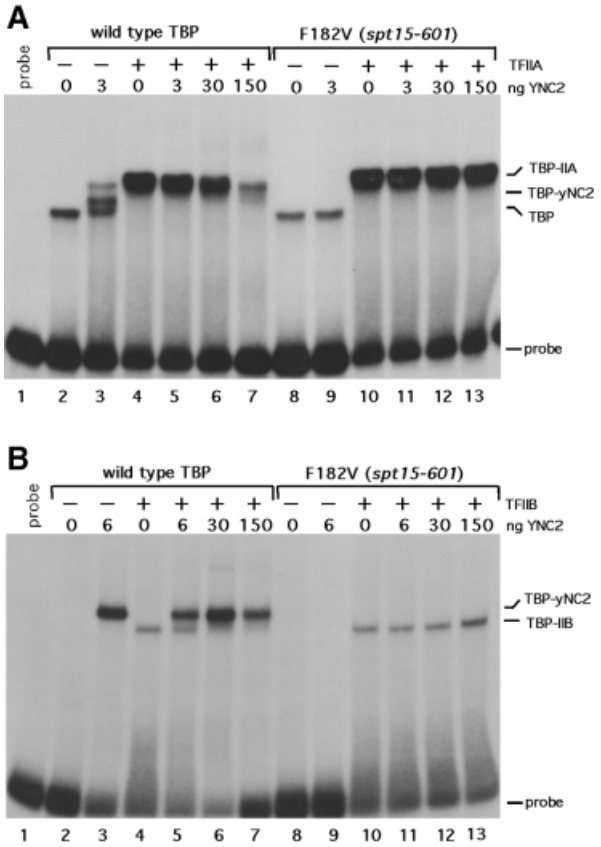


**Fig. 6.** yNC2 fails to compete with TFIIA and TFIIB for binding F182V. (**A**) yNC2 competes with TFIIA for binding wild-type TBP but not for binding F182V. Where indicated, 8 ng of TBP and 0.5 ng of TFIIA were incubated with the indicated amounts of yNC2. (**B**) yNC2 competes with TFIIB for binding wild-type TBP but not for binding F182V. Reactions containing 8 ng of TBP, 100 ng of recombinant core TFIIB, which lacks amino acids 1–119 (a gift from S.Buratowski), and the indicated amounts of yNC2 were resolved in 6% gels in Tris–glycine buffer at room temperature. Electrophoresis in (B) was performed under conditions that destabilize the TBP–DNA complex.

and yNC2 performs a positive role in the binding of V71M *in vitro*, stabilizing its interaction with the TATA box.

### *F182V (spt15-601) is not inhibited by yNC2 in vitro*

Human NC2 was reported to compete with both TFIIA and TFIIB for binding to TBP; this competition is the presumed basis for its inhibitory activity (Goppelt *et al*., 1996; Mermelstein *et al*., 1996). The isolation of TBP mutants defective for binding yNC2 allowed us to test this hypothesis. Gel mobility shift assays were assembled using recombinant yeast proteins and the ability of yNC2 to block association of TFIIA and TFIIB was assayed. Reactions were assembled with constant amounts of TBP and TFIIA (Figure 6A) or core TFIIB (Figure 6B), and increasing amounts of yNC2. yNC2 competes with formation of both the TBP–TFIIA–DNA complex (Figure 6A, lanes 4–7) and the TBP–TFIIB–DNA complex (Figure 6B, lanes 4–7) using wild-type TBP. Consistent with the observation that F182V is defective for binding to yNC2, the F182V–TFIIA–DNA and F182V–TFIIB–DNA complexes were resistant to increasing concentrations of yNC2 (Figure 6A and B, lanes 10–13). This result indicates that the loss of yNC2 binding to TBP caused by F182V results in the inability of yNC2 to block recruitment of TFIIA and TFIIB to the pre-initiation complex, which in turn increases basal transcription.

