GAL4, when GAL4 is overexpressed from the *ADH1* promoter. GAL4 also failed to stimulate binding of TBPE186D to the *GAL1* promoter, explaining the transcription defect and directly demonstrating the importance of the TBP–TFIIB interaction for activator-mediated stimulation of TBP binding.

Srb4 is required for transcription in general[22], and has been implicated as a direct target of certain activators[23]. As expected for a GTF, Srb4 was required for *ADH1*, *RPS5*, *GAL1* and *CUP1* transcription (Fig. 5d). Following inactivation of Srb4, there was also a loss of TBP binding to the *GAL1*, *ADH1*, *CUP1* and, to a lesser extent, the *RPS5* promoters.

On the basis of these results, we infer the following. First, there is a general qualitative and quantitative correlation between transcriptional activity and the association of TBP, as well as other GTFs, with the promoter. Thus, *in vivo* activators function, at least in part, by recruiting GTFs and ultimately RNA polymerase II, as proposed on the basis of *in vitro* assays measuring promoter–GTF interactions[24], and more recently inferred by 'activator bypass' experiments[3]. Second, TBP binding *in vivo* is actively prevented by Mot1. Finally, activator-mediated stimulation of TBP binding requires GTFs other than TFIID and its components. The requirement for multiple GTFs is not readily compatible with models proposing that activators stimulate TBP binding and transcription solely through contact with TFIID[25,26]. The involvement of multiple GTFs in activator-mediated stimulation of TBP binding underscores the cooperative nature of PIC assembly and is consistent with several possible mechanisms, including a pre-assembled 'holoenzyme'[27,28] that is disrupted by inactivation of certain GTFs, or direct contact between the activator and GTFs such as TFIIB[24] or Srb4 (ref. 23), which promote TBP binding and PIC assembly through cooperative interactions. □

Methods

Unless otherwise specified, the yeast strain was W303a and the cells were grown in YP or minimal medium containing glucose. The formaldehyde crosslinking/immunoprecipitation method has been described[7,8]. Briefly, yeast cells were treated with 1% formaldehyde, collected and resuspended in lysis buffer[7]. Following sonication, which generated DNA fragments averaging ~500 bp, cell lysates were precleared by centrifugation; Sarkosyl (1%) was added to the lysate before immunoprecipitation with an anti-yTBP polyclonal antibody[7]. Immunoprecipitated protein–DNA complexes were treated with protease K, the crosslinks reversed, and the DNA purified and analysed by quantitative PCR[8]. PCR reactions contained [α-^{32}P]dATP (2.5 μCi for each 25-μl reaction) and the PCR products were detected by autoradiography after separation on a 6% polyacrylamide gel. Primers corresponded to sequences around the TATA element, and the PCR products ranged from 130 to 200 bp; the control fragments *GAL4* (ORF) and *GAL1* (ORF) are located, respectively, 2,000 and 900 bp downstream of the TATA sequences of their promoters. To analyse transcription, total cellular RNA was prepared and primer extension was carried out using primers close to the RNA cap site.

Received 3 December 1998; accepted 8 April 1999.

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Acknowledgements. We thank R. Young, M. Grunstein, J. Thorner and J. Majors for yeast strains, J. Ma for plasmids, and K. Struhl for discussion and for communicating unpublished results. This work was supported in part by a grant from the NIH to M.R.G. M.R.G. is an investigator of the Howard Hughes Medical Institute.

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Binding of TBP to promoters *in vivo* is stimulated by activators and requires Pol II holoenzyme

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In eukaryotes, transcriptional activators have been proposed to function by recruiting the RNA polymerase II (Pol II) machinery[1–3], by altering the conformation of this machinery[4,5], or by affecting steps after initiation[6–8], but the evidence is not definitive. Genomic footprinting of yeast TATA-box elements reveals activator-dependent alterations of chromatin structure[9] and activator-independent protection[10], but little is known about the association of specific components of the Pol II machinery with promoters *in vivo*. Here we analyse TATA-box-binding-protein (TBP) occupancy of 30 yeast promoters *in vivo*. We find that TBP association with promoters is stimulated by activators and inhibited by the Cyc8–Tup1 repressor, and that transcriptional activity correlates strongly with the degree of TBP occupancy. In a small subset of promoters, TBP occupancy is higher than expected when gene activity is low, and the activator-dependent increase is modest. TBP association depends on the Pol II holoenzyme component Srb4, but not on the Kin28 subunit of the transcription factor TFIIH, even though both proteins are generally required for transcription. Thus in yeast cells, TBP association with promoters occurs in concert with the Pol II holoenzyme, activator-dependent

recruitment of the Pol II machinery occurs at the vast majority of promoters, and Kin28 acts after the initial recruitment.

