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The TATA-Binding Protein Is Required for Transcription by All Three Nuclear RNA Polymerases in Yeast Cells

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Summary

Using temperature- and proteolytically sensitive derivatives to inactivate the function of the yeast TATA-binding protein (TBP) in vivo, we investigated the requirement of TBP for transcription by the three nuclear RNA polymerases in yeast cells. TBP is required for RNA polymerase II (pol II) transcription from promoters containing conventional TATA elements as well as functionally distinct promoters that lack TATA-like sequences. TBP is also required for transcription of the U6 snRNA and two different tRNA genes mediated by RNA pol III as well as transcription of ribosomal RNA mediated by RNA pol I. For all promoters tested, transcription decreases rapidly and specifically upon inactivation of TBP, strongly suggesting that TBP is directly involved in the transcription process. These observations suggest that TBP is required for transcription of all nuclearly encoded genes in yeast, although distinct molecular mechanisms are probably involved for the three RNA polymerase transcription machineries.

Introduction

Eukaryotic organisms contain three nuclear RNA polymerases that are individually responsible for the synthesis of ribosomal RNA (pol I), messenger RNAs (pol II), transfer RNAs (pol III), and small nuclear RNAs (pol II and III). Although these RNA polymerases display considerable sequence similarity in their largest subunits (Allison et al., 1985; Memet et al., 1988) and actually have three subunits in common (Sentenac, 1985; Woychick et al., 1990), it is clear that different molecular mechanisms are involved in the process of transcriptional initiation. The RNA polymerases function at promoters that differ markedly in critical DNA recognition sequences and overall structural organization (Figure 1), and they interact with distinct sets of basic transcription factors that are required for accurate initiation of RNA synthesis (for reviews see Sollner-Webb and Tower, 1986; Geiduschek and Tocchini-Valentini, 1988; Sawadogo and Sentenac, 1990).

Most RNA pol II promoters contain a TATA element upstream of the mRNA start site that binds the basic transcription initiation factor TFIID (Davison et al., 1983; Parker and Topol, 1984; Nakajima et al., 1988). TFIID binding is the essential first step in the stepwise assembly of the transcription complex (Fire et al., 1984; Reinberg and Roeder, 1987; Van Dyke et al., 1988; Buratowski et al., 1989), and it appears to potentiate the promoter for transcription in the context of chromatin (Workman and Roeder, 1987). The TATA element interaction is mediated by the TATA-binding protein (TBP), a protein that is highly conserved across eukaryotic species (>80% identity in the 180 aa C-terminal core domain) (reviewed by Greenblatt [1991]).

TBP can function with the other basic RNA pol II initiation factors in supporting TATA-dependent transcription, but other factors (called adaptors, mediators, or coactivators) appear to be required for responding to transcriptional activator proteins (Kambadur et al., 1990; Kelleher et al., 1990; Meisterernst et al., 1990; Pugh and Tjian, 1990). Yeast and human TBPs, though extremely similar by many functional criteria (Buratowski et al., 1988; Cavallini et al., 1988; Wobbe and Struhl, 1990; Kelleher et al., 1992; Strubin and Struhl, 1992), display species specificity in vivo (Cormack et al., 1991; Gill and Tjian, 1991; Poon et al., 1991) and in vitro (Peterson et al., 1990; Pugh and Tjian, 1990). In cell-free extracts, yeast TBP fractionates as a simple monomeric protein (Buratowski et al., 1988), whereas mammalian and Drosophila TBPs are tightly associated with a number of additional factors in multiprotein TFIID complexes (Nakajima et al., 1988; Dynlacht et al., 1991; Timmers and Sharp, 1991). Two distinct TFIID complexes have been observed in human cells, only one of which can support transcriptional stimulation by proteins containing acidic or glutamine-rich activation domains (Timmers and Sharp, 1991).

Not all RNA pol II promoters contain recognizable TATA sequences, thus provoking questions regarding the role of TFIID and the mechanism by which the transcription complex is brought to DNA. For example, the yeast *HIS3* promoter contains two proximal elements, T_C and T_R, that are responsible for transcriptional initiation from the +1 and +13 sites, respectively (Struhl, 1986; Figure 1). T_R is a consensus TATA element (Chen and Struhl, 1988) that supports TFIID-dependent transcription in vitro (Wobbe and Struhl, 1990) and responds to transcriptional activator proteins such as GCN4 and GAL4 (Ponticelli and Struhl, 1990; Struhl, 1986). In contrast, T_C bears little resemblance to a TATA element (Mahadevan and Struhl, 1990), does not respond to acidic activator proteins (Struhl, 1986), and does not bind TFIID nor support TFIID-dependent transcription in vitro (Ponticelli and Struhl, 1990). Transcription from another TATA-less promoter, *his3*-GG1, depends on GCN4 protein being bound at the TATA position (Chen and Struhl, 1989) and on an upstream element Q (Brandl and Struhl, 1990) that coincides with a nucleosome positioning sequence (Fedor et al., 1988). The artificial *his3*-GG1 promoter strongly resembles the native yeast *TRP3* promoter that also has a GCN4 site in the place of a TATA element and no detectable TATA sequence.

Potential mechanisms that could account for transcription from promoters lacking conventional TATA elements include: TBP binding to nonconsensus sites, distinct TBP-like proteins that interact with alternative proximal ele-

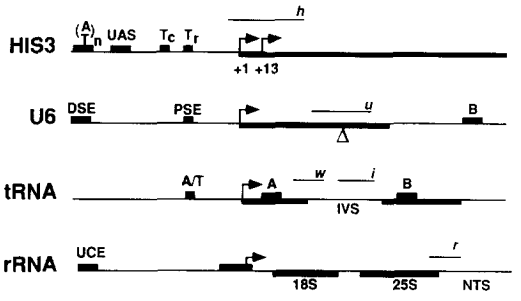

Figure 1. Promoter Structure

Critical promoter elements (closed boxes marked by capital letters), transcriptional initiation sites (arrows), mature RNA-coding sequences (thick black line), processed RNA regions (IVS and ETS), deleted sequences (open triangle for U6), and position of hybridization probe (thin line marked by small italic letter) are shown for each gene. The PSE in the U6 promoter contains a consensus TATA element, and many but not all yeast tRNA genes contain an AT-rich proximal sequence. For further details see: Sollner-Webb and Tower, 1986; Struhl, 1986; Geiduschek and Tocchini-Valentini, 1988; Sawadogo and Sentenac, 1990; Dahlberg and Lund, 1991.

ments, or accessory proteins that act in concert with TBP, perhaps by stabilizing template interactions in the absence of sequence-specific DNA binding. In human cell-free extracts, the partially purified TFIID complex appears to be required for transcription from TATA-less promoters (Carcamo et al., 1990; Smale et al., 1990; Pugh and Tjian, 1991). Interestingly, TBP cannot substitute for TFIID for transcription from these promoters (Pugh and Tjian, 1991), and its precise role is unknown.