### *K145E (spt15-640) is defective for binding Mot1 and TFIIA*

The results described above indicate that the TBP cluster mutants are defective for interactions with yNC2. This is in contrast to previous results (Kim *et al*., 1995), which indicated that TBP residue K145 was a determinant for binding NC2. K145 has also been implicated as being important for binding Mot1 (Auble and Hahn, 1993) and TFIIA (Zhou and Buratowski, 1992), but these studies utilized a double mutant (K133L + K145L) and the contributions of the individual K133 and K145 mutations were not examined. Based on these previous results, the increased transcription from *suc2Δuas* caused by our K145E mutant could have been due to an increased affinity for TFIIA or a reduced affinity for either yNC2 or Mot1. The results presented in Figure 5B, however, indicate that the TBP–yNC2 interaction is unaffected in the K145E mutant. To distinguish between the remaining possibilities, we first examined whether K145E (*spt15-640*) is defective for binding Mot1. Binding of Mot1 to TBP can be assessed in two ways using gel mobility shift assays. In the absence of ATP, Mot1 retards the mobility of a TBP–DNA complex, whereas in the presence of ATP, Mot1 displaces TBP from the TATA box. Wild-type and mutant TBPs were expressed and purified from *E.coli*, and were assayed for binding to Mot1 that had been purified from yeast. Wild-type TBP, F182V and V71M each formed TBP–Mot1 complexes in the absence of ATP (Figure 7A, lanes 2, 5 and 11), and in the presence of ATP those TBPs were displaced from the TATA box by Mot1 (Figure 7A, lanes 3, 6 and 12). The K145E mutant, however, did not form a Mot1–TBP complex, and this mutant TBP was resistant to displacement by Mot1 in the presence of ATP (Figure 7A, compare lanes 2 and 3 with lanes 8 and 9). Resistance to the inhibitory effects of Mot1 is consistent with the Bur$^-$ phenotype of this mutant.

We next examined whether the TBP Bur$^-$ mutants bind TFIIA (Figure 7B). Similar to the results with Mot1, wild-type TBP, F182V and V71M bound TFIIA, whereas the K145E mutant was unable to form a TBP–TFIIA complex; K145E is therefore defective for binding both Mot1 and TFIIA. The reduction of K145E binding to TFIIA is consistent with previous mapping of TFIIA-binding determinants (Bryant *et al*., 1996; Tang *et al*., 1996). Reduced binding of Mot1, and not TFIIA, is most likely the cause of the Bur$^-$ phenotype of K145E (Figure 8); moreover, the defective TFIIA binding provides an explanation for why the phenotypes of this mutant are not as severe as some *mot1* mutations (see Discussion).

### *Allele-specific interactions between TBP Bur$^-$ mutants, MOT1 and BUR6*

Biochemical analysis of our TBP Bur$^-$ proteins demonstrated that V71M (*spt15-627*) is defective for binding DNA, K145E (*spt15-640*) is unable to bind Mot1 and TFIIA, and that F182V (*spt15-601*) and another cluster mutant, Y195C (*spt15-637*), are defective for binding yNC2. To determine whether the defects detected *in vitro* are relevant physiologically, we tested for genetic inter-

