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TBP mutants defective in activated transcription *in vivo*

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The TATA box binding protein (TBP) plays a central and essential role in transcription initiation. At TATA box-containing genes transcribed by RNA polymerase II, TBP binds to the promoter and initiates the assembly of a multiprotein preinitiation complex. Several studies have suggested that binding of TBP to the TATA box is an important regulatory step in transcription initiation *in vitro*. To determine whether TBP is a target of regulatory factors *in vivo*, we performed a genetic screen in yeast for TBP mutants defective in activated transcription. One class of TBP mutants identified in this screen comprises inositol auxotrophs that are also defective in using galactose as a carbon source. These phenotypes are due to promoter-specific defects in transcription initiation that are governed by the upstream activating sequence (UAS) and apparently not by the sequence of the TATA element. The finding that these TBP mutants are severely impaired in DNA binding *in vitro* suggests that transcription initiation at certain genes is regulated at the level of TATA box binding by TBP *in vivo*.

Key words: *SPT15*/TATA box/TBP/transcription/yeast

Introduction

Accurate transcription initiation by RNA polymerase II *in vitro* requires the assembly of a preinitiation complex that contains at least five different general transcription factors (TFIIB, TFIID, TFIIE, TFIIF and TFIIH) and RNA polymerase II (Conaway and Conaway, 1991; Roeder, 1991; Sayre *et al.*, 1992; Zawel and Reinberg, 1993). At most genes transcribed by RNA polymerase II, assembly of the preinitiation complex occurs at a TATA box. The TATA box binding protein (TBP), the central component of the general transcription factor TFIID, plays an early and critical role in transcription initiation. TBP binds directly to the TATA box and initiates the assembly of the preinitiation complex (Buratowski *et al.*, 1989).

A preinitiation complex comprising highly purified TBP, TFIIB, TFIIE, TFIIF and TFIIH is sufficient to support basal or unactivated levels of transcription initiation *in vitro*. Additional factors are required for the response to transcriptional activator proteins that bind to sites upstream of the TATA box (Pugh and Tjian, 1992). Several apparently distinct classes of accessory factors have been shown to play important roles in achieving high levels of transcriptional activation. One class of factors is the TBP-associated factors (TAFs) that, together with TBP, comprise TFIID. The best characterized TAFs, those isolated from human and *Drosophila* cells, have been shown to be essential for activation in highly purified systems (Dynlacht *et al.*, 1991; Tanese *et al.*, 1991; Zhou *et al.*, 1992), and certain TAFs can interact directly with activator proteins (Goodrich *et al.*, 1993; Hoey *et al.*, 1993). TAFs have been also identified in the yeast *Saccharomyces cerevisiae* (Poon and Weil, 1993; Verrijzer *et al.*, 1994). A second class of accessory activation factors has been most thoroughly studied in yeast. Recent studies have shown that a large group of proteins forms a complex that associates with RNA polymerase II and is required for transcriptional activation *in vitro* (Kim *et al.*, 1994b; Koleske and Young, 1994). The components of this complex are not tightly associated with TBP. A third class of factors, such as members of the SNF–SWI complex of yeast, appear to assist activators by counteracting the repressing effect of chromatin structure on gene expression (Winston and Carlson, 1992; Côté *et al.*, 1994; Laurent and Carlson, 1994).

The mechanisms by which these and other accessory proteins permit or enhance activation by activator proteins are not known. Since transcription initiation is a multistep process, different activators and accessory proteins are likely to affect different steps in the initiation process. Indeed, at least three different steps in preinitiation complex assembly can be regulated by activators *in vitro*. As the first step in preinitiation complex assembly, the binding of TFIID to the TATA box is a likely target for regulatory factors and several studies have demonstrated direct and specific interactions between a number of activator proteins and TBP (Stringer *et al.*, 1990; Horikoshi *et al.*, 1991; Ingles *et al.*, 1991; Lee *et al.*, 1991; Lieberman and Berk, 1991; Boyer and Berk, 1993). One of these activators, the Epstein–Barr virus Zta protein, significantly decreases the rate of dissociation of TBP from the TATA box *in vitro* (Lieberman and Berk, 1991). In addition, *in vivo* footprinting techniques have detected activation-dependent footprints at the TATA boxes of the *GAL1* and *GAL10* genes of yeast (Selleck and Majors, 1987). The second step in preinitiation complex assembly, the binding of TFIIB to the TBP–TATA complex, is also facilitated by activators *in vitro* (Lin and Green, 1991; Sundseth and Hansen, 1992; Choy and Green, 1993). Two acidic activators, GAL4-AH and GAL4-VP16, recruit TFIIB to the complex, and amino acid changes in TFIIB that disrupt a physical interaction with GAL4-VP16 eliminate transcriptional activation by this activator *in vitro* (Lin and Green,