Although TFIID has been assumed to be specifically involved in transcription by RNA pol II, recent experiments suggest that it is required in vitro for transcription of the U6 snRNA gene mediated by RNA pol III (Lobo et al., 1991; Margottin et al., 1991; Simmen et al., 1991). In human cell-free extracts, the TATA element upstream of the U6 gene is required for transcription by RNA pol III; deletion of this TATA element generates a TATA-less RNA pol II promoter. Moreover, the TFIIIC/D fraction can be replaced by TFIID to support U6 transcription in vitro, suggesting that TBP may function at RNA pol III promoters (Lobo et al., 1991; Simmen et al., 1991). In yeast extracts lacking TFIIIC, TBP is necessary for efficient transcription of the U6 gene, again pointing to a role in RNA pol III transcription (Margottin et al., 1991). However, the yeast U6 template is deleted for the B box 3′ to the U6 gene that is absolutely required in vivo (Brow and Guthrie, 1990), thus making it unclear whether TBP has any effect in vivo on a wild-type template.

In this paper, we assess the role of TBP in vivo by generating three conditional TBP alleles and analyzing transcription from a variety of promoters under conditions where TBP has been functionally inactivated or destroyed. In vivo analysis has the advantage that unlike in reconstituted cell-free systems, we are assessing the role of the factor in its normal environment. We show that TBP is required for transcription mediated by all three nuclear RNA polymerases.

Results

Temperature-Sensitive Alleles of TBP

We generated temperature-sensitive (ts) alleles of TBP using a plasmid shuffle technique. In brief, a *TRP1* centromeric vector carrying the TBP gene was mutagenized with hydroxylamine, and the resulting DNA molecules were introduced into a yeast strain deleted for the chromosomal copy of TBP, but carrying a *URA3* centromeric vector encoding a wild-type copy of the gene. Transformants were plated onto rich media at 30°C and then screened for the ability to grow at 37°C on plates containing 5-fluoroorotic acid, a compound toxic to *URA3*$^+$ cells. Under these conditions, only cells that have lost the *URA3* plasmid can survive, thus making cell growth dependent on the functional status of the TBP gene carried on the *TRP1* plasmid.

Two ts alleles of TBP were isolated in this screen; strains containing these alleles as the only copy of TBP grow normally at 30°C but fail to grow at 37°C (Figure 2A). Upon shifting a growing culture of either TBP mutant strain to the restrictive temperature, growth ceases within 3–4 hr (Figure 2B). Though growth inhibited, the cells remain viable and can resume growth if shifted back to the permissive temperature. The cells do not display a discrete cell-cycle phenotype, although many appear to arrest as multibudded cells reminiscent of the terminal phenotype observed in ts *cdc4* mutant strains.

The entire TBP-coding regions of both alleles were sequenced, and in each case the temperature sensitivity was due to a single point mutation (Figure 2C). The ts1 mutation, a threonine to isoleucine change at position 111, maps in the first of two direct repeats that have been implicated by mutational studies to be involved in DNA binding (Reddy and Hahn, 1991; Strubin and Struhl, 1992). The ts2 mutation, a change of serine 136 to asparagine, maps in the so-called basic repeat that has been implicated to be important in protein–protein interactions (Buratowski and Zhou, 1992) as well as for the overall structure of the protein (Horikoshi et al., 1990). Both mutant proteins are seriously compromised in their ability to bind a TATA element in vitro, although the ts2 derivative binds very weakly at 23°C (Figure 2D). We do not understand why at 23°C these derivatives bind poorly to TATA elements in vitro but function normally in vivo; however, similar effects have been observed on other mutant TBP derivatives (unpublished data).

Effects of the TBP ts Alleles on Transcription by RNA Pol II

The parental and the two ts TBP strains were grown at 30°C in rich media to the early exponential phase, whereupon the cultures were shifted to 37°C. Aliquots taken at various times after the shift were rapidly collected by centrifugation and frozen in liquid nitrogen. Equal amounts of total RNA prepared from each sample were hybridized to completion with an excess of labeled oligonucleotide complementary to the *HIS3*, *DED1*, and *TRP3* transcripts, and the resulting products were digested with S1 nuclease (Figure 3). Although this procedure measures RNA levels at the various sampling times, RNA synthesized prior to

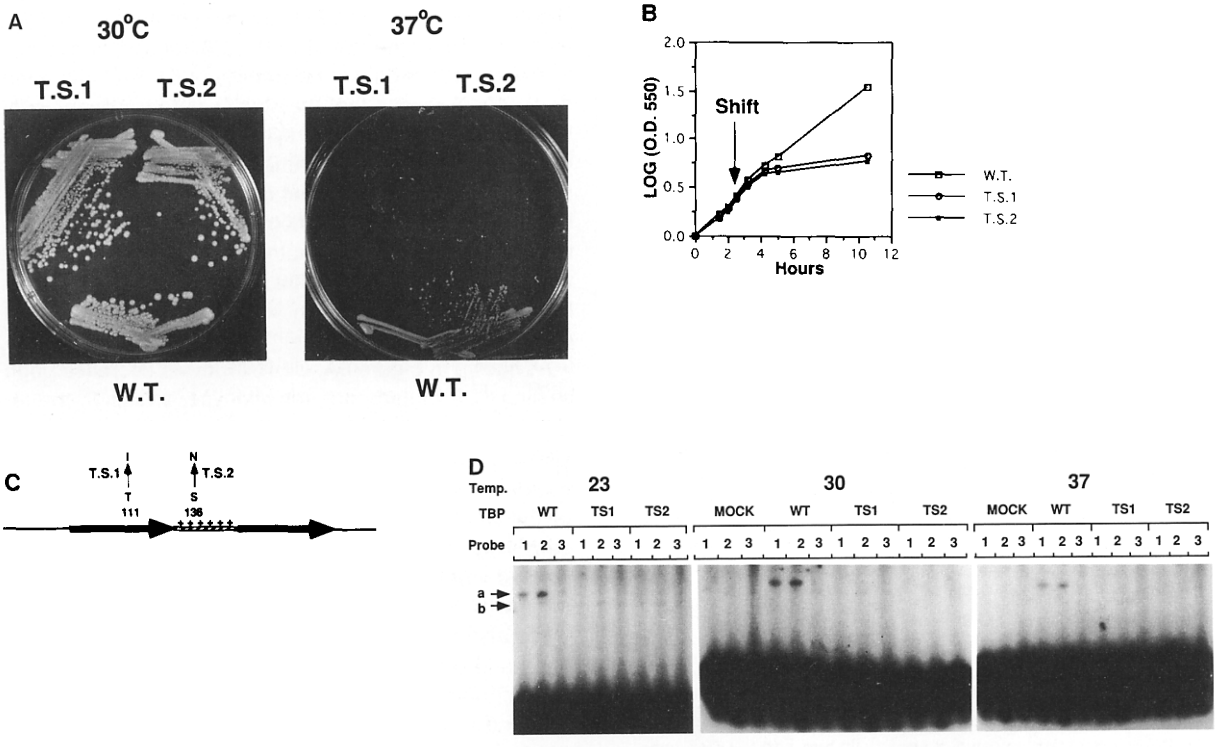

Figure 2. Characterization of ts Derivatives of TBP

(A) Growth of wild-type and TBP mutant strains on plates containing YPD medium at 30°C and 37°C.

(B) Growth curves of wild-type and TBP mutant strains in YPD medium as determined by measuring the OD_{550} at various times; cultures were shifted from 30°C to 37°C at the time indicated by the arrow.

(C) The amino acid changes associated with the ts mutations are indicated above the structure of TBP (direct repeats indicated by thick arrows and basic region shown by striped box marked with plus signs).

