A TATA Binding Protein Mutant with Increased Affinity for DNA Directs Transcription from a Reversed TATA Sequence In Vivo

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The TATA-binding protein (TBP) nucleates the assembly and determines the position of the preinitiation complex at RNA polymerase II-transcribed genes. We investigated the importance of two conserved residues on the DNA binding surface of Saccharomyces cerevisiae TBP to DNA binding and sequence discrimination. Because they define a significant break in the twofold symmetry of the TBP-TATA interface, Ala100 and Pro191 have been proposed to be key determinants of TBP binding orientation and transcription directionality. In contrast to previous predictions, we found that substitution of an alanine for Pro191 did not allow recognition of a reversed TATA box in vivo; however, the reciprocal change, Ala100 to proline, resulted in efficient utilization of this and other variant TATA sequences. In vitro assays demonstrated that TBP mutants with the A100P and P191A substitutions have increased and decreased affinity for DNA, respectively. The TATA binding defect of TBP with the P191A mutation could be intragenically suppressed by the A100P substitution. Our results suggest that Ala100 and Pro191 are important for DNA binding and sequence recognition by TBP, that the naturally occurring asymmetry of Ala100 and Pro191 is not essential for function, and that a single amino acid change in TBP can lead to elevated DNA binding affinity and recognition of a reversed TATA sequence.

Transcription of protein-encoding genes in eukaryotes requires the assembly of a large, multiprotein complex at the promoter. This preinitiation complex contains RNA polymerase II, six different general transcription factors (TFIIA, TFII B, TFIID, TFIIE, TFIIF, and TFI IH), and a large number of accessory proteins that mediate the response to transcriptional activators and repressors (46). Of these proteins, TFIID is unique in its ability to bind DNA in a sequence-specific manner in the absence of other members of the preinitiation complex. Sequence recognition by TF IID is directed by the TATA binding protein (TBP) subunit. As the initial step in the assembly of the preinitiation complex, the binding of TFIID to the TATA box is a highly regulated event. In vivo and in vitro studies have shown that many transcriptional activators stimulate transcription by facilitating the rate-limiting binding of TFIID to the TATA box (35, 41, 48, 50, 67, 79), while certain transcriptional repressors, including histones, impede this step (4, 36, 41, 83).

As the central component of the general transcription machinery, the TBP-TATA box complex has been the subject of intense study. Computational studies have identified an 8-bp consensus sequence for the TATA box [TATA(A/T)A(A/T)N] (9). The importance of this sequence for transcriptional activity has been confirmed by systematic mutational studies (14, 25, 82). These previous experiments, along with crystallographic analyses (62), have demonstrated that base substitutions in the 5' half of the consensus sequence are particularly detrimental to binding by TBP. X-ray crystal structures have been solved for TBP-TATA complexes from diverse species and indicate a remarkable degree of conservation (20, 30, 32–34, 58, 59, 62, 77, 78). In each of these structures, the highly conserved carboxy-terminal core domain of TBP binds the 8-bp TATA sequence as a saddle-shaped molecule with a high degree of pseudodyad symmetry. The symmetry between the two subdomains of the saddle derives from two imperfect, direct repeats that dominate the TBP core domain. TBP binds the TATA box exclusively through minor-groove contacts that are predominantly hydrophobic in nature and by causing a dramatic distortion of the DNA.

For successful transcription of a gene, the preinitiation complex must assemble at the promoter in a way that leads to a properly positioned and oriented RNA polymerase II. X-ray crystallographic studies of all eukaryotic TBP-TATA complexes revealed a unique binding orientation for TBP in which the carboxy-terminal subdomain of the TBP saddle contacts the 5' half of the TATA box and the amino-terminal subdomain contacts the 3' half of the TATA box (20, 30, 32–34, 58, 59, 62, 77, 78). This orientation has also been observed in vivo (3) and is required for the directional assembly of the preinitiation complex and transcription initiation (20, 59, 77, 78). Because of the approximately twofold rotational symmetry of TBP and certain TATA sequences, the underlying basis for the observed orientation remains unclear. Molecular dynamics studies have revealed no unfavorable contacts if TBP is forced to bind in the opposite orientation (55), and there is very little chemical distinction between a T-A base pair and an A-T base pair in the minor groove (71). In fact, experiments designed to assess the directionality of TBP binding on the adenovirus major late promoter (AdMLP) TATA box and the Saccharomyces cerevisiae CYC1 TATA box in solution have demonstrated that TBP is capable of bidirectional binding in vitro but still exhibits a modest preference (60:40 ratio) for the orientation observed in the crystal structures (16). Addition of
TFIIB and TFIIB significantly increases the preference (to approximately 95%) for the “correct” orientation (31).