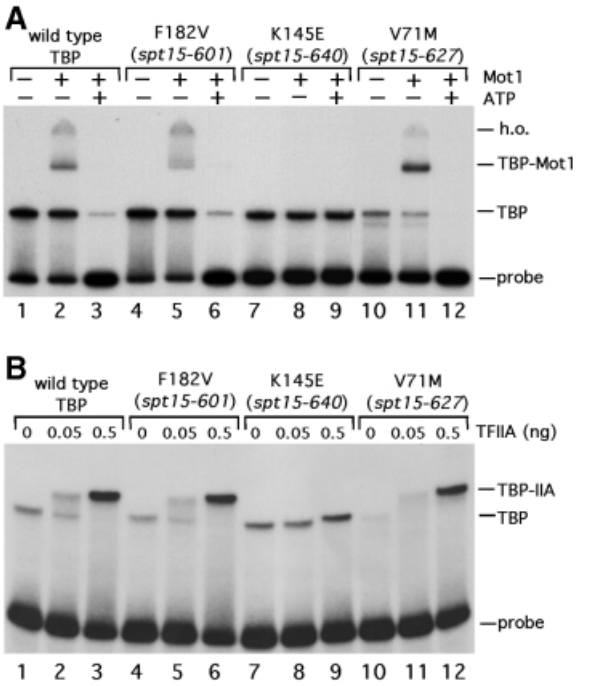


**Fig. 7.** K145E (*spt15-640*) is defective for binding Mot1 and TFIIA. (**A**) K145E (*spt15-640*) fails to bind Mot1. Gel shift reactions were assembled using oligos containing the adenovirus major late promoter and purified recombinant wild-type or mutant TBP as indicated. TBP and DNA were incubated together for 20 min at room temperature, followed by the addition of purified Mot1 expressed in yeast and ATP where indicated. Reactions were analyzed on a 6% non-denaturing polyacrylamide gel. h.o. designates a higher order TBP–Mot1–DNA complex formed in the absence of ATP. (**B**) K145E (*spt15-640*) fails to bind TFIIA. Either 0.05 ng (lanes 2, 5, 8 and 11) or 0.5 ng (lanes 3, 6, 9 and 12) of TFIIA was incubated with 8 ng of wild-type or mutant TBP, as indicated, at 30°C for 30 min and the reactions were analyzed on a 6% non-denaturing gel. The positions of the TBP–IIA–DNA and TBP–DNA complexes are shown on the right.

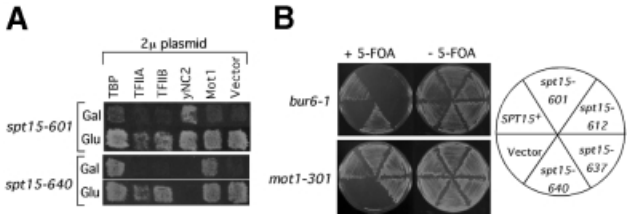


**Fig. 8.** Allele-specific interactions between *SPT15*, *MOT1* and *BUR6*. (**A**) Suppression of *spt15* Gal$^-$ phenotypes by high copy number Mot1 and yNC2 (Bur6 + Ydr1) plasmids. 2μ plasmids expressing either TBP, TFIIA, TFIIB, yNC2 or Mot1 were transformed into *spt15-601* and *spt15-640* strains. The transformants were replica plated onto selective media containing glucose (Glu) or galactose (Gal) as the carbon source. The *spt15-601* Gal$^-$ phenotype is complemented by 2μ yNC2 and the *spt15-640* phenotype is complemented by 2μ *MOT1* and TBP. 2μ yNC2 is lethal in the *spt15-640* strain. (**B**) Allele-specific synthetic lethality between *spt15*, *mot1* and *bur6* mutations. YY86 (*spt15*Δ *bur6-1*) and YY92 (*spt15*Δ *mot1-301*) strains that contain *SPT15*$^+$ on a *URA3 CEN* plasmid were transformed with plasmids carrying the indicated *spt15* alleles. Transformants were streaked onto selective media in the presence or absence of 5-FOA. A 5-FOA-sensitive phenotype indicates a synthetic lethal interaction. Photographs were taken after 3 days.

actions of these *spt15* Bur$^-$ alleles with *MOT1* and *BUR6*. If these defects are the cause of the mutant phenotypes, then two strong genetic predictions can be made. First, the weakened interactions caused by the TBP mutations might be specifically compensated for by overexpression of the interacting protein, and secondly, the phenotypes caused by a weakened interaction should be specifically exacerbated by a mutation in that interacting protein. To test the first prediction, we transformed an *spt15-601* strain (defective for binding yNC2 *in vitro*) and an *spt15-640* strain (defective for binding Mot1 and TFIIA *in vitro*) with high copy number plasmids that overexpress either yNC2, Mot1, TFIIA, TFIIB or TBP (Figure 8A). Specific patterns of suppression were observed: the *spt15-601* (F182V) Gal$^-$ phenotype was suppressed by overexpression of yNC2, but not any of the other factors, and conversely the *spt15-640* (K145E) defect was suppressed by overexpression of Mot1, but not any of the other factors. Thus, the patterns of suppression that were obtained mirror the biochemical effects observed *in vitro*; overexpression of yNC2 compensated for reduced binding of yNC2 to F182V and overexpression of Mot1 compensated for reduced binding between Mot1 and K145E. Interestingly, overexpression of yNC2 was lethal in the *spt15-640* strain (see Discussion). Expression of TBP complemented *spt15-640*, but only weakly complemented *spt15-601*, indicating that *spt15-601* is partially dominant.