(D) DNA binding in vitro. The indicated in vitro translated proteins were incubated at 23°C, 30°C, and 37°C with ^{32}P-labeled probe DNAs corresponding to the following sequences (1, TATAAA; 2, TATATA; 3, TATGAA). The positions of the specific TBP–TATA element complex (a) and a nonspecific complex observed in the absence of TBP (b) are indicated.

the temperature shift should not significantly interfere with the analysis because the half-lives of these mRNA species are very short (<7 min) (Herrick et al., 1990).

HIS3 transcription initiated from the +13 site depends primarily on the canonical TATA sequence T_R, whereas transcription initiated from the +1 site depends almost exclusively on the T_C element that is structurally and functionally distinct from a conventional TFIID interaction site (Struhl, 1986; Mahadevan and Struhl, 1990; Ponticelli and Struhl, 1990). However, upon heat inactivation, both ts TBP strains display a rapid and kinetically similar reduction in the levels of the +1 and +13 transcripts (Figure 3A). Within 30 min, the levels of both transcripts have fallen by 50% and they continue to fall in subsequent time points. The levels of the *DED1* and *TRP3* transcripts are also extremely sensitive to loss of TBP activity. As expected, the levels of all of these transcripts are not affected when the parental strain containing wild-type TBP gene is shifted to 37°C.

To assess the role of TBP in transcription stimulated by acidic activator proteins, the experiment was carried out in a strain constitutively expressing GCN4 protein (Figure 3B). The levels of the *HIS3* +13 and *TRP3* RNAs, which are induced by GCN4, appear equally sensitive to TBP loss as the *HIS3* +1 and *DED1* transcripts, which are not responsive to GCN4. These results demonstrate that canonical TATA-containing promoters (defined by the *HIS3* +13 and *DED1* transcripts) as well as TATA-less promoters (defined by the *HIS3* +1 and *TRP3* transcripts) require TBP for normal and GCN4-activated transcription in vivo.

It could be argued that the above observations are not directly due to the loss of TBP activity, but rather to indirect effects of metabolic mayhem at 37°C. To control for this, we conducted a similar temperature-shift experiment in a strain containing a ts mutation in *CDC28*, the gene encoding the cyclin-associated protein kinase that mediates entry into the cell cycle. No appreciable effect on *HIS3* transcription is observed, and the effect on *DED1* levels is small and confined to later time points (Figure 4A).

An alternative interpretation for the effect of TBP on the TATA-less promoters is that it might reflect loss of a highly unstable protein (e.g., a specific factor for TATA-less promoters) that is encoded by an unstable RNA whose synthesis is dependent on TBP. To address this possibility, we shifted cells to the restrictive temperature in the presence of 100 μg/ml cycloheximide, conditions that block >95% of cellular translation. If such an unstable factor existed, then the cycloheximide block should cause a decrease in

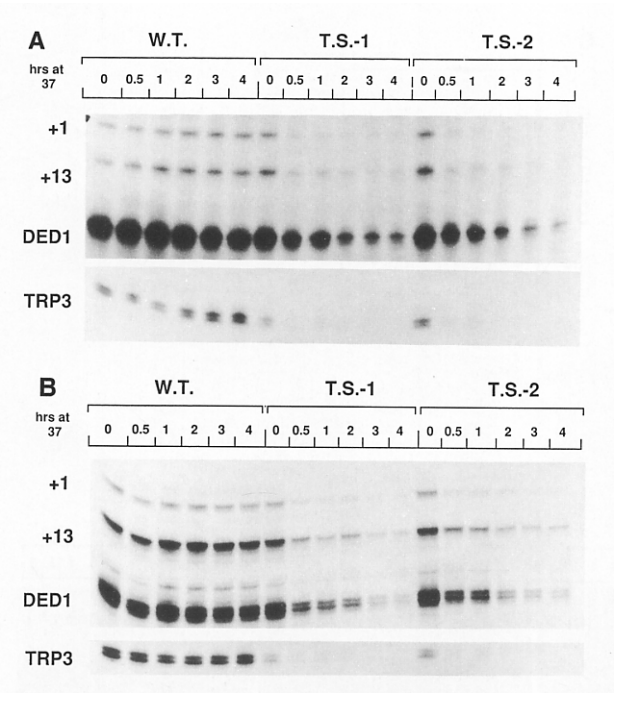

Figure 3. Effect of TBP ts Alleles on Basal and GCN4-Activated Transcription by RNA Pol II

(A) RNA levels of the indicated genes in wild-type and ts TBP strains at various times (in hours) after shifting the cultures to 37°C.

(B) RNA levels of the indicated genes in wild-type and ts TBP strains containing a plasmid that constitutively expresses GCN4 at various times after shifting the cultures to 37°C.

transcription from TATA-less transcripts in both the wild-type and TBP mutant strains. As shown in Figure 4B, there is no decrease in *TRP3* or *DED1* RNA levels in the parental strain, whereas synthesis of those transcripts ceases as usual in the TBP mutant strains. These control experiments and the rapidity of the decrease in RNA levels provide compelling evidence that transcription from TATA-less promoters requires the direct action of TBP.

Role of TBP in Transcription by RNA Pol III

The yeast and mammalian U6 genes are transcribed by RNA pol III, but they contain a TATA sequence upstream of the RNA start and they appear to require TBP for transcription in vitro (Dahlberg and Lund, 1991; Lobo et al., 1991; Margottin et al., 1991; Simmen et al., 1991). However, analysis of the role of TBP in U6 transcription in vivo is problematic because the stability of U6 RNA (Moenne et al., 1990) means that the steady-state transcript level does not reflect the rate of synthesis. We therefore analyzed transcription of a nonfunctional U6 derivative (kindly provided by David Brow) that contains a 12 base internal deletion and presumably encodes an unstable RNA species (see Figure 1). This mutant U6 RNA is synthesized with wild-type efficiency in vitro, but is only present at about 5% of the normal levels in vivo (David Brow, personal communication).

As shown in Figure 5A, U6 RNA levels decrease upon shifting TBP mutant, but not wild-type, strains to the restrictive temperature, suggesting that U6 transcription in vivo requires TBP activity. However, levels of U6 RNA do not decrease as rapidly or to the same extent as those of the *DED1* transcript. This may reflect either a lower sensitivity to the loss of TBP or a longer half-life of the deleted version of the U6 RNA as compared with *DED1* mRNA.

We also investigated transcription of the yeast tryptophan and isoleucine tRNA genes and circumvented the problem associated with the extreme stability of tRNAs by using a hybridization probe complementary to the 5′ intron–exon junction (see Figure 1). tRNA introns are processed rapidly (half-life of less than 3 min; Knapp et al., 1978), so that measurement of their levels should reflect the rate of initiation from the promoter. As shown in Figure 5A, transcription of both tRNA genes is strikingly sensitive to the loss of TBP activity, with kinetics that are similar to that observed for the TATA-containing *DED1* promoter.