Several hypotheses invoking the intrinsic properties of TBP and the TATA box have been proposed to explain the binding orientation detected by crystallography and preferred in solution. The proposed sources of directional binding include asymmetric deformability of the two subdomains of TBP and the two halves of the TATA box (22, 32, 34, 62, 76), small electrostatic charge differences between the DNA binding surfaces of the TBP subdomains (34), and slight breaks in the symmetry of the amino acid-base contacts along the TBP-DNA interface (30, 33). One prominent break in the pseudodyad symmetry of the TBP-DNA interface involves a highly conserved proline residue, Pro191 of yeast TBP, which directly contacts base A1' of the TATA box (position A1' denotes the A of the first T·A base pair) (Fig. 1) (30, 32–34, 58, 62). The side chain of the equivalently positioned residue in the amino-terminal repeat, Ala100, makes no contact with the 3' end of the TATA box. Juo et al. proposed that this proline-alanine asymmetry plays a dominant role in establishing the binding orientation of TBP (30). According to the “proline clash” hypothesis, Pro191 effectively determines binding orientation because its side chain would sterically clash with bases other than an A at position 1' (30). The small side chain of alanine imposes no restrictions on base pair 8, and therefore this position of the TATA sequence is the least well conserved.

To test the importance of Pro191 in dictating TBP binding orientation, we mutated this residue to an alanine and tested the ability of the mutant TBP (TBP-P191A), to direct transcription in vivo from a TATA sequence of reversed orientation. We also generated a TBP mutant with the reciprocal change, A100P (TBP-A100P). Our results suggest that Pro191 and Ala100 influence TATA box recognition by TBP, although not by the mechanism proposed. Whereas TBP-A100P efficiently utilized the reversed TATA sequence, TBP-P191A did not. We show that a TBP mutant, TBP-A100P, with two prolines on the DNA binding surface functions dominantly to wild-type TBP, has increased affinity for DNA in vitro, and efficiently directs transcription from core promoter and variant TATA sequences, including a reversed TATA sequence, in vivo.

MATERIALS AND METHODS

Media and yeast strains. The S. cerevisiae strains used in this study are listed in Table 1. All KY strains were derived from FY2 (MATa ura3-52, a GAL2 derivative of S288C (81). Standard methods of mating, sporulation, tetrad dissection, and transformation were used (68). Rich medium (yeast extract-peptone-dextrose), synthetic complete (SC) medium, and synthetic minimal (SD) medium were made as described previously (68). YP succinate medium contained 2% sucrose and 1 μg of ampicillin A/mL. The aminotriazole media included SD medium with the indicated concentrations of 3-amino-1,2,4-triazole (AT; Sigma) and all necessary amino acids.

Yeast strains containing variant HIS3 promoters were constructed as previously described (3, 25). Briefly, complementary pairs of oligonucleotides (Table 2) were designed to contain the desired TATA box, its flanking sequence, and terminal 5' EcoRI and 3' SacI restriction sites. After annealing, the duplex fragment was inserted at the EcoRI and SacI sites of a URA3-marked, integrating plasmid, thereby replacing the regulatory TATA sequence upstream of the HIS3 gene. The constitutive HIS3 TATA element had been deleted in these constructs; however, the Gcn4 binding site and poly(dA·dT) sequence were retained.