We analysed TBP occupancy of promoters in yeast cells by using a modified version of a chromatin immunoprecipitation procedure[11,12]. Initially, we analysed the *PGK1* promoter and genomic regions that are centrally located within large (5 kilobase (kb)) structural genes (*RPO21* and *POL1*) and hence as far as possible from promoters. As expected, the product of the polymerase chain reaction (PCR) corresponding to the *PGK1* promoter is easily detected in cells containing the epitope-tagged TBP but not in control cells containing non-tagged TBP (Fig. 1). Crosslinking efficiency at the *PGK1* promoter is 0.9% (hereby defined as 10 units; see Methods), which is in accord with results obtained with other yeast DNA-binding proteins[12,13]. TBP appears to crosslink at low efficiency to the *RPO1*, *POL1*, and *GLT1* (results not shown) structural genes (compare strains containing tagged rather than non-tagged TBP). This observation suggests that TBP weakly and non-specifically associates with most (perhaps all) chromosomal DNA *in vivo*.

To investigate transcriptional activation mechanisms, we determined TBP occupancy of various promoters that respond to specific DNA-binding activators. Ga14-dependent promoters are transcriptionally active in cells grown in galactose, but not in glucose or raffinose. TBP strongly associates with all four *GAL* promoters tested (40–60 units) in galactose-grown cells, but is not detectably associated in glucose- or raffinose-grown cells (Fig. 1a). Strong TBP occupancy requires a functional promoter, as evidenced by the weak signal corresponding to UAS_G, the region just upstream of the *GAL7* enhancer. These results extend the previous observation that photofootprinting *in vivo* detects Gal4- and transcription-dependent changes at the *GAL1* and *GAL10* TATA elements[9]. Similarly, increased TBP occupancy is observed at four *MET* promoters that are activated by heteromeric complexes containing the Met4 activator when cells are grown in medium lacking methionine[14] (Fig. 1b). Thus, Gal4 and Met4 stimulate TBP recruitment at several different core promoters.

Yeast cells exposed to a transient heat shock rapidly induce transcription of various genes using distinct molecular mechanisms. For genes directly activated by Hsf1 (*SSA3*, *SSA4*, *HSP82*, and probably *HSP104*) or the redundant Msn2 and Msn4 activators (*CTT1*, *HSP12*, and probably *HSP104*), heat shock dramatically increases TBP occupancy *in vivo* (Fig. 2a). In addition, heat shock causes increased transcription (Fig. 2c) and increased TBP occupancy of the glycolytic genes *ENO1* and *PGK1*, but not of *PYK1*. Finally, in another rapid stress response that involves the Ace1 activator, copper-dependent induction of *CUP1* transcription (40-fold; data not shown) is associated with a concomitant 40-fold increase in TBP occupancy at the *CUP1* promoter (Fig. 2b). Taken together, these results demonstrate that Ace1, Hsf1, Msn2, Msn4, and unknown activators increase TBP recruitment, and they strengthen the correlation between transcriptional activity and TBP occupancy.

The transition between growth on glucose and non-fermentable carbon sources such as ethanol requires the induction of many genes, including those responsive to Adr1, Hap1 and unknown activators. In accord with their transcriptional activities, TBP is strongly associated with the *ADH2*, *ICL1*, *PCK1*, and *CYB2* promoters when cells are grown in ethanol medium, but not when cells are grown in glucose medium (Fig. 3). In addition, *PGK* transcription is reduced threefold in ethanol medium, and TBP occupancy decreases by a comparable extent.