To exclude the possibility that these observed effects are due to an unstable subunit or auxiliary factor of the RNA pol III machinery whose transcript(s) requires TBP for synthesis, we determined levels of U6 and tryptophan tRNA in a strain containing a ts allele of *RPB1*, the largest subunit of RNA pol II (Nonet et al., 1987). If the effects on the tRNA and U6 genes are an indirect consequence of a defect in RNA pol II transcription, the same indirect effect should be observed in the *rpb1* mutant strain. Conversely, if TBP is directly involved in transcription by RNA pol III, then a defect in RNA pol II should not affect the synthesis of U6 or tRNA. After shifting the *rpb1* mutant cells to the restrictive temperature, *HIS3* RNA levels decrease at least

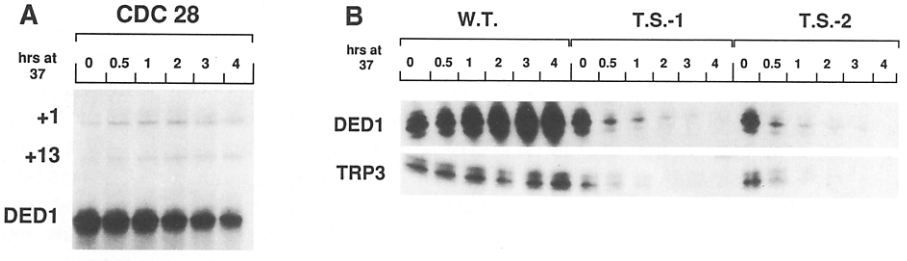

Figure 4. Effect of *cdc28* Mutations and Cycloheximide Treatment

(A) RNA levels of the indicated genes in a *cdc28* ts strain at various times (in hours) after shifting the cultures to 37°C.

(B) RNA levels of the indicated genes in wild-type and ts TBP strains at various times (in hours) after adding cycloheximide to the cultures and shifting them to 37°C.

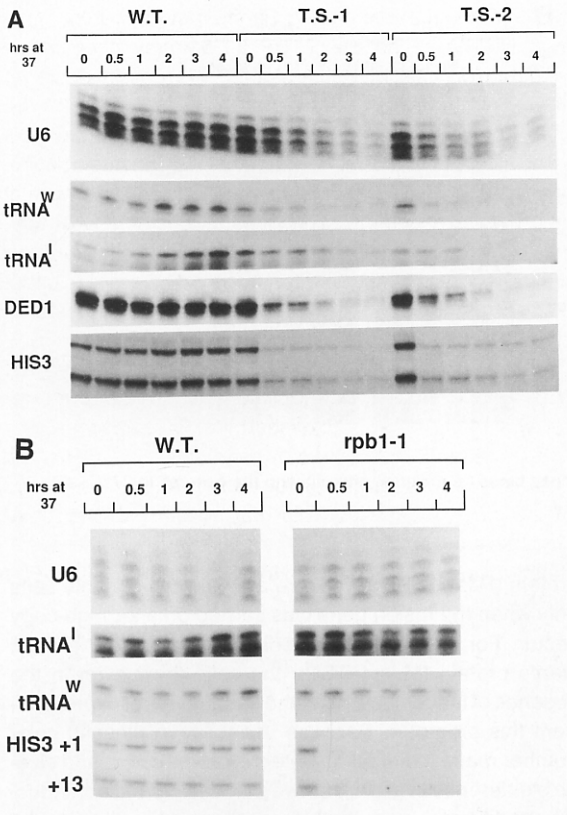

Figure 5. Effect of TBP ts Alleles on Transcription by RNA Pol III

(A) RNA levels of the indicated genes in wild-type and ts TBP strains at various times (in hours) after shifting the cultures to 37°C.

(B) RNA levels of the indicated genes in wild-type and ts *rpb1* strains at various times after shifting the cultures to 37°C.

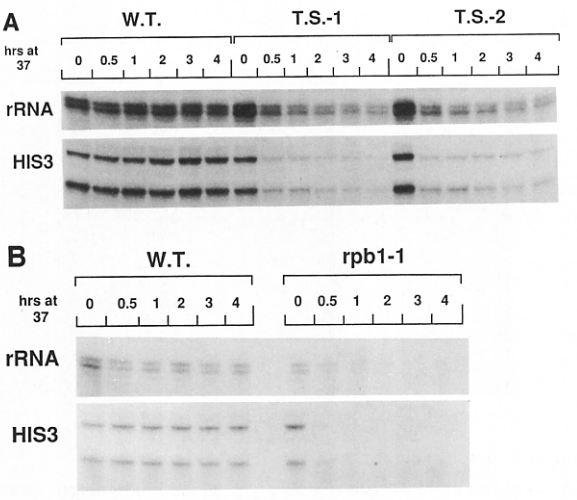

Figure 6. Effect of TBP ts Alleles on Transcription by RNA Pol I

(A) rRNA and *HIS3* levels in wild-type and ts TBP strains at various times (in hours) after shifting the cultures to 37°C.

(B) RNA levels in wild-type and ts *rpb1* strains at various times after shifting the cultures to 37°C.

as rapidly as observed in the TBP mutant strains; in contrast, there is no appreciable effect on the synthesis of tRNA or U6 RNA (Figure 5B). These results strongly suggest that the effect seen on U6 and tRNA is a direct consequence of abrogating TBP activity, and they point to an important in vivo role for TBP in transcription initiated by RNA pol III.

Role of TBP in Transcription by RNA Pol I

To examine the role of TBP in transcription by RNA pol I, we utilized an oligonucleotide probe complementary to the junction between the mature 25S rRNA and the 3′ nontranscribed spacer of the ribosomal precursor RNA (see Figure 1). The 3′ end of the mature 25S rRNA is produced by rapid processing of a longer transcript (Kempers-Veenstra et al., 1986). Thus, levels of this precursor species should not reflect accumulated ribosomal RNA but rather the level of transcription of the rRNA genes at a given time. Upon the shift to the nonpermissive temperature, rRNA levels show a marked and rapid decrease in both TBP mutant strains but not in the wild-type strain (Figure 6A), suggesting that TBP is required for RNA pol I transcription.

To determine whether this effect was specific to loss of TBP activity, similar experiments were performed in the *rpb1*-1 strain (Figure 6B). Unlike the U6 and tRNA genes whose transcript levels are largely unaffected in *rpb1*-limiting conditions, rRNA levels decrease quite significantly. However, the decrease in rRNA transcription is more rapid in the TBP mutant strains than in the *rpb1* mutant strain. After 30 min at the restrictive temperature, rRNA levels are markedly reduced in the TBP mutant strains, but remain at near wild-type levels in the *rpb1*-1 strain. Moreover, at this time point, levels of the RNA pol II transcripts are severely diminished in both TBP and *rpb1* mutant strains (Figures 5B and 6B). Thus, loss of TBP activity results in a kinetically similar decrease in RNA pol I– and pol II–mediated transcription, whereas loss of *RPB1* function affects mRNA synthesis more rapidly than rRNA synthesis. Our observation that loss of *RPB1* function causes a surprisingly rapid decrease in rRNA synthesis (but less rapid than the decrease in mRNA synthesis) is entirely consistent with earlier results that depended upon a completely different method for analyzing transcription (Nonet et al., 1987). Presumably, there exists a sensitive feedback mechanism, perhaps analogous to the stringent response in enteric bacteria (Cashel, 1975; Gallant, 1979), that shuts off rRNA synthesis in response to conditions where gene expression is otherwise compromised.