TABLE 1. S. cerevisiae strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype*</th>
</tr>
</thead>
<tbody>
<tr>
<td>KY423</td>
<td>MATa his3-Δ290 ura3-52 trp1Δ63</td>
</tr>
<tr>
<td>KY430</td>
<td>MATa his3-250(CTTTTATAG) trp1Δ63 ura3-52</td>
</tr>
<tr>
<td>KY787</td>
<td>MATa his3-250(CTTTTATAG) hy2-128 leu2Δ1 ura3-52 trp1Δ63 sp1Δ102::LEU2</td>
</tr>
<tr>
<td>KY788</td>
<td>MATa his3-250(CTTTTATAG) hy2-128 leu2Δ1 ura3-52 trp1Δ63 sp1Δ102::LEU2</td>
</tr>
<tr>
<td>KY789</td>
<td>MATa his3-252(CATATAAG) trp1Δ63 ura3-52</td>
</tr>
<tr>
<td>KY909</td>
<td>MATa his3-251(TATAAAG) trp1Δ63 ura3-52</td>
</tr>
<tr>
<td>KY791</td>
<td>MATa his3-253(TATAGAG) trp1Δ63 ura3-52</td>
</tr>
<tr>
<td>KY792</td>
<td>MATa his3-254(TATAGAG) trp1Δ63 ura3-52</td>
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<tr>
<td>KY793</td>
<td>MATa his3-255(CTTTTATAG) ura3-52 trp1Δ63 sp1Δ102::LEU2</td>
</tr>
<tr>
<td>KY794</td>
<td>MATa his3-255(CTTTTATAG) ura3-52 trp1Δ63 sp1Δ102::LEU2</td>
</tr>
<tr>
<td>YY1</td>
<td>MATa his3-4976 lys2-128 leu2Δ1 ura3-52 trp1Δ63 stc2Δ1Δ51::(−1900)−390 sp1Δ102::LEU2</td>
</tr>
</tbody>
</table>

* his3 alleles 250 to 255 have variant TATA elements, shown in parentheses, directing transcription of a wild-type HIS3 open reading frame. These promoter mutants are integrated at the normal HIS3 locus, replacing the wild-type gene.
The final plasmids contained a single TATA element positioned 23 bp downstream of the Gcn4 binding site and 83 bp upstream of the transcription initiation site. Plasmids were confirmed by DNA sequencing, linearized with XhoI, and transformed into KY423. After a two-step gene replacement (70), His+ recombinants were identified and proper integration of the plasmid was confirmed by Southern analysis. The following oligonucleotides (Table 2) and integrating plasmids were used to generate strains harboring variant HIS3 promoters (the top strand of the 8-bp TATA box is indicated from 5′ to 3′): CTTTATA (his3-250), KA142, and pJVS50; TATAAAAA (his3-251), JVS50, JVS51, and pJVS73; CATAAAAG (his3-252), JVS44, JVS45, and pJVS66; TATAAGAG (his3-253), JVS54, JVS55, and pJVS75; TATAAGGT (his3-254), JVS65, JVS66, and pJVS76; TATAATAG (his3-255), JVS66, JVS67, and pJVS77; and CATAAAAG (his3-256), JVS44, JVS45, and pJVS66.

**Plasmid construction.** Plasmid construction was performed using standard cloning techniques (5). Plasmids pJVS49 (for TBP-A100P) and pJVS48 (for TBP-P191A) were made by transforming KY423. After a two-step gene replacement (70), His+/H11001 plasmids were used to generate strains harboring variant promoters (the top strand of the 8-bp TATA box is indicated from 5′ to 3′): CTTTATA (his3-250), KA142, and pJVS50; TATAAAAA (his3-251), JVS50, JVS51, and pJVS73; CATAAAAG (his3-252), JVS44, JVS45, and pJVS66; TATAAGAG (his3-253), JVS54, JVS55, and pJVS75; TATAAGGT (his3-254), JVS65, JVS66, and pJVS76; TATAATAG (his3-255), JVS66, JVS67, and pJVS77; and CATAAAAG (his3-256), JVS44, JVS45, and pJVS66.