To test the second prediction, we crossed *bur6-1* and *mot1-301* mutations into *spt15* mutant backgrounds and examined the double mutant phenotypes (Figure 8B). Once again, allele-specific reciprocal interactions were detected that were consistent with the biochemical results; *bur6-1* was lethal in combination with three cluster mutants, *spt15-601*, *spt15-612* and *spt15-637*, whereas *mot1-301* was lethal in combination with *spt15-640*, which was defective for Mot1 interaction. The specific patterns of synthetic lethality observed indicate that lethality is not merely due to combined sickness of the strains, but rather is due to specific functional defects shared between *mot1-301* and *spt15-640*, and between *bur6-1* and the cluster mutants *spt15-601*, *spt15-612* and *spt15-637*. Combined, these results provide strong genetic evidence that reduced binding of yNC2 and Mot1 is the direct cause of the *spt15-601* and *spt15-640* phenotypes.

### *Combinatorial effects of TBP Bur$^-$ mutations on core promoter activity*

If the TBP Bur$^-$ mutants are not inhibited by yNC2 and Mot1, then they should have general effects, increasing transcription from core promoters other than *suc2*Δ*uas*. To test this prediction, the effects of the *spt15* mutations on expression from the *cyc1*Δ*uas*, *ho*Δ*uas* and *gal1*Δ*uas* core promoters were determined. Reporter plasmids containing *LacZ* fusions to these three core promoters were transformed into *SPT15*$^+$, *spt15-601* and *spt15-640* strains, and β-galactosidase levels were quantitated (Figure 9). All three core promoters reproducibly directed 2.5- to 6.5-fold greater *LacZ* expression in the *spt15-601* strain (defective for yNC2–TBP interactions); in contrast, the *spt15-640* mutation (defective for Mot1 and TFIIA interactions) exhibited virtually no effect on these core promoters (see Discussion). To determine whether simultaneous loss of both Mot1- and yNC2-dependent repression resulted in combinatorial effects, we created a double mutant TBP allele, *spt15-643*, which contains the mutations present in *spt15-601* and *spt15-640*, and is therefore defective for binding Mot1, yNC2 and TFIIA.

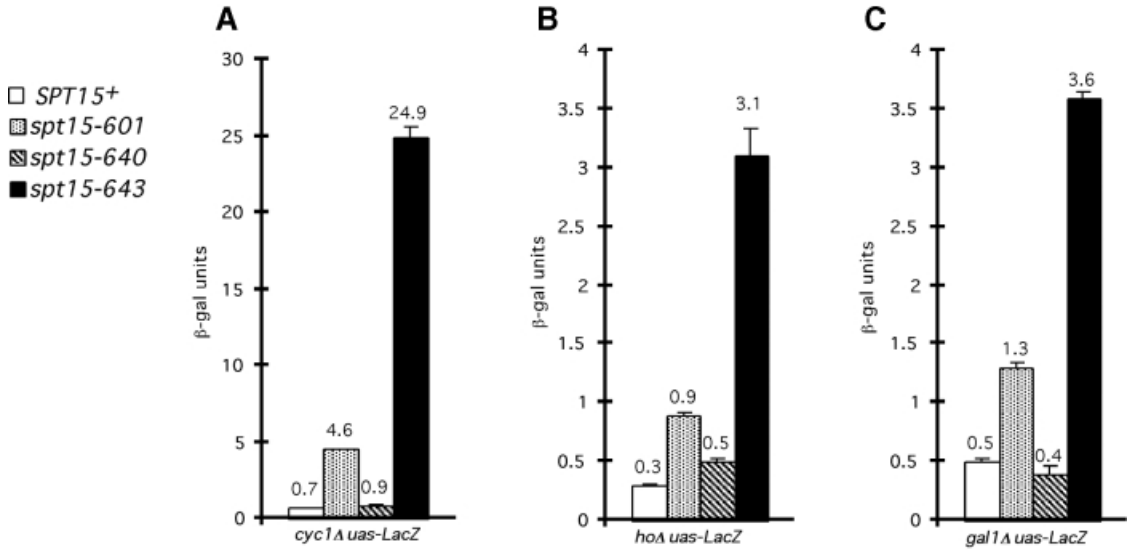


**Fig. 9.** Combinatorial effects of *spt15* mutations on expression from other core promoters. *SPT15*$^{+}$, *spt15-601*, *spt15-640* and *spt15-643* strains were transformed with reporter plasmids carrying *cyc1Δuas-LacZ* (**A**), *hoΔuas-LacZ* (**B**) and *gal1Δuas-LacZ* (**C**). Individual transformants were grown in either SC-URA plus raffinose (for *gal1Δuas* and *cyc1Δuas*) or SC-Ura plus glucose (for *hoΔuas*), extracts were prepared and β-galactosidase (β-gal) activities were quantitated. Levels of expression from the *SPT15*$^{+}$ strain are represented by white boxes, levels from the *spt15-601* strain by dotted boxes, levels from the *spt15-640* strain by hatched boxes and levels from the *spt15-643* strain by black boxes. β-gal levels are the mean of three experiments using independent transformants, with standard errors (T bars) generally <15%.