In contrast to the other 28 promoters tested, TBP clearly associates with the *CYC1* and *COX5a* promoters in glucose medium even though transcription levels are very low (Fig. 3). Moreover, TBP occupancy at these promoters is barely stimulated (less than twofold) when cells are grown in ethanol medium, even though transcription is stimulated by a factor of 10. These results are partially consistent with a DNase I footprint over the *CYC1* TATA element in isolated nuclei, even under conditions where transcription was not observed[10]. However, DNase I footprints imply a high degree of protein occupancy (at least 50%), whereas TBP occupancy at the *CYC1* promoter is considerably lower (5–10%; see below);

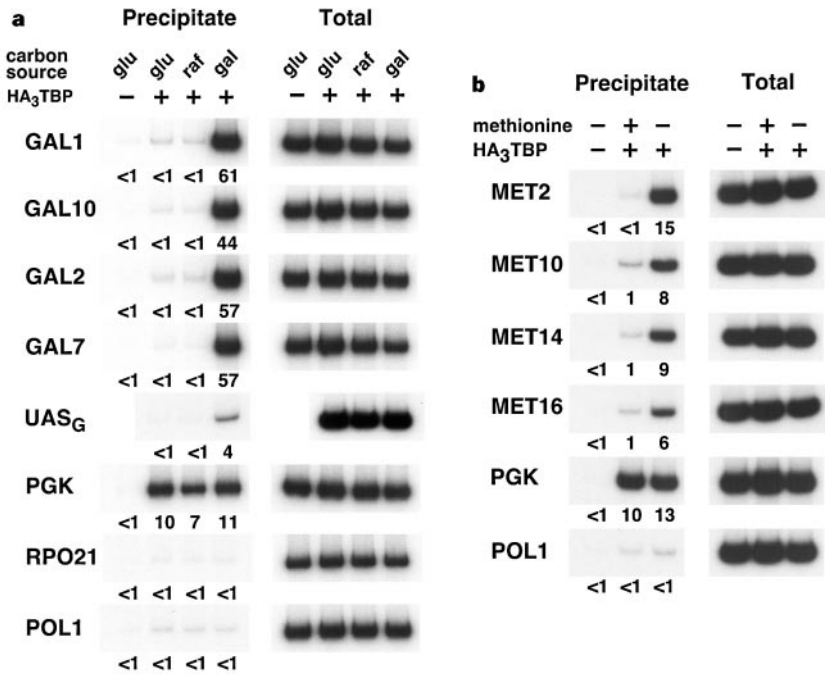

Figure 1 TBP occupancy at *GAL* and *MET* promoters. **a**, Cells containing HA_3-tagged or untagged TBP were grown in: **a**, YP medium containing glucose (glu), raffinose (raf) or galactose (gal) as carbon source; or **b**, glucose minimal medium in the presence or absence of 1 mM methionine. PCR products corresponding to the indicated promoters, the *RPO21* and *POL1* structural genes, or the region just upstream from UAS_G at *GAL7* were generated from total chromatin or immunoprecipitated DNA. The amount of immunoprecipitated DNA assayed by PCR varied slightly depending on the promoter examined, and this was considered in determining units of TBP occupancy (indicated under the corresponding panels).

hence, it is unclear whether the previously observed DNase I footprint corresponds to TBP association.

Finally, we examined whether the Cyc8–Tup1 co-repressor exerts its effects at the level of TBP occupancy. The Cyc8–Tup1 complex is recruited by pathway-specific DNA-binding proteins to promoters that are regulated by cell-type (*MFA1*), glucose (*SUC2*) and oxygen (*ANB1*)[15,16]. In wild-type strains, *MFA1*, *ANB1* and *SUC2* are essentially not expressed, and TBP occupancy occurs at background level (Fig. 4). However, in a *tup1* strain, transcription of these genes occurs to various extents, and TBP associates with the promoters. Thus, Cyc8–Tup1 inhibits TBP association with three promoters that respond to different DNA-binding repressors and activators and contain unrelated core promoter regions.

The maximal level of TBP occupancy (50–100 units) is observed for several highly active genes, and hence is likely to correspond to full occupancy of the promoter by TBP. This suggests that our measurements reflect TBP occupancy *per se* and so are not generally affected by conformational differences at TATA elements that might affect crosslinking efficiency. In support of this, TBP occupancy at all five transfer-RNA promoters tested occurs at this maximal value (our unpublished results), even though TBP plays distinct roles at Pol II and Pol III promoters. Moreover, the TATA-element sequence has no significant effect on the crystal structure of the TBP–TATA complex[17]. These considerations, and the very strong correlation between transcription levels and TBP occupancy, strongly suggest that we have quantitatively measured TBP occupancy (estimated experimental error is ±30%).