**Protein purification and DNA binding assays.** TBP, TBP-A100P, TBP-P191A, and TBP-A100P, P191A were purified from *E. coli* BL21(DE3) expression strains essentially as described previously (64). In all cases, the TBPAs were approximately 95% pure, as judged by Coomassie blue staining of sodium dodecyl sulfate (SDS)-acrylamide gels. DNase I protection and electrophoretic mobility shift assays (EMSAAs) were performed with a 32P-labeled DNA probe from plasmid pHW and containing the AdMLP TATA box as described previously (10, 18).

**Ethylation interference footprinting** was performed as described previously (47). Briefly, 5 nmol of complementary oligonucleotides pJVS88 and JVS99 (Table 2) were individually ethylated in 200-μl reaction mixtures with 100 μg of N-ethyl-N-nitrosourea (Sigma) in 25 mM cacodylate, pH 8.0. A 200-μg/ml stock solution of N-ethyl-N-nitrosourea was prepared in 100% ethanol. After ethylation, the DNA was dialyzed into water and then end labeled using T4 polynucleotide kinase and [γ-32P]ATP. Ethylated, kinase oligonucleotides were purified over NENSORB columns (DuPont) according to the manufacturer’s directions. Complementary oligonucleotides, only one of which was ethylated and radiolabeled, were annealed in 20-μl reaction mixtures containing 0.2 M NaCl and 10 mM Tris-Cl, pH 7.5, by heating the reaction mixtures in a water bath to 95°C for 30 min and then allowing them to cool slowly overnight. EMSAs were performed with bacterially expressed wild-type and mutant TBPs as described above. Bands corresponding to bound and unbound DNA were excised from the gel, and the DNA was eluted in 0.5 M NaCl and purified over NENSORB columns. Cleavage of the DNA was carried out in 10 mM sodium phosphate (pH 7.0)–1 mM EDTA–150 mM NaOH at 90°C for 30 min. After cleavage, the oligonucleotides were analyzed on a 20% polyacrylamide–8.3 M urea sequencing gel with a 2-in. 8% polyacrylamide–8.3 M urea cushion.

**β-galactosidase assays.** Strain KY787 was transformed with plasmids pKA75 (SP5T’/TRP/CEN/ARS/2) and pJVS49, and Trp’ transformants were selected, purified, and streaked to medium containing 5-fluoroorotic acid (5-FOA) to select against the PDE28-6 plasmid. The resulting Ura- strains were transformed with one of the following URA3-marked lacZ reporter plasmids: pLGA-178 (cyl1/ΔAS-lacZ [23]), pLG669-Z, (Cyl1-lacZ [24]), pM740 (hoa1/ΔAS-lacZ [29]), and p1701 (HO-lacZ, obtained from David Stillman). Transformants were grown to a concentration of 1 × 107 to 2 × 107 cells/ml in SC medium lacking uracil and tryptophan (SC-ura-Trp). Cells were harvested, extracts were prepared, and β-galactosidase assays were performed as described previously (56, 65). Protein concentrations were determined by the method of Bradford (8). β-galactosidase unit calculations were done as described previously (56).
from 107 cells was loaded in each lane of an SDS-polyacrylamide gel. Following electrophoresis, proteins were transferred to nitrocellulose and processed by standard procedures for immunoblotting (26). Anti-TBP polyclonal antiserum was the gift of Steve Buratowski. The secondary antibody was donkey anti-rabbit immunoglobulin G conjugated to horseradish peroxidase (American Biosystems). Immunoblots were developed with a chemiluminescent detection system (Amersham). Mot1 and TAND interaction assays. Experiments to test the ability of purified Mot1 (provided by David Auble) to disrupt TBP-DNA complexes were performed with reaction mixtures containing 2 ng of wild-type or mutant TBP and the TATA-containing 32P-labeled probe from PRW as described previously (17). Where indicated, ATP was added to a final concentration of 5 mM. Glutathione S-transferase (GST)–Taf1 N-terminal domain (TAND) fusion proteins were expressed in E. coli from previously described plasmids (37) and prepared and quantitated by published procedures (39). GST pull-down assays were performed as described previously (39).