When the effects of *spt15-643* on these *Δuas LacZ* reporters were tested, strong combinatorial effects were observed; *cyc1Δuas* expression, for example, was unaffected in *spt15-640*, increased 6.5-fold in *spt15-601*, but a 36-fold increase was observed in the *spt15-643* double mutant (Figure 9A). Similar patterns were observed for the *hoΔuas* and *gal1Δuas* promoters (Figure 9B and C). Primer-extension analysis demonstrated that the *spt15-643* double mutant also caused combinatorial effects on *suc2Δuas* transcription (Figure 3A, lanes 4, 5 and 8). These results indicate that TBP is repressed by both yNC2 and Mot1 *in vivo* at all four core promoters tested and that the combined effects of yNC2 and Mot1 result in significant transcriptional repression.

## Discussion

### *A new class of TBP mutants*

Here we describe a new class of TATA-binding protein mutants that result in increased transcription from core (UAS-less) promoters *in vivo*. Most previous TBP mutants were identified based upon loss of TBP activity, resulting in changes in start site selection (Eisenmann *et al*., 1989; Arndt *et al*., 1992; Strubin and Struhl, 1992), temperature-sensitive lethality (Cormack and Struhl, 1993), loss of activation (Arndt *et al*., 1995; Lee and Struhl, 1995; Stargell and Struhl, 1996) or polymerase-specific transcriptional defects (Schultz *et al*., 1992; Cormack and Struhl, 1993). In addition, two large-scale, site-directed mutagenesis efforts generated mutations at 106 TBP surface residues that have been tested for loss of specific biochemical activities (Bryant *et al*., 1996; Tang *et al*., 1996). Despite this extensive mutational bombardment, very few TBP mutants with increased transcriptional activity have been described thus far (Blair and Cullen, 1997; Jackson-Fisher *et al*., 1999). Here we isolated a large number of mutants, based on a unique phenotype, that provide two important insights into TBP function. First, the Bur$^{-}$ TBP alleles provide the first direct evidence that TBP is repressed *in vivo*, and secondly, they reveal a previously unrecognized surface domain important for interaction with yNC2 *in vivo* and *in vitro*.

### *Mechanisms underlying the TBP Bur$^{-}$ phenotype*

We identified three classes of Bur$^{-}$ TBP mutants that increase transcription by different mechanisms; the largest class, comprising 34 of our 36 isolates, forms a cluster on the exposed TBP surface adjacent to the TFIIB interaction domain. The clustered mutations affect seven amino acids that were not identified by any previous selections and which were not targeted in the large-scale directed mutagenesis efforts. Multiple alleles have been identified at five of these positions: His179, Phe182, Tyr195, Val198 and Val233. It is unlikely that mutations at these sites cause the gain of a novel interaction because several different amino acid substitutions at these sites increase transcription from *suc2Δuas*; gain-of-function mutations would demand limited, specific changes. Instead, we propose that these residues identify the site of action for a repressor of TBP, and that loss of interaction with the repressor increases TBP activity. Single mutations identified at two nearby positions, Ser183 and Pro232, involve proline substitutions, and thus may cause conformational changes that reduce direct repressor contacts within the cluster. Five of these seven residues are conserved absolutely in organisms ranging from yeast, plants and *Drosophila* to humans (Nikolov *et al*., 1992), suggesting the highly conserved nature of the TBP–NC2 interaction surface.