The promoters analysed here differ in the quality, location and number of activator-binding sites and TATA elements, as well as in nucleosome position and phasing. Despite these differences, our results show that the predominant, although not exclusive, limitation for transcriptional activity in wild-type cells correlates with the ability of TBP to associate stably with the promoter. As the remainder of the Pol II machinery cannot associate with promoters in the absence of TBP (or TFIID), our results strongly suggest that the entire Pol II machinery is absent from the vast majority of promoters in the absence of functional activator proteins, although they do not indicate which components of the Pol II machinery are important for TBP recruitment or whether partial preinitiation complexes can stably associate with promoters *in vivo*.

To investigate these issues, we examined TBP occupancy under conditions in which Pol II transcription was generally reduced by temperature-sensitive mutations in the following components: Srb4, a component of the mediator and Pol II holoenzyme complexes[18]; Rpb1, the largest subunit of Pol II (ref. 19); and Kin28, the subunit of TFIIH that phosphorylates the C-terminal tail of Pol II (ref. 20) (Fig. 5). Unexpectedly, loss of Srb4 function significantly reduces TBP occupancy at all six promoters tested. TBP occupancy is also decreased in the *rpb1* mutant strain, although the effect is less marked and not seen at all promoters. In contrast, TBP occupancy is not affected upon inactivation of Kin28, even though the reduction in transcription is comparable to that from the *rpb1* and *srb4* strains. Thus, TBP association with promoters requires the Pol II holoenzyme, but not the Kin28 subunit of TFIIH.

The Srb4 requirement for TBP occupancy strongly suggests that

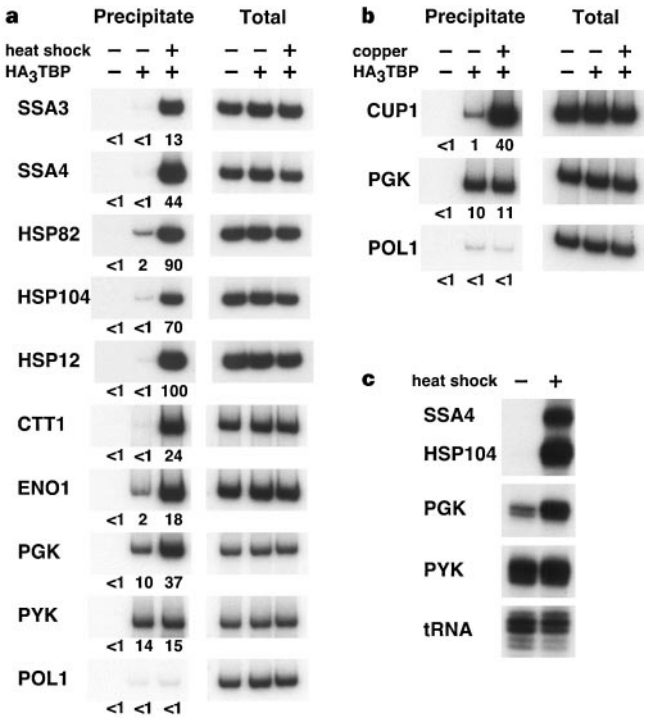

Figure 2 TBP occupancy in response to heat shock and copper induction. Cells containing HA_3-tagged or untagged TBP were grown in glucose minimal medium at 24 °C and subjected to: **a**, a 15-min heat shock at 39 °C; or **b**, a 20-min treatment with 1 mM copper sulphate. TBP occupancy was analysed as for Fig. 1. **c**, Quantitative S1 analysis.