These results were confirmed and extended by measuring rRNA synthesis by an in vitro run-on assay in which wild-type and mutant cells prepared at various times after the temperature shift were permeabilized and incubated with ^{32}P-labeled UTP. As shown in Figure 7, rRNA synthesis in the TBP mutant strains decreased noticeably within 5 min of the temperature shift and reached very low levels within 30 min. In contrast, in the *rpb1*-1 strain, rRNA synthesis was maintained at approximately wild-type rates for 30 min after which time it decreased. Wild-type strains subject to the same experimental protocol maintained constant levels of rRNA synthesis throughout the course of

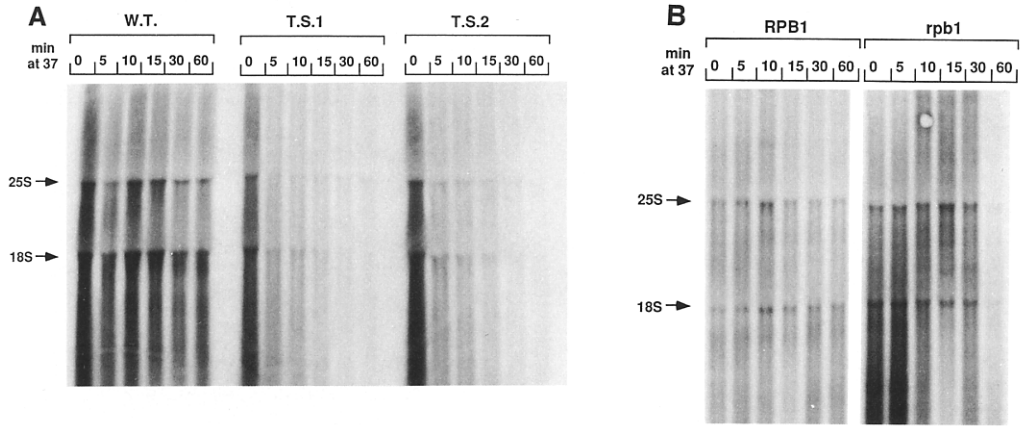

Figure 7. In Vitro Run-On Assays for rRNA Transcription

Levels of 18S and 25S rRNAs in wild-type and ts TBP and *rpb1* strains at various times (in minutes) after shifting the cultures to 37°C.

the experiment. Thus, these results strongly suggest that TBP is directly required for RNA pol I transcription in vivo.

Conditional Expression of TBP under *CUP1* Control

As an independent confirmation of the results obtained with the ts mutants, we wished to generate a different kind of conditional allele in which the TBP-coding region was subject to the control of an inducible promoter. Under inducing conditions, such a promoter would direct sufficient TBP transcription for cell growth, whereas the level of TBP expression in the absence of the inducer would be insufficient for cell viability. We utilized the *CUP1* promoter because the presence or absence of Cu^{2+} is unlikely to affect global cellular metabolism significantly as would promoters whose activity is regulated by the choice of carbon source or the level of inorganic phosphate.

It was also desirable to design an unstable version of TBP, so that protein levels would rapidly fall after removal of the inducer and corresponding decrease in the level of the transcript encoding TBP. For this purpose, we exploited a system based on the effects of the N-terminal amino acid residue on proteolytic sensitivities to the ubiquitin-dependent degradation pathway (Bachmair et al., 1986; Park et al., 1992). Four ubiquitin–*lacI* cassettes, differing in the single amino acid at the junction between the ubiquitin and *lacI* moieties, were fused to the entire TBP-coding region. In the cell, the ubiquitin moiety is rapidly cleaved, thereby exposing the amino acid at the junction that dramatically influences protein stability (Figure 8A). The four protein derivatives contain arginine, tyrosine, isoleucine, and methionine residues at the junction; these correspond to predicted half-lives of <2, <10, <30, and >500 min, respectively (as determined by analysis of lacZ protein derivatives).

Twelve yeast strains were generated in which the four TBP variants were carried on 2μ plasmids, *CEN* plasmids, or integrated at the *URA3* locus. The growth of the various strains correlates well with predicted protein levels (Figure 8A). For the construct predicted to encode the least stable protein (R.lacI–TBP), we were able to obtain viable cells only when the fusion gene was carried on a 2μ high-copy vector. For the construct predicted to encode the most stable protein (M.lacI–TBP), the cells grew even in the absence of inducer, owing to the basal levels of expression from this promoter. Because variability in plasmid copy number made it difficult to detect a reproducible and sizeable difference in growth between inducing and noninducing conditions, it was ultimately necessary to utilize strains in which the constructs were integrated into the genome. For one such strain (Y.lacI–TBP), the presence of 100 μM Cu^{2+} is absolutely required for cell growth (Figure 8B).

Using this Cu^{2+}-dependent conditional allele, we examined several transcripts in order to assess the role of TBP in their transcription (Figure 9). Cells grown in the presence of Cu^{2+} were washed and resuspended in fresh medium lacking Cu^{2+}, and total RNA was prepared at various times afterward. The levels of the *HIS3* +1 and +13 transcripts decline with nearly identical kinetics, whereas the *DED1* transcript falls off more slowly and linearly. The *TRP3* transcript shows a dramatic and rapid decline, corresponding closely to the drop in the TBP transcript itself. As a control, Cu^{2+} was added back to part of the washed culture; no decrease in any of the assayed transcripts was observed (data not shown).

There is a slight increase in the *TRP3* and the +13 *HIS3* transcripts starting about 4 hr after Cu^{2+} removal. This probably reflects induction of the GCN4 protein synthesis as a result of cell stress associated with decreasing TBP levels, especially because the same effect is observed in other strains with subfunctional TBP alleles (M. Strubin, B. P. C., and K. S., unpublished data). This hypothesis was verified by conducting the same experiment in a strain deleted for the *GCN4* gene. The *TRP3* and both *HIS3* transcripts all declined as before, but with no subsequent increase in levels at later time points (data not shown).

These experiments independently demonstrate that TBP is required for transcription from promoters lacking TATA elements. We also looked at tRNA levels after removal of Cu^{2+}. Levels of tRNA synthesis show a decline

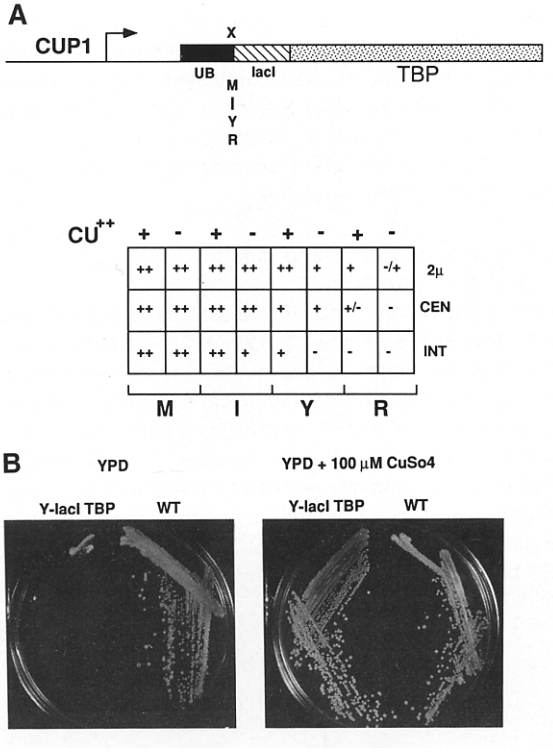

Figure 8. Generation of a Cu^{2+}-Dependent Allele of TBP

(A) The Cu^{2+}-dependent TBP allele contains the *CUP1* promoter driving the expression of a fusion protein consisting of sequences from ubiquitin (UB; closed box), *lacI* (striped box), and the entire TBP-coding region (stippled box). The four derivatives are distinguished by the amino acid residue at the ubiquitin–*lacI* junction (M, I, Y, R). See Experimental Procedures for details. Shown below are the growth phenotypes of strains containing the four fusion proteins (defined by the M, I, Y, R junction residues) expressed from 2μ, *CEN*, and integrating plasmids in the presence (+) or absence (−) of Cu^{2+}. Phenotypes are designated as follows: ++, wild-type growth; +, grows somewhat more slowly than wild type; +/−, very weak growth; −, no growth).