The cluster mutations identify residues required for interactions with yNC2. This is supported by five lines of evidence obtained primarily by detailed analysis of a strong allele from the cluster *spt15-601* (F182V). First, yNC2 co-immunoprecipitates with wild-type TBP, but not

with F182V. Secondly, purified yNC2 associates with wild-type TBP, but not with F182V or Y195C by gel mobility shift assays. Thirdly, purified yNC2 inhibits the association of TFIIA and TFIIB with wild-type TBP, but not with F182V. Fourthly, overexpression of yNC2 suppresses the Gal⁻ phenotype of *spt15-601* in an allele-specific manner. Fifthly, *spt15-601* and two other cluster mutants are synthetically lethal in combination with a *bur6-1* mutation, which encodes a subunit of yNC2, but not with a phenotypically similar *mot1* mutation. The identification of these residues as important yNC2-binding determinants may appear to be in contradiction to a previous study (Kim *et al*., 1995), which determined that residues K133, K145 and K151 in the basic region, on the opposite face of TBP, are required for NC2 interactions. This apparent discrepancy is most likely due to the use of Dr1 in the previous study rather than the physiologically relevant NC2 heterodimer, since our results indicate that yNC2 binds to the TBP K145E mutant as effectively as to wild-type TBP. Residues within the basic region may be important for contact with monomeric Dr1, but there is no evidence that those TBP mutations block NC2-dependent repression *in vivo* or *in vitro*. Alternatively, there may be two regions important for NC2 binding, only one of which was detected in our study because of the demands of the genetic selection. Finally, the previous study utilized human factors, whereas our analysis has been restricted to analyzing yeast proteins. We think it unlikely that species differences account for the discrepancy, however, since human and yeast NC2 inhibits TBP isolated from either species (Goppelt and Meisterernst, 1996; Kim *et al*., 1997). Confirmation that the clustered Bur⁻ residues of TBP contact NC2 awaits structural analysis of the TBP–NC2 complex.

Our selection also identified a single mutation at K145, a residue implicated previously in contacting TFIIA, Mot1 and hDr1. The increased transcription from *suc2Δuas* and other core promoters caused by K145E could have been due to an increased affinity for TFIIA or a reduced affinity for Mot1 or yNC2. We now believe that the Bur⁻ phenotype is due to reduced affinity for Mot1 based on the following evidence. First, K145E (*spt15-640*) is defective for binding Mot1 *in vitro*; in the absence of ATP, Mot1 forms a stable complex with wild-type TBP, but not with K145E. K145E is thus resistant to ATP-dependent displacement from the TATA box by Mot1. Secondly, K145E (*spt15-640*) displays reduced, not increased, binding of TFIIA, and binds yNC2 as well as wild-type TBP. Thirdly, the Gal⁻ phenotype of *spt15-640*, but not other *spt15* alleles, is suppressed by overexpression of Mot1. TFIIA overexpression did not suppress the *spt15-640* Gal⁻ phenotype, indicating that the Gal⁻ phenotype is due to loss of the TBP–Mot1 interaction and not loss of the TBP–TFIIA interaction. Finally, *spt15-640* is synthetically lethal with the *mot1-301* mutation, but not with *bur6-1*. In general, the phenotypes of *spt15-640* are not as strong as *mot1* mutations; this is possibly due to the simultaneous defect of K145E for binding TFIIA. TFIIA and Mot1 bind to the same surface of TBP, compete with each other, and TFIIA stabilizes TBP from displacement by Mot1 (Auble and Hahn, 1993; Auble *et al*., 1994). *spt15-640* is thus partially defective for interaction with both a repressor (Mot1) and a positive factor (TFIIA), resulting in a net weaker phenotype than *mot1* alleles.

The final Bur⁻ TBP class consists of a single allele, V71M. This mutation is on the DNA-binding surface, resulting in reduced affinity for the AdMLP TATA box. Two possibilities may account for the V71M Bur⁻ phenotype. Structural analysis demonstrates that Val71 directly contacts $dTAF_{II}230$ (D.Liu *et al*., 1998), the *Drosophila* homolog of $yTAF_{II}145$; V71M may therefore disrupt $TAF_{II}145$-mediated inhibition. Alternatively, TBP dimerization may be disrupted by the protruding methionine side chain now present in V71M, resulting in more active monomeric TBP. In support of this model, overexpression of TBP mutants that disrupt dimerization was recently found to increase basal transcription from certain promoters (Jackson-Fisher *et al*., 1999). Further studies will be required to examine the specific effects caused by this mutation.