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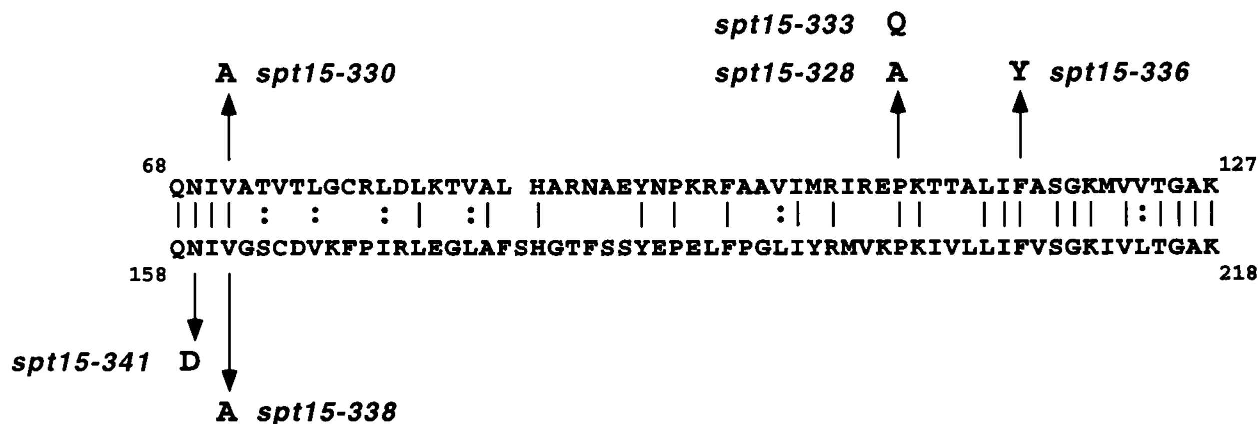

Fig. 1. Amino acid substitutions encoded by *spt15* mutations that confer Ino⁻ and Gal⁻ phenotypes. The two direct repeats in the carboxy-terminal domain of *S.cerevisiae* TBP are shown aligned to one another. Amino acid identities (|) and conservative differences (•) are indicated.

1991; Choy and Green, 1993; Roberts *et al.*, 1993). Finally, when preinitiation complex assembly assays are performed with TFIID instead of TBP, GAL4-AH also mediates the recruitment of a general transcription factor(s) that enters the complex after TFIIB (Choy and Green, 1993).

Since current models for transcriptional activation are derived largely from *in vitro* studies that have employed highly purified general transcription factors and synthetic activator proteins, we sought to identify the steps in transcription initiation that might be regulated *in vivo*. Because TBP binds to co-activator molecules (TAFs) and because TBP plays a pivotal role in preinitiation complex assembly, we reasoned that, through the application of yeast genetics, we might be able to identify TBP mutants that are defective for transcriptional activation at specific promoters. By directly mutagenizing the *S.cerevisiae* gene for TBP, *SPT15*, and then screening the resulting mutations for those that cause specific mutant phenotypes, we have identified a class of TBP mutants that exhibit promoter-specific defects in transcription *in vivo*. While activated transcription at some promoters is greatly affected by these TBP mutants, activated transcription at other promoters is largely unaffected. Biochemical analysis of two of the mutant proteins revealed that they are severely impaired in TATA box binding. Taken together, our findings suggest that activators may enhance the formation or stability of the TBP–TATA complex at some promoters *in vivo*.

Results

Isolation of the mutants

To identify TBP mutants defective in transcription initiation at specific promoters, we performed a genetic screen in *S.cerevisiae*. A plasmid shuffle method (Boeke *et al.*, 1987) was used to introduce random *spt15* mutations into cells deleted for the chromosomal copy of *SPT15*. *SPT15* was mutagenized by the polymerase chain reaction (Zhou *et al.*, 1991) and amplified fragments were subcloned into an autonomously replicating plasmid for yeast to create a library of random mutations. To identify recessive mutations, transformants that contained the mutagenized plasmid but lacked a wild type copy of *SPT15* were selected and then screened for a variety of mutant phenotypes that included nutrient auxotrophies and growth on various carbon sources. Our choice of phenotypes was guided by existing information on transcriptional regulation in yeast, and we screened for mutant phenotypes that might be caused by the altered expression of genes that have been well studied at the transcriptional level.

Of 38 000 transformants, 13 mutants were identified as inositol auxotrophs that were also defective for growth on galactose-containing media (Ino⁻ Gal⁻ phenotype). To confirm that the mutant phenotypes were due to plasmid-encoded *spt15* mutations, plasmid DNA was recovered from the 13 transformants, passaged through *Escherichia coli* and then retransformed into the original strain. For each of the 13 candidates, the mutant phenotype was conferred by the plasmid. Among 10 mutations that have been sequenced, six different amino acid changes in the conserved C-terminal domain of TBP are represented (Figure 1). Interestingly, *spt15* mutations that change some of these same amino acids were identified previously in a genetic screen for dominant negative *spt15* mutations (Reddy and Hahn, 1991).