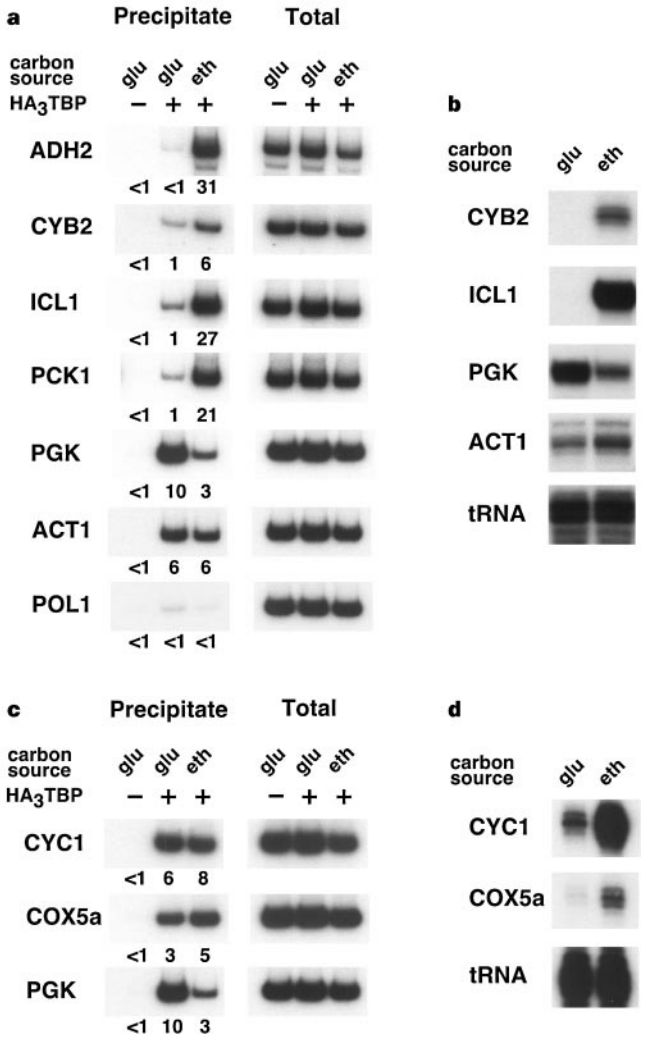

Figure 3 TBP occupancy at promoters induced during non-fermentative growth. **a**, **c**, Cells containing HA_3-tagged or untagged TBP were grown in synthetic complete medium containing 4% glucose as carbon source. Half of the culture was washed with medium lacking glucose and then transferred to medium containing 3% ethanol for 6 h (eth). TBP occupancy was analysed as for Fig. 1. **b**, **d**, Quantitative S1 analysis.

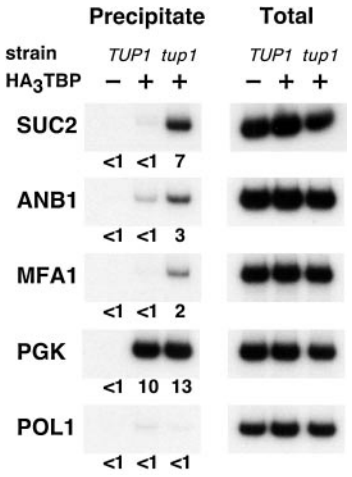

Figure 4 TBP occupancy at promoters repressed by the Cyc8–Tup1 corepressor complex. *TUP1* or *tup1*Δ::*LEU2* cells containing HA_3-tagged or untagged TBP were grown in YPD medium, and TBP occupancy was analysed as for Fig. 1.

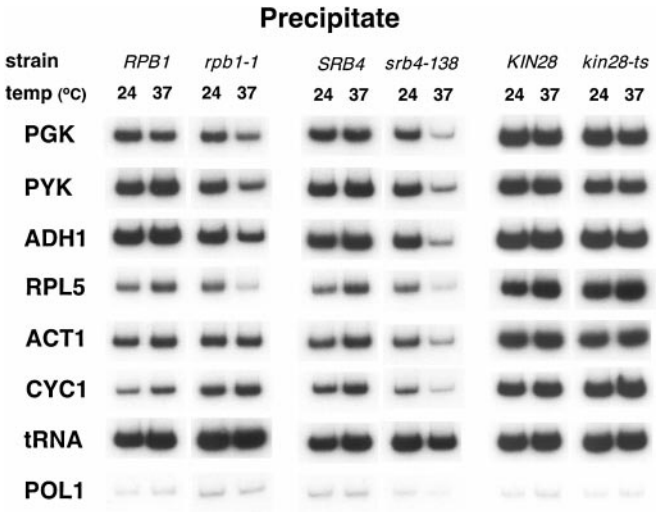

Figure 5 Role of general transcription factors for TBP occupancy at promoters. Isogenic wild-type or the indicated mutant cells (all containing untagged TBP) were grown in YPD medium and shifted to 37 °C, the restrictive temperature, for 1 h (or 75 min in the case of the *kin28* strain). PCR products corresponding to the indicated promoters, including tRNA, which responds to Pol III (or *POL1* structural gene), were generated from total chromatin (not shown) or DNA immunoprecipitated with antibodies against TBP.