### *Functional overlap between yNC2 and Mot1*

By some criteria, transcriptional activity from core promoters in Bur⁻ strains is not extremely robust; *bur6-1 suc2Δuas* expression is ~10% of fully induced *SUC2* levels, and the effects of individual TBP Bur⁻ mutations are even less. Does this mean that repression of TBP plays an insignificant role in transcription *in vivo*? Based on three main considerations, we believe that the opposite is true: the effects that we detect are substantial, and TBP repression by NC2 and Mot1 is critical for cell viability. First, by definition, UAS-less promoters do not contain activator binding sites and therefore should not be expected to attain fully activated levels of transcription. Secondly, complete deletion of *BUR6*, *YDR1* or *MOT1* causes lethality (Davis *et al*., 1992; Kim *et al*., 1997; Lee *et al*., 1998) or extreme sickness (Prelich, 1997), indicating that yNC2 and Mot1 have non-overlapping functions, each essential for cell viability; that essential function is presumed to be regulation of TBP. Quantitating the magnitude of the effects is, therefore, only possible using alleles that still possess some repression activity. Although we do not detect binding of Mot1 to K145E or of yNC2 to F182V using gel shift assays *in vitro*, these TBP mutants must still retain some activity *in vivo* since they are suppressed by overexpression of Mot1 and yNC2, respectively. In support of this conclusion, very low levels of F182V co-immunoprecipitated with yNC2 from crude extracts. Finally, yNC2 and Mot1 are partially redundant for inhibiting core promoters, resulting in diminished effects of mutations that abolish only one of these pathways. For example, a TBP double mutant (*spt15-643*) that is defective for interaction with both yNC2 and Mot1 shows combinatorial effects on transcription compared with the single mutations, and *bur6 mot1* double mutants are nearly lethal (G.Prelich, unpublished data), indicating that yNC2 and Mot1 have some overlapping functions. In addition, other repressors of the basal transcription machinery, such as nucleosomes, $TAF_{II}145$, the NOT complex (Collart, 1996; H.Y.Liu *et al*., 1998) and the other *BUR* genes, are still intact in those strains. In light of these considerations, detecting 10–20% of fully induced transcription in the absence of the *SUC2* UAS in individual *bur* mutants (Prelich, 1997) is quite remarkable. We are left with an emerging picture of an essential, elaborate, and partially

redundant, regulatory system that has evolved to ensure that basal promoter elements are inactive under the appropriate conditions.

## Materials and methods

### Strains and growth conditions

The *S.cerevisiae* strains used in this study were derived from an S288C background: YY1 (*MATa his4-917δ lys2-173R2 suc2Δuas(-1900/-390) ura3-52 trp1Δ63 leu2Δ1 spt15Δ102::LEU2* [*pDE28-6*]), YY86 (*MATa his4-917δ lys2-173R2 suc2Δuas(-1900/-390) ura3-52 trp1Δ63 leu2Δ1 bur6-1 spt15Δ102::LEU2* [*pDE28-6*]) and YY92 (*MATa his4-917δ lys2-173R2 suc2Δuas(-1900/-390) ura3-52 trp1Δ63 leu2Δ1 bur3-1 spt15Δ102::LEU2* [*pDE28-6*]). All media used, including rich (YPD), synthetic complete drop-out, minimal and sporulation media, were made as described previously (Rose *et al.*, 1990). Sucrose and galactose media contained the respective carbon source at 2% plus 1 μg antimycin A/ml. DNA transformation was performed using the lithium acetate method.

### Isolation of TBP mutants

An *spt15* mutant library generated using the PCR method (Arndt *et al*., 1995) was transformed into strain YY1, which contains a genomic *spt15* deletion and plasmid pDE28-6 (*SPT15*$^+$ *URA3 CEN*). Trp$^+$ transformants were replica-plated to media containing 5-fluoroorotic acid (5-FOA) to select for colonies that had lost the *SPT15*$^+$ plasmid. 5-FOA-resistant colonies were replica-plated to YPsucrose media. Plasmid DNA was recovered from Suc$^+$ candidates (Hoffman and Winston, 1987) and transformed into *E.coli*. The *SPT15* open reading frame (ORF) from these candidates was sequenced on both DNA strands. A double mutant allele, *spt15-643*, was constructed by replacing the *Bsm*I–*Eco*RI fragment of pYC1 (*spt15-601*) with the *Bsm*I–*Eco*RI fragment of pYC46, which contains *spt15-640*.

### RNA analysis

Total RNA was prepared from derepressed cultures, and primer extensions using *SUC2*- and *U6*-specific primers were performed as described previously (Prelich and Winston, 1993).