The Ino⁻ Gal⁻ TBP mutants are defective in DNA binding

Localization of the amino acids altered by the *spt15* mutations on the TBP–TATA co-crystal structures revealed that all of the amino acids, with the exception of Pro109, directly contact DNA (Kim *et al.*, 1993a,b). Pro109 lies in a turn that connects two of the β-strands that form the DNA binding surface of TBP (Kim *et al.*, 1993a,b). To determine whether these mutations affect the ability of TBP to bind DNA, we performed electrophoretic mobility shift assays on two of the TBP mutants, *spt15-328* protein (TBP-P109A) and *spt15-341* protein (TBP-N159D). For these experiments, proteins were prepared by purification from *E.coli* overexpressing strains (Figure 2) or by *in vitro* translation in rabbit reticulocyte extracts (data not shown). The mobility shift assays demonstrate that the TBP mutant proteins are severely defective in binding to two different TATA elements *in vitro*, the adenovirus major late TATA box (Figure 2 and data not shown) and the yeast *HIS4* TATA box (data not shown). In control experiments, addition of recombinant TBP-N159D to binding reactions containing wild type TBP did not inhibit binding by the wild type protein, arguing against the presence of an inhibitor in the TBP-N159D

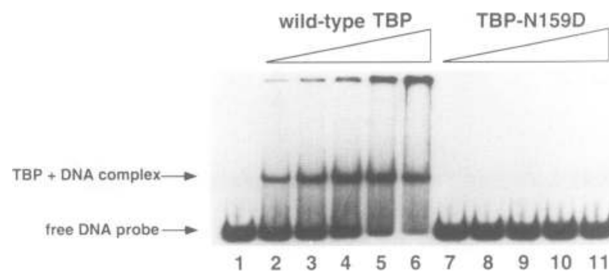

Fig. 2. Electrophoretic mobility shift analysis of wild type TBP and TBP-N159D binding to the adenovirus major late promoter (AdMLP) TATA box. A ^{32}P-labelled DNA probe containing the AdMLP TATA box (Buratowski *et al.*, 1989) was incubated with bacterially produced wild type TBP (lanes 2–6) and TBP-N159D (lanes 7–11): lane 1, no TBP; lanes 2 and 7, 0.5 ng TBP; lanes 3 and 8, 1 ng TBP; lanes 4 and 9, 2 ng TBP; lanes 5 and 10, 4 ng TBP; lanes 6 and 11, 8 ng TBP.

preparation (data not shown). In addition, recombinant TBP-N159D was able to restore transcriptional activity (14% of wild type TBP levels) to a yeast *in vitro* transcription system that was depleted of endogenous TBP (Flanagan *et al.*, 1992), demonstrating that the purification process did not completely inactivate the mutant protein (data not shown).

The Ino$^-$ Gal$^-$ TBP mutants exhibit promoter-specific defects in transcriptional activation

To determine whether the Ino$^-$ and Gal$^-$ phenotypes caused by TBP-P109A and TBP-N159D are due to specific defects in transcription, we analyzed transcription of several inducible genes in yeast. As measured by Northern analysis, activated transcription of the *GAL1*, *GAL10* and *INO1* genes is dramatically reduced in strains that contain TBP-P109A or TBP-N159D (Figure 3). The well studied acidic transcription factor GAL4 activates *GAL1* and *GAL10* transcription, and the helix–loop–helix proteins INO2 and INO4 are required for derepression of the *INO1* gene (Hoshizaki *et al.*, 1990; Nikoloff *et al.*, 1992). The dramatic reduction in *GAL1* and *GAL10* transcription in strains that contain TBP-P109A and TBP-N159D cannot be explained by a reduction in GAL4 levels. *GAL4* transcription is decreased <2-fold by these TBP mutants and overexpression of GAL4 protein from a high copy number vector, as confirmed by Western analysis, did not significantly alter the Gal$^-$ phenotype of the mutant strains (data not shown). In addition, Western analysis demonstrated that TBP levels are unaffected in these strains (data not shown).

In contrast to regulation by GAL4 and INO2/INO4, transcriptional activation of two genes, *HIS4* and *HIS3*, by the acidic factor GCN4 is not significantly altered by TBP-P109A and TBP-N159D (Figure 3). To induce GCN4 synthesis, strains containing wild type TBP, TBP-P109A or TBP-N159D were exposed to 3-aminotriazole, a competitive inhibitor of the *HIS3* gene product. The degree of induction of *HIS4* and *HIS3* (+13 initiation) transcription achieved by 3-aminotriazole was similar in the three strains. [A 3- to 4-fold reduction in *HIS4* transcription under non-activating conditions presumably stems from a defective response of the TBP mutants to the *BAS1* and *BAS2* gene products (Arndt *et al.*, 1987).] Moreover, the TBP mutant strains exhibit no significant decrease in