(B) Growth of wild-type and a strain containing an integrated Y.lacI–TBP construct in YPD medium in the presence or absence of 100 μM $CuSO_4$.

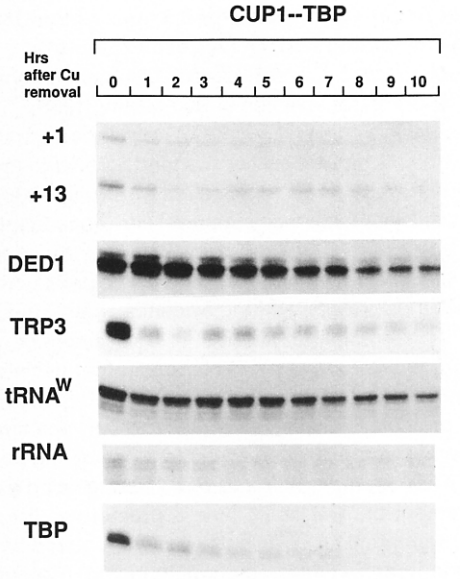

Figure 9. Effect of the Cu^{2+}-Dependent TBP Allele on Transcription

RNA levels of the indicated genes in the strain containing the integrated Y.lacI–TBP allele at various times (in hours) after shifting the cultures to medium lacking 100 μM $CuSO_4$.

similar to that for *DED1* RNA, consistent with a role for TBP in tRNA synthesis (Figure 9). Finally, rRNA transcription also decreased upon Cu^{2+} removal, although the effect appeared to be somewhat less pronounced. We do not fully understand why the levels of the various RNA species appear to decrease with different kinetics, but suspect that it reflects differential responses to suboptimal (and continually decreasing) TBP concentrations. In contrast, we suspect that the kinetically similar profiles observed in the temperature-shift experiments reflect the rapid and general inactivation of TBP.

Discussion

Previous investigations on the role of TBP have almost exclusively involved in vitro transcription assays using heat-treated nuclear extracts or partially purified initiation factors. These biochemical analyses have been extremely useful, but are limited by the heterogeneity of the various protein fractions and by experimental conditions that are far from physiological. In this paper, we utilize conditional TBP alleles to analyze the role of TBP in transcription of various yeast genes in vivo. This approach offers the unique ability to assess the TBP requirement for various promoters under biologically significant conditions. However, as in all genetic experiments, the concerns are that the mutant alleles may not simply inactivate gene function and that the observed effects on transcription might be indirect.

It is unlikely that the ts alleles are misleading as to the elucidation of normal TBP function, because transcription effects are observed with two different TBP mutations but not with a *cdc28* mutation. Moreover, the results are confirmed by Cu^{2+} conditional transcription, an entirely independent method for generating TBP-depleted conditions. Thus, the observed effects on cellular transcription almost certainly result from loss of TBP function.

Although the question of whether the observed effects are directly or indirectly a result of loss of TBP function can never be demonstrated unambiguously, the evidence strongly suggests that the effects are direct. First, the observed effects are rapid, indicating that any indirect effect must be even more rapid. Second, the transcriptional effect caused by the TBP ts alleles cannot be mimicked with either cycloheximide or (in the cases of RNA pol I and III promoters) *rpb1*-1, conditions that are at least as deleterious to cell physiology. Moreover, decreases in transcription occur more rapidly upon loss of TBP function than upon blocking protein synthesis, indicating that the TBP-dependent effect is not mediated through proteins synthesized after the temperature shift.

TBP Is Required for RNA Pol II Transcription from TATA-Containing and TATA-Less Promoters

Although the *his3* T_C and T_R elements support comparable basal levels of transcription in vivo (Struhl, 1986), T_C does not resemble a conventional TATA sequence (Mahadevan and Struhl, 1990), does not respond to acidic activator proteins (Struhl, 1986; Ponticelli and Struhl, 1990), and does not bind TFIID nor support TFIID-dependent transcription in vitro (Ponticelli and Struhl, 1990). Here we show that T_C–dependent transcription depends critically on TBP, thus providing strong evidence against models in which T_C interacts with novel TFIID-like factors or generates an unusual structure in chromatin that obviates the need for protein binding. Moreover, the T_C–dependent and *TRP3* promoters display an equal and perhaps stronger requirement for TBP than promoters containing conventional TATA elements (T_R and *DED1*), suggesting a direct involvement of TBP at TATA-less promoters. Taken together with previous results that TBP is required for transcription from TATA-less promoters in vitro (Carcamo et al., 1990; Smale et al., 1990; Pugh and Tjian, 1991), these observations suggest that a single TBP in the cell is required for transcription from all RNA pol II promoters. Nevertheless, the clear functional distinctions between T_R and T_C strongly suggest that TBP behaves differently at TATA-containing and TATA-less promoters.

Given that TBP has substantial ability to bind and function efficiently at nonconsensus TATA sequences (Hahn et al., 1989; Singer et al., 1990), one possible model for transcription from TATA-less promoters is that TBP recognizes weak consensus sites. Although we have been unable to obtain DNAase I footprints of T_C by purified TBP (A. S. Ponticelli and K. S., unpublished data), T_C is defined by an AT-rich region of 25 bp (Mahadevan and Struhl, 1990) and hence might contain one or more very weak binding sites. A potential weak site in the *TRP3* promoter could also account for the extreme sensitivity of the *TRP3* RNA levels to small decreases in TBP concentration. However, this model does not simply explain why T_R and T_C are comparable in supporting basal *his3* expression in vivo; perhaps T_C is more accessible than T_R in the context of chromatin. Nor does the model easily account for why T_C fails to support GCN4 and GAL4 activation; perhaps TBP is bound too transiently or in a different conformation so as to preclude a functional response to acidic activator proteins.

An alternative model is that TATA-less promoters are recognized by TBP in the context of a larger complex that contains other proteins. Such TBP-associated factors might modulate the binding properties of TBP itself or might themselves contact the DNA. Human and Drosophila TBPs are tightly associated with other proteins (Dynlacht et al., 1991; Timmers and Sharp, 1991), and a heat-labile "tethering" factor appears to be required in vitro for the function of TATA-less promoters (Pugh and Tjian, 1991). Although yeast TBP fractionates as an autonomous protein in cell-free extracts (Buratowski et al., 1988), it might associate with other factors under physiological conditions. In this model, functional distinctions between TATA elements (Struhl, 1986; Homa et al., 1988; Simon et al., 1988; Wefald et al., 1990) would be explained not by multiple TBPs (as is the case for prokaryotic σ factors) but rather by multiple TBP complexes that are distinguished by their associated proteins. Distinct TBP complexes might recognize a larger repertoire of promoters than possible by TBP alone and/or respond differentially to upstream activator proteins.