the association of TFIID (the biologically relevant form of TBP) and the Pol II holoenzyme are concerted events. Although these events might be mechanistically and temporally distinct, it appears that neither TFIID nor the Pol II holoenzyme can individually associate stably with promoters *in vivo*. As a corollary, our results argue that biochemically stable subcomplexes (such as TBP–TFIIB–TATA), as well as functional preinitiation complexes corresponding to the minimal transcription machinery, are relatively unstable *in vivo*. As such, our results favour (although they do not prove) the idea that preinitiation complexes are formed by recruitment of a preassembled Pol II holoenzyme, rather than by stepwise recruitment of individual factors.

The dispensability of the Kin28 subunit of TFIIH for TBP occupancy suggests that, *in vivo*, phosphorylation of the C-terminal tail of Pol II occurs after the concerted (and presumably stable) association of TFIID and the Pol II holoenzyme. This would be consistent with the observations that Kin28-dependent phosphorylation of the Pol II tail occurs only on a preassembled complex[21] and that formation of the preinitiation complex requires unphosphorylated Pol II (ref. 22). Our results do not address whether TFIIH association occurs together with or subsequent to the association of the Pol II holoenzyme, although we note that TFIIH is not a component of the Pol II holoenzyme[23,24] and is not absolutely required for transcription under certain biochemical conditions[22]. The modest decrease in TBP occupancy in the *rpb1* strain is hard to interpret, although it suggests that the mutated Pol II is partially functional in preinitiation complex formation and defective, to some extent, at a later step such as promoter clearance or elongation.

Our results constitute direct physical evidence that the predominant mechanism by which activators stimulate (and repressors inhibit) transcription in yeast cells involves recruitment of the Pol II machinery to promoters. At more than 90% of the natural promoters we examined, there appear to be two states: a transcriptionally inactive state, in which TBP and hence the entire Pol II machinery are not associated, and an activator-dependent state, in which the level of transcription correlates with the amount of TBP occupancy. However, the concerted association of TFIID and the Pol II holoenzyme with promoters makes it impossible to determine whether activators directly recruit TFIID, Pol II holoenzyme, or both. In addition, activators could function by recruiting chromatin-modifying activities, thereby generating an altered chromatin structure that increases the association of TBP and Pol II holoenzyme. Finally, although recruitment is the predominant mechanism, TBP occupancy is not strictly limiting for activation of *CYC1* and *COX5a*. It may be that, in a small subset of promoters, the transcriptionally inactive state loosely resembles the situation in Kin28-deficient cells, in which it is presumed that TFIID and Pol II holoenzyme are associated, but a late step such as TFIIH recruitment or promoter clearance is defective; in this regard, TFIIB is associated with the *CYC1* and *COX5a* promoters in glucose medium (P. Kosa and K.S., unpublished observations). Although the detailed mechanisms remain to be determined, our findings explain observations suggesting that activators can function at distinct steps in yeast cells[25–27]. □

Methods

Yeast strains. The haemagglutinin (HA)-tagged TBP molecule contains three copies of the HA epitope inserted after codon 3 of the TBP open reading frame. For the experiment in Fig. 1, *URA3* centromeric plasmids expressing TBP or HA_3-TBP from the TBP promoter were introduced into BYΔ2 by plasmid shuffling[28]. Otherwise, *URA3* integrating plasmids containing N-terminal portions (residues 1–172) of TBP or HA_3-TBP were introduced into the *TBP* locus of W303-1A (Figs 2, 3) or FT5 (ref. 16) (Fig. 4) by integrative disruption. For the experiment in Fig. 5, the isogenic *RPB1* and *rpb1*-1 (ref. 19) and *KIN28* and *kin28*-ts16 (ref. 20) strains have been described; the *SRB4* and *srb4*-138 alleles[18] were present on centromeric plasmids in a strain deleted for the chromosomal locus.