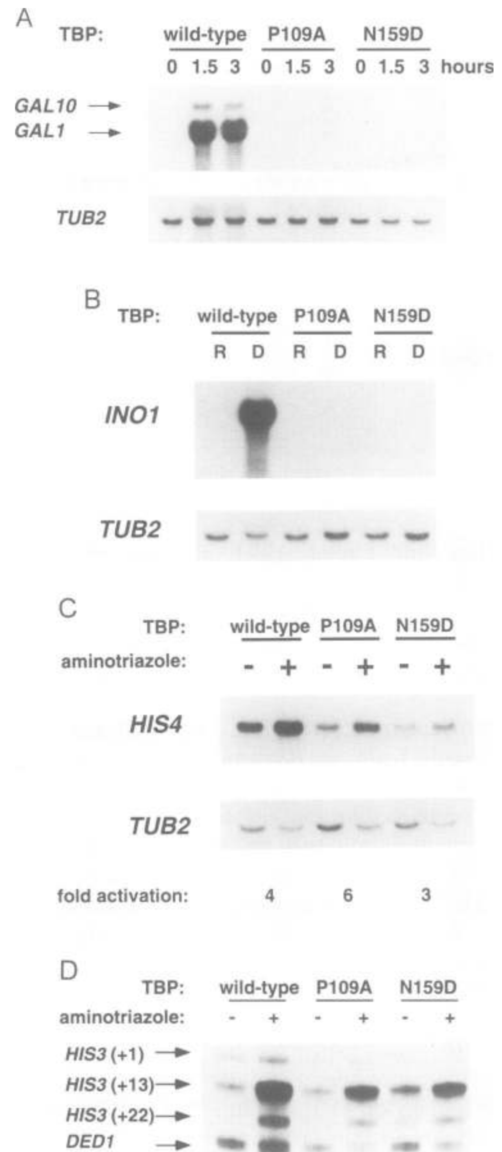

Fig. 3. TBP-P109A and TBP-N159D exhibit gene-specific defects in transcription *in vivo*. (**A**) Northern analysis of *GAL1* and *GAL10* transcription in non-inducing (raffinose) and inducing (galactose) conditions. RNA was prepared after the indicated times in galactose. The 0 h sample was taken prior to addition of galactose. (**B**) Northern analysis of *INO1* transcription in repressing (R, 200 μM inositol) and derepressing (D, 10 μM inositol) conditions. (**C**) Northern analysis of *HIS4* transcription in non-inducing (no 3-aminotriazole) and inducing (10 mM 3-aminotriazole) conditions. The values for fold activation by 3-aminotriazole are the average from three experiments. All filters were probed for *TUB2* message for normalization. (**D**) S1 hybridization analysis of *HIS3* transcription. *DED1* transcript levels serve as a normalization control. The induction ratio for the +13 transcript in each strain was as follows: wild type, 4.4; P109A, 4.1; N159D, 2.4.

transcription initiation at *HIS3* arising from either the GCN4-regulated T_R TATA element (+13 initiation), which has a consensus TATA sequence, or the constitutive T_C TATA element (+1 and +13 initiation), which lacks an identifiable TATA box (Mahadevan and Struhl, 1990).

Basal transcription is unaffected in one mutant

To determine whether TBP-P109A and TBP-N159D affect basal (i.e. unactivated) transcription at a promoter where

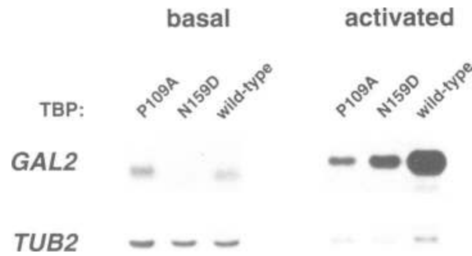

Fig. 4. Basal and activated transcription of *GAL2* in TBP-P109A and TBP-N159D mutant strains. Northern analysis of *GAL2* transcription in non-inducing conditions (raffinose) and after 1.5 h in inducing (galactose) conditions. To analyze basal transcription (left panel), 10 μg total RNA were loaded in each lane and the filter was exposed to film for ~16.5 h. To analyze activated transcription (right panel), 2 μg total RNA were loaded in each lane and the filter was exposed to film for ~4 h. *TUB2* transcript levels serve as a normalization control.

Fig. 5. *GAL1* and *GAL10* transcription is reduced in a *gal80Δ* strain containing TBP-N159D. Northern analysis of *GAL1* and *GAL10* transcription in strains containing a deletion of the *GAL80* gene and either wild type TBP or TBP-N159D as indicated. Strains were grown in non-inducing conditions (glycerol lactate media) and harvested for RNA analysis. The two lanes of the Northern blot were from the same exposure of the same filter and were spliced together for presentation. Levels of rRNA, as determined by staining with ethidium bromide, serve as a normalization control.

the activated transcription level is significantly reduced, we assayed transcription of the GAL4-regulated gene *GAL2* in both non-inducing and inducing conditions. A similar analysis of *GAL1* and *GAL10* transcription was not possible, because these genes give rise to undetectable transcript levels in the absence of galactose (Figure 3). In contrast, *GAL2*, which encodes galactose permease (Tschopp *et al.*, 1986), is transcribed at a low but measurable level in non-inducing media (raffinose). Upon addition of galactose to the media, *GAL2* transcription is stimulated ~500-fold (Figure 4). Interestingly, one of the two TBP mutants, TBP-P109A, does not decrease the basal level of *GAL2* transcription but does reduce the activated level ~8-fold. In contrast, TBP-N159D causes a reduction of both basal and activated transcription of *GAL2*. Given the similar mutant phenotypes and transcriptional properties at some genes conferred by TBP-P109A and TBP-N159D (Figure 3), the basis for their different effects on *GAL2* basal expression is not clear. However, the observation that TBP-P109A is defective in activated but not basal transcription for at least one promoter suggests that there may be a fundamental difference in the requirements for basal and activated transcription with respect to the strength of the TBP–TATA interaction or the conformation of the TBP–TATA complex.