TBP Is Required for Transcription by RNA Pol I and III

We examined three genes transcribed by RNA pol III and found that all are affected by loss of TBP activity. In the case of the U6 gene, our observations in vivo are consistent with and establish the biological relevance of in vitro transcription experiments that implicate TBP in the synthesis of yeast and human U6 RNA (Lobo et al., 1991; Margottin et al., 1991; Simmen et al., 1991). The human and yeast U6 genes contain a TATA element at position −30 that is required for transcription in vitro. Thus, it seems likely that TBP binding to the TATA elements in the U6 promoters is critical for transcription by RNA pol III.

The unexpected requirement for TBP in yeast tRNA synthesis is particularly interesting because tRNA promoters differ considerably from the U6 promoter in that the critical elements lie within the coding region and sequences upstream of the initiation site are relatively unimportant (reviewed by Geiduschek and Tocchini-Valentini, 1988; Dahlberg and Lund, 1991). However, many yeast tRNA genes have upstream TATA sequences, and it is possible that TBP binding is required for transcription. At least some of the tryptophan tRNA promoters and presumably other tRNA promoters appear to lack canonical TATA sequences. How TBP might function at such TATA-less tRNA promoters is unclear, but it could involve weak binding to non-TATA sequences or protein–protein interaction with some other component(s) that binds the promoter. In this sense, TATA-less RNA pol III promoters may be analogous to TATA-less RNA pol II promoters that also require TBP for transcription in vivo.

Despite the fact that the rRNA promoter is structurally very different from promoters responding to RNA pol II and III (Figure 1), our results very strongly implicate TBP as being directly involved in transcription by RNA pol I. Shown in two independent measurements, rRNA synthesis decreases very rapidly upon loss of TBP activity. Moreover, this rapid reduction in rRNA transcription specifically occurs during conditions of TBP inactivation. Although rRNA synthesis does decrease upon inactivation of the RNA pol II machinery, this reduction occurs considerably after the effect, owing to TBP loss, and hence is likely to reflect a more indirect effect. Although surprising, our results are consistent with very recent in vitro transcription experiments that suggest that human SL1, an RNA pol I accessory factor, is a TBP complex (Comai et al., 1992). Although they contain a common TBP subunit, the human SL1 and TFIID activities are chromatographically separable, functionally distinct (in terms of RNA polymerase specificity), and contain different associated protein factors (Comai et al., 1992).

The role of TBP in transcription by RNA pol I is obscure

at present, especially because rRNA promoters lack TATA-like sequences and SL1 does not have inherent DNA binding activity. It is therefore possible that the DNA binding activity of TBP is not required for RNA pol I transcription. For example, TBP-associated proteins might be the critical RNA pol I transcription factors, with TBP being required only for the formation or stability of a protein complex. Alternatively, TBP might contact DNA in a non-sequence-specific manner; presumably such an interaction would be stabilized by protein–protein contacts to other DNA-bound components of the RNA pol I transcription machinery such as the upstream binding factor (Bell et al., 1988).

Taken together, the above observations strongly suggest that TBP is important for some general aspect of transcription by RNA pol I and III, in addition to its role in transcription by RNA pol II. One possibility is that TBP interacts with proteins shared by the RNA pol II and III transcription machineries such as the common subunits of the enzymes (Sentenac, 1985; Woychick et al., 1990). Alternatively, TBP might interact with distinct auxiliary proteins that are specific to the different transcription machineries, a model supported by the existence of physically distinct TBP complexes (Timmers and Sharp, 1991; Comai et al., 1992) and the extreme species specificity of the RNA pol I transcription factor SL1 (Grummt et al., 1982; Bell et al., 1989). In this regard, although yeast TBP has so far been found only as a simple monomeric protein in cell-free extracts (Buratowski et al., 1988), it seems very likely that it will associate with other proteins in vivo. Finally, it should be noted that human TBP cannot substitute for the essential function(s) of yeast TBP in vivo (Cormack et al., 1991; Gill and Tjian, 1991) even though it is sufficient for transcriptional activation from RNA pol II promoters in vivo (Strubin and Struhl, 1992) and in vitro (Kelleher et al., 1992). Perhaps the inability of human TBP to support yeast cell growth reflects a species-specific difference in transcription mediated by RNA pol I and/or III.

TBP May Be Necessary for Transcription of All Nuclearly Encoded Genes in Yeast

In this paper, we have shown that TBP is required for transcription mediated by all three nuclear RNA polymerases under physiological conditions. These observations are consistent with and establish the biological relevance of biochemical studies using partially fractionated cell-free extracts (Lobo et al., 1991; Margottin et al., 1991; Simmen et al., 1991; Comai et al., 1992). Moreover, our in vivo studies indicate that potential non-TBP transcriptional initiation mechanisms are unlikely to exist (or are very inefficient). As a wide variety of structurally distinct promoters require TBP for activity in vivo, it seems very likely that TBP is necessary for transcription of all nuclear genes (although the possibility of a small subset of genes transcribed without the direct involvement of TBP cannot be excluded). Moreover, this "universal" transcriptional role for TBP appears to be conserved from yeast to human. In this sense, TBP is likely to be the primordial eukaryotic transcription factor that evolved to function with distinct basic transcription machineries. However, in TATA-containing RNA pol II genes, TBP plays a critical role in promoter recognition, whereas other DNA-binding proteins appear to be more predominant in selecting promoter regions utilized by RNA pol I and III.

Experimental Procedures

Strains and Plasmids

The strain used for all these experiments is a derivative of BY2 (Cormack et al., 1991) that contains a chromosomal deletion of TBP and carries a wild-type TBP gene on a *URA3* centromeric vector. All derivative strains were generated by using the plasmid shuffle (Boeke et al., 1987) to replace the wild-type TBP gene with the desired TBP alleles, which were cloned in the *TRP1* centromeric vector YCplac22 (Gietz and Sugino, 1988). The *CUP1–TBP* constructs (described below), cloned in the integrating vector YIplac211, were cleaved with ApaI and introduced into BY2 containing a *TRP1* plasmid carrying TBP. Integrants were grown in YPD supplemented with 100 μM $CuSO_4$, and individual colonies were screened on minimal plates supplemented with casamino acids (6 g/l) and 100 μM Cu^{2+}. The resulting trp colonies had lost the plasmid-borne TBP gene and retained only the *CUP1–TBP* construct integrated at the *URA3* locus. The U6 deletion construct (lacking bases 59–72), obtained from David Brow, was subcloned as a PstI–BamHI fragment into YCplac33 (Gietz and Sugino, 1988), a *URA3* centromeric vector. This U6-containing plasmid was transformed into the three BY2 strains carrying either the wild-type or ts TBP alleles on a *TRP1* plasmid. The experiments involving GCN4 activation were carried out by transforming the three BY2 strains with YCp88 GCN4, which expresses GCN4 constitutively from the *DED1* promoter (Hope and Struhl, 1986). The *rpb1*-1 strain N15 and the RPB1 parent N1 were a gift of R. Young.