Chromatin immunoprecipitation. Chromatin was prepared as described[11], with several modifications. Cells (400 ml) grown to an absorbance at 600 nm (A_{600}) of ~0.5 were treated with 1% formaldehyde for 20 min at room temperature, with occasional swirling. Glycine was added to a final concentration of 330 mM and the incubation continued for 5 min. Cells were collected, washed twice with cold TBS (20 mM Tris-HCl, pH 7.5, 150 mM NaCl), once with cold FA-lysis buffer (50 mM HEPES-KOH, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% sodium deoxycholate, 0.1% SDS, 1 mM PMSF), and resuspended in 1 ml cold FA-lysis buffer containing 0.5% SDS. An equal volume of glass beads (of diameter 0.5 mm) was added, and cells were disrupted by vortexing for 15 min on ice. The lysate was diluted into 8 ml FA-lysis buffer, and the glass beads were discarded as described[11]. The crosslinked chromatin was pelleted by centrifugation at 200,000*g* for 20 min, washed for 1 h with FA-lysis buffer, resuspended in 1.5 ml FA-buffer for 1 h at 4 °C, and sonicated to yield an

average DNA fragment size of 350 base pairs (bp) (range, 100–850 bp). Finally, the sample was adjusted to 8 ml with FA-lysis buffer, clarified by centrifugation at 200,00*g* for 20 min, and aliquots of the resultant chromatin solution were stored at −80 °C.

Chromatin solution (800 μl), adjusted to 275 mM NaCl, was incubated with 10 μl anti-HA monoclonal antibody (Figs 1–4) or 25 μl anti-TBP antibody (Fig. 5) coupled to protein-A–Sepharose beads. After 90 min at room temperature on a rotator, beads were washed twice for 4 min in 1.4 ml FA-buffer, twice in 1.4 ml FA-buffer with 500 mM NaCl, once in 1.4 ml of 10 mM Tris-HCl, pH 8.0, 0.25 M LiCl, 1 mM EDTA, 0.5% N-P40, 0.5% sodium deoxycholate, and once in 1.4 ml TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). Immunoprecipitated material was eluted from the beads by heating for 10 min at 65 °C in 400 μl of 25 mM Tris-HCl, pH 7.5, 10 mM EDTA, 0.5% SDS. To reverse crosslinks, samples were adjusted to 0.8 mg ml^{-1} Pronase and incubated for 1 h at 42 °C and for 5 h at 65 °C. After extraction with phenol–chloroform–isoamyl alcohol and chloroform, DNA was ethanol-precipitated overnight at −20 °C in the presence of 20 μg glycogen, and resuspended in TE buffer.

Quantitative analysis. Precipitated DNA was analysed by quantitative PCR using primer pairs (24–26mers with ~45% G+C content) for specific regions. PCR was first performed with decreasing amounts of template to determine the linear range for each combinant of primer sets and DNA. Typically, 1/100 to 1/500th of the immunoprecipitated DNA and 1/30,000 of the total DNA input were used. Reactions were carried out in 10 μl and contained 1 μM primers, 0.1 mM dNTPs and 0.1 mCi ml^{-1} of ^{32}P-dATP (specific activity, 3,000 Ci $mmol^{-1}$). Cycling was for 90 s at 94 °C, followed by 26 cycles (24 cycles in the case of the *CUP1* primers) with 30 s at 94 °C, 30 s at 55 °C, 1 min at 72 °C, then 4 min at 72 °C. PCR products (typically, 200–300 bp) were quantified by a Fujix BSA 2040 PhosphorImager. The fraction of immunoprecipitated material for a specific fragment was calculated by dividing the amount of PCR product obtained with immunoprecipitated DNA by the amount obtained with total DNA. The value obtained for the *PGK* promoter (0.9%) was arbitrarily defined as 10 units of promoter occupancy, and this value was used for direct comparison among experiments. We estimate that the overall error is ±30%, apart from values below 2 units, where the error is greater.

Received 18 February; accepted 8 April 1999.

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Acknowledgements. We thank M. Benson, J. Geisberg, M. Harrison and S. Reed for DNAs and yeast strains; L. Stargell for TBP antibodies; M. Green for communicating unpublished results; and O. Aparicio, S. Bell and J. Lis for discussion. This work was supported by postdoctoral fellowships to L.K. from the Association pour la Recherche sur le Cancer and the Human Frontier Science Program, and by research grants to K.S. from the NIH.

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