Decreased *GAL2* transcription indicated that the dramatic reduction in *GAL1* and *GAL10* transcription in the TBP mutant strains may be due in part to decreased expression of GAL4-regulated genes 'upstream' of *GAL1* and *GAL10* in the induction pathway. Consistent with this idea, we have observed an ~3-fold reduction in activated transcription of *GAL3* in strains containing TBP-P109A and TBP-N159D (data not shown). The *GAL3* gene product is required for transduction of the galactose signal and the consequent inactivation of the GAL80 transcriptional repressor (Bajwa *et al.*, 1988). A similar cumulative effect of reduced GAL4-dependent expression of several genes may also contribute to the defects in *GAL1* and *GAL10* transcription reported for histone H4 (Durrin *et al.*, 1991) and RNA polymerase II mutants (Scafe *et al.*, 1990). That the defect in *GAL1* and *GAL10* transcription in at least one of the TBP mutants, TBP-N159D, is only partly due to diminished *GAL2* and *GAL3* expression was determined by deleting *GAL80*, the target of galactose induction. In a *gal80Δ spt15-341* double mutant strain, *GAL1* and *GAL10* mRNA levels are still reduced 3- to 4-fold relative to a *gal80Δ SPT15*$^+$ strain (Figure 5). Although the more moderate reduction of *GAL1* and *GAL10* transcription in the double mutant may stem from an elimination of the requirement for inducer, it also seems quite plausible that the physiologically relevant activator, the GAL4–GAL80 complex (Chasman and Kornberg, 1990; Leuther and Johnston, 1992; Parthun and Jaehning, 1992) may be more sensitive to the TBP mutant than GAL4 alone.

The promoter-specific activation defects of the TBP mutants are determined by the UAS

As a first step to determining the mechanism by which TBP-P109A and TBP-N159D impair activation of certain genes, we measured expression of *lacZ* reporter plasmids that contain different upstream activating sequences (UAS) and the same heterologous TATA element (*CYC1* TATA). Surprisingly, the pattern of expression observed for these promoters is dependent on the UAS and apparently not on the TATA sequence (Table I). The high levels of *lacZ* expression driven by the *HIS4* UAS in the mutant strains demonstrate that TBP-P109A and TBP-N159D are able to utilize the *CYC1* TATA element with high efficiency. (Partial induction by GCN4 in the mutants is probably due to an inducing effect of the minimal media used in this particular experiment.) For plasmids containing the *GAL10* UAS or the *INO1* UAS, *lacZ* expression is greatly reduced in the TBP mutants. Therefore, the promoter-specific transcriptional defects caused by TBP-P109A and TBP-N159D appear to be dictated by the upstream activating sequence and not by the TATA element.

To test directly the role of the transcriptional activators GAL4 and GCN4 in mediating this UAS-dependent specificity, we assayed β-galactosidase production from *lacZ* reporter plasmids that carried synthetic activator binding sites [a single 17-mer binding site for GAL4 (Giniger *et al.*, 1985) or two tandem GCN4 binding sites (Hinnebusch *et al.*, 1985) (Table II)]. In contrast to the patterns of expression observed by Northern analysis of endogenous genes (Figure 3) or by *lacZ* reporter analysis of complete UAS elements (Table I), the GAL4- and GCN4-responsive templates behaved similarly in this assay. That is, activation by both GCN4 and GAL4 is impaired in the TBP mutants when these activators are

Table I. Expression of *CYC1* promoter-*lacZ* reporter plasmids that contain complete UAS elements

UAS	TBP	Uninduced expression		Induced expression	
		β-galactosidase activity (U)	% wild type	β-galactosidase activity (U)	% wild type
GAL10	wild-type	0.1	100	640	100
GAL10	P109A	0.2	200	17	3
GAL10	N159D	0.5	500	4	0.6
INO1	wild-type	0.1	100	450	100
INO1	P109A	0.6	600	13	3
INO1	N159D	0.4	400	3	0.7
HIS4	wild-type	1800	100	5100	100
HIS4	P109A	2700	150	3800	75
HIS4	N159D	570	32	700	14

Table II. Expression of *CYC1* promoter-*lacZ* reporter plasmids that contain synthetic activator binding sites

UAS	TBP	Uninduced expression		Induced expression	
		β-galactosidase activity (U)	% wild type	β-galactosidase activity (U)	% wild type
GAL4 site	wild-type	27	100	900	100
GAL4 site	P109A	4	15	10	1
GAL4 site	N159D	10	37	19	2
GCN4 site	wild-type	810	100	5500	100
GCN4 site	P109A	440	54	450	8
GCN4 site	N159D	110	14	56	1

directed to isolated binding sites outside the context of naturally occurring UAS elements. This finding suggests that additional positively acting factors at the *HIS4* and *HIS3* UAS elements compensate for a defective TBP–TATA interaction and permit communication between GCN4 and the preinitiation complex.