**RESULTS**

TBP-A100P directs transcription from a reversed TATA box. To test the involvement of Pro191 and Ala100 of yeast TBP in the regulation of the directionality of transcription, plasmids expressing TBP-P191A, TBP-A100P, and the double mutant TBP-A100P, P191A were transformed into the yeast strain KY788. In this strain, transcription of the chromosomal HIS3 gene is driven by a TATA box of reversed orientation (RTATA box) with the sequence 5′CCCTTATATAGG3′ (the underlined sequence denotes the reversed orientation of a consensus TATA box, TATAAAAG). Recognition of the RTATA sequence maintains the transcriptionally relevant orientation of TBP with respect to the HIS3 gene (Fig. 2), a requirement imposed by the asymmetric positioning of the other general transcription factors and RNA polymerase II in a manner necessary for HIS3 transcription. C and N refer to the carboxy- and amino-terminal subdomains of the TBP saddle, respectively.

Phenotypes included slightly impaired growth on galactose, sucrose, and raffinose media and weak temperature sensitivity for growth at 37°C.

To confirm and quantify the transcriptional effect at HIS3, S1 nuclease protection assays were performed on strains that express the TBP mutants in the presence of endogenous wild-type TBP. RNA was prepared from cells that were grown in SD medium that maintained selection for the TBP-expressing plasmid. Relative to a strain expressing only wild-type TBP, a strain expressing TBP-A100P showed a twofold increase in HIS3 mRNA levels, providing a transcriptional explanation for the increased AT resistance (Fig. 3B). Strains expressing TBP-P191A and TBP-A100P, P191A showed HIS3 mRNA levels that were comparable to and 1.3-fold higher than that of the wild type, respectively. DED1, a constitutively expressed RNA polymerase II transcript, and SNR6, a stable RNA polymerase III transcript, were used as internal loading controls for this experiment (Fig. 3B and data not shown). Taken together with our phenotypic results, these data suggest that Pro191 and Ala100 contribute in important ways to TATA site recognition.

To address the possibility that altered TBP levels were affecting TATA utilization, Western blot analysis of strains expressing a chromosomal HA3-tagged version of wild-type TBP and a plasmid-expressed copy of TBP, TBP-A100P, TBP-P191A, or TBP-A100P, P191A was performed. These strains showed no significant difference in TBP levels (Fig. 3C). In addition, overexpression of wild-type TBP from a 2-μm plasmid in an RTATA-HIS3 strain did not lead to a level of AT resistance greater than that of the vector control strain (data not shown). These results indicate that TBP levels do not play a role in determining the level of AT resistance observed in these strains.
TBP-A100P does not recognize a cryptic TATA box within the TATA sequence. Embedded in the RTATA sequence is a potentially cryptic TATA box (CTTTTATAGGGT). The 5' end of this element contains the consensus TATA sequence, while the 3' end differs extensively from the preferred AAAAN. However, the deformability of the 5' end of the TATA box has been proposed as a key determinant of TBP binding directionality (22), and binding of yeast TBP to a similar nonconsensus TATA box, TATAGGGT, has been observed in vitro (49). Therefore, we used two different strategies to investigate the possibility that TBP-A100P recognized this sequence instead of the RTATA box. First, we substituted the sequence CTATTAGGGT for the RTATA sequence upstream of the HIS3 gene and assayed expression of this reporter gene by growth of yeast strains on AT medium. The sensitivity of this in vivo test is high, because transcription from a very poor TATA box can be detected as growth on low concentrations of AT (25, 82). As shown in Fig. 4A, neither TBP nor TBP-A100P used this TATA sequence sufficiently to allow growth on 10 mM AT. This finding contrasts with the results of our experiments involving the full RTATA box construct (Fig. 3A) and strongly suggests that TBP-A100P recognizes the RTATA sequence.

To determine the precise position of TBP on an RTATA-containing DNA probe, we performed ethylation interference footprinting with recombinant TBP and TBP-A100P. Complementary 26mer oligonucleotides, JVS58 and JVS59, encoding the RTATA sequence, CTTTTATAGGGT, were individually ethylated and radiolabeled as described in Materials and Methods. Ethylation of phosphate groups that are critical for the TBP-TATA interaction prevents binding by TBP, and the corresponding DNA fragments are excluded from the TBP-bound population of an EMSA. Since the ethyl groups project about 4.5 Å from the phosphate (54), this procedure allows fine structure mapping of the TBP-TATA complex. The position of the ethylation interference footprint of TBP-A100P on both strands was similar to that of wild-type TBP (Fig. 4B and data not shown), indicating that both proteins protected the same DNA sequence, CTTTTATA. Taken together, these data suggest that TBP-A100P recognizes and directs transcription from a reversed TATA sequence in vivo.