The fusion genes for the *CUP1* depletion experiments were made by using the polymerase chain reaction to introduce an XbaI site at the AUG initiation codon of TBP and then fusing that to a BamHI–XbaI fragment, obtained from J. Park (Park et al., 1992), carrying *lacI* (residues 318–346) and the HA1 epitope from influenza virus (Wilson et al., 1984). This was cloned downstream of 1 of 4 EcoRI–BamHI fragments encoding ubiquitin and the amino acid ultimately at the ubiquitin–*lacI* junction (Bachmair et al., 1986). The *CUP1* promoter was obtained from YEp46 (Butt et al., 1988) as a SmaI–EcoRI fragment and was cloned upstream of the ubiquitin fragment. The whole fusion gene was cloned into either YCplac22, YEplac111, or YIplac211 (Gietz and Sugino, 1988).

Isolation of Mutants

A YCplac22 plasmid containing the 2.4 kb EcoRI–BamHI TBP fragment was mutagenized in vitro by treatment with 1 M hydroxylamine (pH 6.0) at 65°C for 5 hr. This mutagenized DNA was introduced into Escherichia coli, and the resulting library (50,000 independent colonies) was used to transform yeast strain BY2. Of the 20,000 yeast transformants screened, approximately 40 were unable to grow in the presence of 5-fluoroorotic acid, indicating that the *TRP1* plasmid-borne TBP copy had a lethal mutation. Two colonies grew on 5-fluoroorotic acid plates at 30°C but not at 37°C and contain ts TBP alleles. The *TRP1* plasmids from these strains were recovered, the TBP genes sequenced, and the plasmids reintroduced into BY2 to verify the ts phenotype. By appropriate subcloning experiments, it was also verified in each case that the mutant phenotype was carried in the SpeI–XbaI fragment (position −150 to +750) of the mutagenized gene.

DNA Binding Experiments

The wild-type TBP gene and both ts derivatives were cloned downstream of an SP6 promoter in pGEM72. SP6 run-off transcripts were generated in vitro and used in in vitro translation reactions with rabbit reticulocyte lysate to generate ^{35}S-labeled proteins. The quantity and integrity of these proteins were verified by SDS–polyacrylamide gel electrophoresis and autoradiography. The DNA probes were labeled with ^{32}P by amplification with the polymerase chain reaction from templates containing various TATA elements (TATAAA, TATATA, TATGAA) as described previously (Wobbe and Struhl, 1990). Equal amounts of the three TBP derivatives were combined with 1 ng of each probe in a binding buffer consisting of 50 mM KCl, 10 mM $MgCl_2$, 15

mM HEPES (pH 7.9), 1 mM dithiothreitol, 0.5 mM EDTA, 1 μg of dG–dC, and 10% glycerol. The reactions were incubated for 20 min at 23°C, 30°C, and 37°C, after which 2 μg of single-stranded salmon sperm DNA was added and incubated for an additional 10 min (Strubin and Struhl, 1992). The reactions were stopped on ice, and the products were analyzed on nondenaturing polyacrylamide gels.

Temperature-Shift Experiments

Cells were grown in YPD medium to the early exponential phase at 30°C (A_{600} = 0.15), shifted to 37°C for 15 min, and returned to 30°C for 1 hr. After this pre-heat shock, the cultures were shifted back to 37°C and aliquots were taken at the appropriate times. The pre-heat shock induces the expression of heat shock proteins and prevents the transient (roughly 30 min) decrease in all transcripts that occurs under these mild heat shock conditions (Nicolet and Craig, 1991). This protocol ensures that the effect observed at early time points is due to the ts TBP allele and is not a consequence of a general heat shock response. For each time point, the cells were rapidly collected by centrifugation and frozen in liquid nitrogen, the entire process taking between 1 and 2 min.

Depletion of TBP Using the *CUP1* Promoter

The strain containing the integrated Y.lacI–TBP allele was grown at 30°C in YPD medium containing 100 mM $CuSO_4$ to an optical density of 0.2. Cells were collected by centrifugation, washed once with YPD containing 25 mM EDTA, twice with YPD, and finally resuspended in YPD at 30°C to an optical density of 0.03. Aliquots were taken at various times, and cells were rapidly collected by centrifugation and frozen in liquid nitrogen.

RNA Analysis

Total RNA was prepared from each sample and quantitated by absorbance at 260 nM and by ethidium bromide staining of 18S and 28S RNAs after separation by agarose gel electrophoresis. Ten micrograms of each RNA sample was hybridized to completion with a 10- to 100-fold excess of the appropriate ^{32}P-labeled oligonucleotide and treated with S1 nuclease as described previously (Chen et al., 1987; Chen and Struhl, 1988). The resulting products were analyzed on denaturing polyacrylamide gels and quantitated using a Betagen B detector. The oligonucleotides all contain 6 residues at their 3′ ends that are not complementary to the RNA, thereby permitting an easy distinction between bands due to appropriate RNA–DNA hybrids and undigested probe. Conditions of hybridization probe excess were verified by showing that the observed band intensities were unchanged upon decreasing the amount of probe by a factor of 5. The *HIS3* and *DED1* oligonucleotides have been previously described (Chen et al., 1987), and the sequences of others employed here follow below.

TRP3: GGTAAAGGAATCGTAGTTGTCAATTAGAACCACATGCTTACCTTAG.
rRNA: TGCCAGTACCCACTTAGAAAGAAATAAAAAACAAATCGTTAGG.
tRNA^{I}: AAAGGCCTGTTTGAAAGGTCTTTGGCACAGAAACTTCGGAAACCGTCGTGG.
tRNA^{W}: GGAATTTCCAAGATTTAATTGGAGTCGAAAGCTCGCCTTA.
U6: CTTTGTAAAACGGTTCATCCTTATGCAGGGGAACTGCTGATCATCTCTGTATTGTTTCAAATTGACCTGGCCG.
TBP: GGAAACAATTCTGGCTCATAGGAGGAGAAAGTACCATGACTGAATGCGACTCT.

In Vitro Run-On Transcription Assays

The run-on transcription assays were performed by a method described previously (Elion and Warner, 1986). Cultures were treated as described above, and for each time point after the temperature shift 15 ml samples were collected by pouring over sterile ice; all subsequent steps were carried out at 0°C–4°C. The cells were collected by centrifugation, washed in TMN (10 mM Tris [pH 7.4], 100 mM NaCl, 5 mM $MgCl_2$), and resuspended in 1 ml of 0.5% sodium N-lauroyl sarcosine. After 15 min, the cells were pelleted and then resuspended in 120 μl of reaction mix (50 mM Tris [pH 7.9], 100 mM KCl, 5 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM dithiothreitol, 0.5 mM ATP, 0.25 mM each GTP and CTP, 10 mM phosphocreatine, 1.2 mg of creatine phosphokinase, 10 μCi of [α-^{32}P]UTP at 300 Ci/mmol). After incubation for 10 min at 25°C, 1 ml of ice-cold TMN containing 0.5 mM nonradioactive UTP was added and RNA was prepared. For each time point equal amounts of RNA (determined by ethidium bromide staining of 18S and 25S rRNAs) were separated by agarose gel electrophoresis, transferred to a nylon filter, and analyzed by autoradiography and quantitated using a Betagen B detector.

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Note Added in Proof

After this paper was submitted, White et al. provided biochemical evidence implicating the TATA binding protein in transcription of tRNA genes by RNA polymerase III. (Proc. Natl. Acad. Sci. USA *89*, 1949–1953, 1992).