Discussion

Transcription initiation by RNA polymerase II is a multi-step process that requires the coordinated and controlled activities of many different proteins. Each step in this process is a potential target for the action of transcriptional regulatory factors, and different regulatory factors are likely to act at different steps. Here we provide evidence that the first step in preinitiation complex assembly, the formation of a TBP–TATA box complex, can be a rate limiting and regulated step at certain promoters *in vivo*.

Using a plasmid shuffle screen in yeast we have identified a novel class of TBP mutants that exhibit specific mutant phenotypes, inositol auxotrophy and defects in galactose metabolism. These phenotypes are caused by promoter-specific effects on transcription. Activated transcription of the *INO1* gene and four different GAL4-regulated genes, *GAL1*, *GAL10*, *GAL2* and *GAL3*, is greatly reduced in strains that contain these TBP mutants. In contrast, transcription of five other genes, *HIS4*, *HIS3*, *TUB2*, *GAL4* and *ACT1* (data not shown) is largely unaffected. A similar pattern of expression is observed when the TATA boxes of the *GAL10*, *INO1* and *HIS4* genes are replaced by a heterologous TATA element from the *CYC1* gene, suggesting that the promoter specificity is governed by the UAS.

DNA sequence analysis of the *spt15* mutations revealed that, with two exceptions, all of the mutations change amino acids known to contact the TATA box (Kim *et al.*, 1993a,b). The two exceptions encode substitutions for a proline residue (Pro109) that lies in a turn between two DNA binding β-strands. Two of these mutants, TBP-P109A and TBP-N159D, were chosen for further study. The DNA binding defects exhibited by TBP-P109A and TBP-N159D are severe; we estimate that the affinity of these TBP mutants for the adenovirus major late TATA box is reduced >100-fold *in vitro*. Given the severity of this DNA binding defect and the relatively specific phenotypes conferred by the *spt15* mutations *in vivo* it seems likely that additional factors may compensate for an impaired TBP–TATA interaction *in vivo*. The extent to which these factors are required for normal transcriptional regulation may vary at different promoters. Candidates for these factors include the general transcription factors TFIIA and TFIIB, which increase the affinity of TBP for the TATA box *in vitro* (Imbalzano *et al.*, 1994).

Taken together, our results suggest that, *in vivo*, transcriptional activation at some promoters is critically dependent upon the intrinsic DNA binding activity of TBP, while activation at other promoters is relatively insensitive to the affinity of TBP for the TATA box. The mechanism underlying this promoter specificity probably relies on properties of the upstream activating sequences and associated factors. However, this specificity appears not to be governed entirely by the activator proteins themselves. Thus, when transcription of endogenous genes or reporter constructs containing intact UAS elements is assayed, activation by GAL4 is impaired by the TBP DNA binding mutants, whereas activation by GCN4 is relatively unaffected. In contrast, when GCN4 is bound to a synthetic binding site outside the context of the *HIS4* or *HIS3* UAS, it is unable to activate transcription in the TBP mutant strains. This finding strongly suggests that certain *cis*- or *trans*-acting factors at the *HIS4* and *HIS3* UAS elements help overcome the DNA binding defects of the TBP mutants.

Several mechanisms can explain our findings, including

Table III. Yeast strains

Strain	Genotype
FY114	*MATa lys2–173R2 ura3–52*
FY630	*MATα his4–917δ lys2–173R2 leu2Δ1 ura3–52 trp1Δ63*
FY654	*MATα spt15Δ102::LEU2 his4–917δ lys2–173R2 leu2Δ1 trp1Δ63 ura3–52 [pDE28–6]*
KY214	*MATα spt15–328 his4–917δ lys2–173R2 leu2Δ1 ura3–52 trp1Δ63*
KY231	*MATα spt15–341 his4–917δ lys2–173R2 leu2Δ1 ura3–52 trp1Δ63*
KY233	*MATa spt15–328 lys2–173R2 ura3–52*
KY234	*MATa spt15–341 lys2–173R2 ura3–52*
KY244	*MATa spt15–341 gal80Δ his4–917δ lys2–173R2 leu2Δ1 ura3–52 trp1Δ63*
KY247	*MATα gal80Δ his4–917δ lys2–173R2 leu2Δ1 ura3–52 trp1Δ63*