TBP-A100P directs transcription from several variant TATA sequences. We also tested TBP-A100P for its ability to recognize other near-consensus TATA boxes. Plasmids encoding TBP and TBP-A100P were transformed into strains that expressed an endogenous, wild-type copy of SPT15 and a HIS3 gene that was driven by one of three variant TATA boxes, CATAAAAG, TATAAGAG, or TATAATAG. In previous studies of wild-type TBP, similar TATA boxes supported a range of in vitro transcription activities from approximately 10% (TATAATGT) to 30% (CATAAAAGT) to 58% (TATAAGGT) relative to TATAATAGG (82). For all of the sequences tested, TBP-A100P supported a higher level of HIS3 expression than did wild-type TBP (Fig. 5). Similar results were observed for variant TATA boxes in two different flanking sequence contexts (data not shown). These results suggest that usage of the RTATA sequence by TBP-A100P stems from a general change in TBP activity rather than from a specific and restricted alteration in sequence recognition.
A100P and P191A substitutions alter the DNA binding affinity of TBP. Since the A100P and P191A substitutions change residues on the DNA binding surface of TBP (32–34), we explored the possibility that the in vivo effects of the TBP mutants were due to an alteration in DNA binding. To test the DNA binding activities of the mutant TBPs, DNase I footprinting and EMSAs were performed with bacterially expressed and purified TBP, TBP-A100P, TBP-P191A, and TBP-A100P, P191A. A probe containing the AdMLP TATA box (TATA-AAAG) was used in these experiments. Quantitation of the DNase I footprinting and EMSA results showed that TBP-A100P had approximately twofold higher affinity for the TATA-containing probe than did wild-type TBP (Fig. 6 and data not shown). In contrast, TBP-P191A exhibited a striking defect in TATA box binding, indicating that this highly conserved proline residue plays a critical role in the formation or stabilization of the TBP-TATA complex (Fig. 7A). X-ray crystallographic studies have demonstrated that proline 191 in yeast TBP makes direct contact with the TATA box (Fig. 1) (32–34). Addition of recombinant TFIIA strongly suppressed the DNA binding defect of TBP-P191A, indicating that the recombinant protein was not denatured or otherwise rendered inactive by our purification procedure (data not shown). Similar suppressive effects of TFIIA have been reported for other TBP mutants defective in DNA binding (43, 52). Like several previously described TBP mutants (2, 45), TBP-P191A supports viability despite a severe TATA box binding defect in vitro. Interestingly, the double-mutant protein, TBP-A100P, P191A, bound the AdMLP TATA probe with nearly wild-type affinity (Fig. 7B). Therefore, replacement of Ala100 with a

### FIG. 4. TBP-A100P does not use a cryptic TATA box within the RTATA promoter. (A) A HIS3 gene driven by the sequence TATAGGGT was integrated at the chromosomal HIS3 locus to generate strain KY794 as described in Materials and Methods. KY794 was transformed with plasmids pKA75 and pJVS49 expressing TBP and TBP-A100P, respectively. Two independent transformants for each plasmid were replica plated to selective medium containing 0 or 10 mM AT. Plates were photographed after 2 days of incubation at 30°C. Longer times of incubation did not lead to significant levels of growth or reveal differences between the transformants. (B) Ethylation interference footprints of TBP and TBP-A100P on the RTATA box sequence. Oligonucleotide JVS58 (Table 2) was ethylated, radiolabeled, and annealed to oligonucleotide JVS59. Recombinant TBP or TBP-A100P was incubated with the annealed probe, and the reaction mixtures were resolved by EMSA. DNA fragments from the TBP-bound (B) and unbound (U) populations were excised from the EMSA gel, eluted, and cleaved as described in Materials and Methods. Products of the cleavage were resolved on a denaturing 20% polyacrylamide gel. Lane 2 contains the cleavage products of a naked DNA sample. Lane 1 contains 32P-labeled oligonucleotide size markers JVS67 (A; 23 nucleotides [nt]), JVS66 (B; 17 nt), JVS65 (C; 13 nt), and JVS64 (D; 10 nt).
proline intragenically suppressed the severe DNA binding defect of TBP-P191A. These results indicate that the presence of a proline at either position 100 or 191 and an alanine at the other position is sufficient for wild-type levels of DNA binding in vitro.