### Protein overexpression and purification

Wild-type TBP, F182V, K145E and V71M were expressed in *E.coli* under the control of the T7 promoter and were purified as described previously (Petri *et al*., 1995). TBPs were >95% pure, except for TBP–V71M, which was ~50% pure. Bur6 was expressed as a C-terminally tagged $His_6$ fusion in pET8c (pGP288), and Ydr1 was expressed as an N-terminally tagged fusion in pET15b (pGP415). Both proteins were bound to Ni–NTA columns (Qiagen) and eluted with a 10–500 mM imidazole gradient, resulting in >95% homogeneity. Core TFIIB was provided by S.Buratowski, and TFIIA was provided by T.Imbalzano. Mot1 was purified from a yeast overexpression strain (Auble *et al*., 1997).

### Co-immunoprecipitation of TBP and Bur6

Flag-Bur6 and Ydr1 were co-expressed divergently from the *GAL1,10* UAS in plasmid pGP468. pGP468 was transformed into YY39 (*SPT15*$^+$) and YY34 (*spt15-601*) strains and the transformants were grown to an $A_{600}$ of 1.0 in SC-Ura media with 5% galactose as the carbon source to induce Flag-Bur6 and Ydr1 overexpression. Extracts were prepared by bead-beating in lysis buffer [20 mM Tris–HCl pH 8.0, 5 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 0.1% Triton X-100] plus 0.15 M KOAc. All protein manipulations were carried out at 4°C and in the presence of the following protease inhibitors: aprotinin (1.72 μg/ml), pepstatin A (1 μg/ml), chymostatin (0.1 μg/ml), E64 (7.2 μg/ml), phosporamidon (1.1 μg/ml) and phenylmethylsulfonyl fluoride (PMSF) (1 mM). Immunoprecipitations were performed by mixing 2 mg of protein extract (pre-spun at 16 000 *g* for 15 min at 4°C in a microcentrifuge to remove protein aggregates) in lysis buffer plus 0.6 M KOAc with 2 μl of anti-TBP antibody or pre-immune serum in a final volume of 1 ml. After 2 h incubation with gentle mixing, the reactions were transferred into fresh tubes containing 40 μl of pre-washed protein A–Sepharose beads (Pharmacia) for an additional 2 h incubation. The beads were pelleted, washed three times by addition of 1 ml of lysis buffer plus 0.6 M KOAc, followed by 5 min of gentle rocking. After the final wash, the beads were resuspended in SDS–sample loading buffer for gel electrophoresis. Western blot analysis of TBP and Bur6 was performed as described previously (Prelich, 1997).

### Gel mobility shift assays

All binding reactions, which contained 2000–4000 c.p.m. 60 bp of adenovirus major late promoter (positions –50 to +10) and various factors as indicated in the figure legends, were incubated at 30°C for 30 min in a buffer containing 20 mM K-HEPES pH 8.2, 5 mM $MgCl_2$, 60 mM KCl, 6% glycerol, 10 ng/ml poly(dG–dC), 1 mM PMSF, 0.5 ng/ml bovine serum albumin (BSA) and 5 mM DTT. The reactions were analyzed in 6% (50:1) native polyacrylamide gels in 1× TBE buffer containing 2 mM $MgCl_2$, 0.5 mM DTT and 5% glycerol. Electrophoresis was performed at 35–40 mA at 4°C in an identical buffer but lacking DTT and glycerol. Reactions involving the core TFIIB were performed using the same conditions as above, except that 0.5 μl of serum was included, and products were resolved in a 6% gel in 1× TGE buffer (25 mM Tris, 190 mM glycine, 1 mM EDTA) at room temperature. Addition of yNC2 buffer slightly stimulated TBP–IIB complex formation at the highest volumes added.

### β-galactosidase assays

Five milliliter yeast cultures were grown to a density of 2–4 × $10^7$ cells/ml in SC-Ura media to select for the reporter plasmid. Protein extract was made and β-galactosidase activity was assayed as described previously (Prelich, 1997). All reported β-galactosidase levels are the means from at least three independent transformants with standard errors generally <15%. The Δuas reporters used in these experiments were pLR1Δ1 (*gal1Δuas*), pLGD178 (*cyc1Δuas*) and M740 (*hoΔuas*).

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