some where both GAL4 and GCN4 regulate the TBP–TATA interaction. For example, GAL4 may facilitate formation of a stable TBP–TATA complex by an indirect route, such as the removal of repressors (e.g. chromatin components) that compete with TBP for the TATA region. Indeed, mutations that alter the amino-terminal domain of yeast histone H4 also cause a reduction in *GAL1* transcription (Durrin *et al.*, 1991). A TBP mutant defective in DNA binding might be particularly sensitive to such repressors. At *HIS4* and *HIS3*, factors other than GCN4 may maintain the accessibility of the TATA box. Strong candidates for these factors include RAP1, which has been shown previously to alter chromatin structure at the GCN4, BAS1 and BAS2 binding sites of the *HIS4* promoter (Devlin *et al.*, 1991), and the poly(dA-dT) element of the *HIS3* promoter, which may be refractory to nucleosome formation (Struhl, 1985). In an alternative model, GCN4, with the assistance of other factors, may directly contact TBP when it is bound to the *HIS4* and *HIS3* promoters, thereby stabilizing even a weakened TBP–TATA interaction. Support for such models has come from the identification of TATA box point mutations that support GCN4- but not GAL4-mediated activation *in vivo* (Harbury and Struhl, 1989) and from a recent kinetic analysis of TBP recruitment by GCN4 (Klein and Struhl, 1994). Moreover, it should be noted that the phenotypes and transcriptional effects caused by the TBP mutants described here are very similar to those caused by mutations that shorten the length of the C-terminal domain (CTD) of the large subunit of RNA polymerase II (Scafe *et al.*, 1990). Thus, the CTD or factors that interact with the CTD may bridge the interaction between the activator and TBP (Koleske *et al.*, 1992; Usheva *et al.*, 1992) or may stabilize the TBP–TATA complex (Peterson *et al.*, 1991).

Finally, it should be emphasized that the findings and conclusions of this study are consistent with previously described models for transcriptional activation. A number of studies have described interactions between TBP (Stringer *et al.*, 1990; Horikoshi *et al.*, 1991; Lee *et al.*, 1991; Lieberman and Berk, 1991; Boyer and Berk, 1993) or TFIID (Horikoshi *et al.*, 1988a,b) and activator proteins, and at least one study demonstrated a direct and quantitative effect of an activator on TATA-box binding by TBP (Lieberman and Berk, 1991). Significantly, in a recent report, Kim *et al.* (1994a) described two TBP mutants that are defective in transcriptional activation *in vitro* and DNA binding *in vitro* (Yamamoto *et al.*, 1992). Our data may also be consistent with models in which steps subsequent to TBP binding are rate limiting. Several studies have provided strong evidence that activators stimulate transcription by recruiting TFIIB to the preinitiation complex (Lin and Green, 1991; Lin *et al.*, 1991; Sundseth and Hansen, 1992; Choy and Green, 1993; Roberts *et al.*, 1993) and by affecting a TAF-dependent step subsequent to TFIIB binding (Choy and Green, 1993). If TFIIB (or some other general transcription factor) stabilizes or facilitates the formation of the TBP–TATA complex *in vivo*, a promoter that is regulated via TFIIB recruitment may also be sensitive to a severely compromised TBP–TATA interaction. Further genetic and biochemical characterization of mutations that affect TBP or other components of the preinitiation complex should provide additional insights into the mechanisms of transcriptional activation employed by activators *in vivo*.

Materials and methods

Yeast strains and media

The *S.cerevisiae* strains used in this study (Table III) are isogenic to FY2, a *GAL2*$^+$ *ura3-52* derivative of S288C (Winston *et al.*, 1995) and were constructed by standard procedures of transformation and tetrad analysis (Rose *et al.*, 1990).

Rich (YP), minimal (SD), synthetic complete (SC) and inositol starvation media were prepared as described previously (Sherman *et al.*, 1981; Rose *et al.*, 1990). For the selection of *ura3* strains in the plasmid shuffle, SC media containing 5-fluoro-orotic acid (5-FOA) (Rose *et al.*, 1990) was used.

Isolation of TBP mutants

The *SPT15* gene was amplified from pFW218 (Eisenmann *et al.*, 1989) by the polymerase chain reaction under mutagenic conditions (Zhou *et al.*, 1991). Oligonucleotides that annealed to sequences −110 to −87 (5′-CGACTACTAGTTAGACTGCTCTGC-3′) and to sequences +1134 to +1157 (5′-GCCAGTGACTTGGGATCCAAGTTC-3′) relative to the translational initiation site were used as primers. To generate a library, amplified fragments were digested with *Spe*I and *Bam*HI and subcloned into pKA75 to replace the *SPT15*$^+$ gene. Plasmid pKA75 contains the 2.4 kb *Eco*RI–*Bam*HI fragment of *SPT15*$^+$ and is a derivative of pRS314 (Sikorski and Hieter, 1989) in which the *Spe*I site in the polylinker has been destroyed. Strain FY654, which contains plasmid pDE28-6 (*SPT15*$^+$, *URA3*) (Eisenmann *et al.*, 1989), was transformed with the *spt15* library to Trp$^+$, transformants were replica plated to media containing 5-FOA, and 5-FOA resistant colonies were replica plated to inositol starvation media or YP media containing 2% galactose and 1 μg/ml antimycin A. Plasmid DNA was recovered from 13 Ino$^-$ Gal$^-$ candidates by a standard procedure (Hoffman and Winston, 1987) and used to transform *E.coli*. Retransformation of these plasmids into FY654 demonstrated that the Ino$^-$ Gal$^-$ phenotypes of the 13 candidates were due to plasmid-encoded *spt15* mutations. For 10 of the candidate plasmids, the complete *SPT15* open reading frame was sequenced on both DNA strands. Nine of the mutant *SPT15* genes encoded only a single amino acid change. One mutant gene, *spt15-338*, in addition to encoding the change V161A, also encodes a second change, E27G, in the non-essential and non-conserved amino-terminal domain. Three of the mutations, *spt15-328*, *spt15-336* and *spt15-341*, were integrated into yeast strains to replace the chromosomal *SPT15* gene by standard methods for two-step gene replacement (Scherer and Davis, 1979). In