TBP-A100P confers a Bur+/H11546 phenotype but interacts with the negative regulators Mot1 and TAND. In genetic screens and selections for mutations that bypass the transcriptional requirement for an upstream activation sequence (Bur+/H11002 phenotype), mutations in several genes related to TBP function, including SPT15 itself, have been identified (11, 65, 66). Included among these genes are those that encode inhibitors of the TBP-TATA interaction, such as histones and Mot1. TBP-A100P has increased affinity for TATA-containing DNA in vitro and could potentially counter the effects of these negative regulators in vivo. Therefore, we tested whether TBP-A100P could bypass the transcriptional requirement for the upstream activation sequences of SUC2, CYC1, and HO.

TRP1-marked plasmids expressing wild-type TBP and TBP-A100P were transformed into strain YY1 which contains a chromosomal copy of SUC2, suc2ΔUAS(−1900/+390), lacking −1,500 bp from the SUC2 promoter, including the entire UAS (11, 69). A URA3-marked plasmid expressing wild-type TBP was evicted from these transformants by growth on medium containing 5-FOA. Expression of the suc2ΔUAS gene was measured as growth on medium containing sucrose as the sole carbon source. As shown in Fig. 8A, strains containing wild-type TBP failed to grow on sucrose media. Previous studies
showed that this Suc− phenotype correlates with a dramatic defect in SUC2 transcription (66). In contrast, a strain containing TBP-A100P and suc2ΔUAS−1900/−390 grew well on sucrose media (Fig. 8A).

To test the ability of TBP-A100P to direct transcription from other core promoters, plasmids bearing UAS-less derivatives of the CYC1 and HO genes, cyc1ΔUAS-lacZ and ho2ΔUAS-lacZ, were transformed into strains containing wild-type TBP or TBP-A100P as the sole TBP source, and the β-galactosidase levels were measured. TBP-A100P supported a small but statistically significant increase in β-galactosidase levels from both reporter genes (Fig. 8B). Therefore, the increased DNA binding affinity of TBP-A100P correlates with increased expression from three different core promoters in vivo.

In two earlier studies, TBP mutants with amino acid substitutions on the DNA binding surface were found to be defective in supporting activated transcription (2, 45). Therefore, we asked whether TBP-A100P also had an activation defect. Strains expressing TBP and TBP-A100P were transformed with CYC1-lacZ and HO-lacZ reporter plasmids containing UAS elements and were assayed for β-galactosidase activity. TBP-A100P and TBP directed similar levels of expression for both reporter genes, suggesting that TBP-A100P is not generally activation defective (Fig. 8C).

The ability of TBP-A100P to drive transcription from core promoters and sub-optimal TATA sequences in vivo may arise from several factors, not limited to the greater DNA binding affinity of the mutant TBP. For example, TBP-A100P might be defective in its interaction with inhibitors of the TBP-TATA interaction. We tested this possibility for two different negative regulators, Mot1 and the TAND region of Taf1. Using the energy provided by ATP hydrolysis, Mot1 can disrupt TBP-TATA complexes and the products of this reaction can be visualized by EMSA (4). In a manner dependent on ATP, TBP-A100P–DNA complexes were as susceptible as wild-type TBP-DNA complexes to disruption by Mot1, indicating that Mot1 interacts effectively with the mutant TBP (Fig. 8D).