addition to conferring the Ino⁻ and Gal⁻ phenotypes, the *spt15-328* mutation causes temperature sensitive growth at 37°C, and this phenotype was significantly enhanced upon integration of the mutation.

Electrophoretic mobility shift assays

Wild type TBP and TBP-N159D were expressed in *E.coli* under the control of T7 RNA polymerase (Rosenberg *et al.*, 1987) and were purified by standard methods (D.Chasman and R.Kornberg, personal communication). Wild type TBP and TBP-N159D were ~95% and 45% pure, respectively. Reaction and electrophoresis conditions for mobility shift assays with TBP and a probe containing the adenovirus major late TATA box (Buratowski *et al.*, 1989) were as previously described (Eisenmann *et al.*, 1992).

Growth of strains and RNA analysis

RNA isolations before and after inductions, Northern analyses and S1 hybridizations were performed as previously described (Chen and Struhl, 1988; Swanson *et al.*, 1991). Total RNA used in each S1 hybridization reaction or loaded in each lane for Northern analyses was 10 μg unless otherwise indicated in the figure legend.

Northern analysis of GAL1, GAL10 and GAL2. Strains FY630, KY214 and KY231 were grown at 30°C in SC media containing 2% raffinose to 1–2×10^7 cells/ml. Galactose was then added to a final concentration of 5% and cells were incubated for the indicated times.

Northern analysis of INO1. FY630, KY214 and KY231 were grown at 30°C in inositol starvation medium supplemented with 200 μM inositol to 1–2×10^7 cells/ml, washed, resuspended and incubated for 10 h in the same media containing 10 μM inositol.

Northern analysis of HIS4 and S1 analysis of HIS3. Strains FY114, KY233 and KY234 were grown at 30°C to 1–2×10^7 cells/ml in SD media and then incubated in the presence of 10 mM 3-aminotriazole for 4 h.

Northern analysis of GAL1 and GAL10 transcription in gal80Δ strains. Strains KY244 and KY247 were grown at 30°C in SC media containing 3% glycerol and 2% potassium lactate (pH 5.7) to 1–2×10^7 cells/ml. Cells were then harvested for RNA isolation. *GAL2*, *HIS4* and *HIS3* transcript levels were quantitated on a Molecular Dynamics PhosphorImager and normalized to *TUB2* or *DED1* transcript levels. Relative *TUB2* and *DED1* transcript levels correlated well with rRNA levels determined from ethidium-bromide stained gels. For Figure 5, *GAL1* and *GAL10* transcript levels were quantitated and normalized to rRNA levels, because the *GAL1* probe could not be adequately stripped from the filter to allow normalization with a *TUB2* probe.

β-galactosidase assays

Reporter plasmids used in β-galactosidase assays were pLGSD5 (365 bp *GAL10* UAS fragment; Guarente *et al.*, 1982), pJH359 (205 bp *INO1* UAS fragment; Scafe *et al.*, 1990), pHYC3 (169 bp *HIS4* UAS fragment; Hinnebusch *et al.*, 1985), pSV14 (one GAL4 17-mer binding site; Giniger *et al.*, 1985) and pHYC2(14×2) (two GCN4 binding sites; Hinnebusch *et al.*, 1985). These 2μ plasmids contain the indicated UAS elements inserted at the same position 5′ to the *CYC1* TATA region and transcription initiation site. A more detailed description of these plasmids has been presented elsewhere (Scafe *et al.*, 1990). Plasmids were transformed by standard methods into strains FY630, KY214 and KY231 for plasmids pLGSD5, pJH359 and pSV14 or into strains FY114, KY233 and KY234 for plasmids pHYC3 and pHYC2(14×2). Growth and induction of transformants were performed as described for Northern analyses except that uracil was omitted from the media to maintain selection for the plasmid. For pLGSD5 and pSV14 transformants, induction in galactose was allowed to proceed for 1.5 h. Extract preparations, β-galactosidase assays and unit calculations were performed as previously described (Miller, 1972; Rose and Botstein, 1983). For each plasmid, two amounts of extract prepared from three independent transformants were assayed; each experiment was repeated at least twice. Mean values are given. Standard errors were typically 5–25% of the reported value.

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