TAND has been shown to inhibit formation of the TBP-TATA complex by interacting with amino acids on the DNA binding surface of TBP (37). Using a GST pull-down assay, we found that TBP and TBP-A100P interacted to approximately the same extent with a GST-TAND derivative, GST-TAND(10-88), which has previously been reported to bind TBP (Fig. 8E) (37). Neither TBP nor TBP-A100P was able to interact with GST alone (data not shown) or a truncated TAND derivative, GST-TAND(10-58). Therefore, these results suggest that the transcriptional effects of TBP-A100P in vivo are not due to impaired interactions with at least two transcriptional repressors, Mot1 and TAND.

**DISCUSSION**

**Properties of the TBP-A100P and TBP-P191A mutants.** In this study, we investigated the importance of two highly conserved amino acids in yeast TBP, Ala100 and Pro191, for TATA box binding and discrimination. These two residues occupy equivalent positions on the DNA binding surface of TBP and represent one of the few breaks in the twofold symmetry of the TBP-DNA interface. Accordingly, these residues were previously proposed to govern the binding orientation of TBP and the directionality of transcription (30). By constructing and analyzing TBP mutants in which Ala100 was changed to a proline and Pro191 was changed to an alanine, we have shown that these residues do indeed play important roles in DNA binding and sequence recognition. However, contrary to previous predictions (30), TBP-P191A cannot efficiently direct transcription from a TATA box of reversed orientation. Surprisingly, TBP-A100P can utilize the RTATA sequence and several other variant TATA boxes with an efficiency greater than that of the wild type. DNA binding assays and measurements of transcription from core promoters strongly suggest that TBP-A100P has increased affinity for DNA in vivo.

TBP-A100P appears to represent a novel class of TBP point mutant, having increased affinity for a consensus TATA box and relaxed specificity for a number of near-consensus TATA sequences. While most previously described TBP mutants have reduced activity, a number of gain-of-function mutants have been reported (1, 7, 11, 21, 28, 75). Two studies have described dominantly acting TBP mutants that specifically enhance transcription from weak or repressed RNA polymerase II promoters (7, 21). Unlike TBP-A100P, these mutants cannot support cell viability when present as the only source of TBP and they have reduced (21) or wild-type affinity for DNA in vitro (7). Other gain-of-function TBP mutants are disrupted in their interactions with negative regulators of preinitiation complex assembly (11) or in dimerization (28). Two mutants with altered DNA binding specificity have been characterized (1, 75). However, these mutants are not broadly relaxed in their sequence recognition properties and do not exhibit elevated affinity for consensus TATA sequences in vitro (1, 3, 75).

The increased DNA binding affinity of TBP-A100P could arise through several mechanisms that are not mutually exclusive. Introduction of a proline side chain on the DNA binding surface of TBP may create a new amino acid-base contact that makes favorable energetic contributions to the stability of the complex. Alternatively, the presence of a proline at position 100 may significantly alter the conformation of the amino-terminal TBP stiirrup or the ability of TBP to bend the TATA box. Ala100 is immediately preceded by Phe99, one of the two phenylalanines that intercalate between base pairs 7 and 8 and introduce a dramatic kink at the 3′ end of the TATA box (34). Since prolines are known to restrict the possible ϕ and ψ angles of the preceding amino acid (53), a proline at position 100 could significantly impact, and perhaps favor, intercalation by Phe99. Previous studies have demonstrated enhanced affinity of TBP for prebent DNA (61) and correlations between TATA box bending and TBP-TATA complex stability (73) or transcriptional activity (84).

The DNA binding surface of TBP also serves as the interaction site for proteins that regulate transcription. In particular, the TAND region of Taf1 and the mammalian counterpart of Mot1, TAF170, have been shown to modulate the DNA binding activity of TBP by interacting with amino acids on the TBP-TATA interface (37, 63). While defective interactions with these negative regulators could lead to increased transcription from weak or core promoters by TBP-A100P, we found that TBP-A100P interacted as well as wild-type TBP with Mot1 and TAND in vitro. The DNA binding surface of TBP can also engage in dimer formation with a second molecule of TBP in a way that sequesters amino acids needed for
TBP-A100P bypasses the requirement for UAS elements. (A) CEN/ARS plasmids expressing TBP (pKA75) or TBP-A100P (pJVS49) were transformed into the suc2ΔUAS strain YJ1. Following 5-FOA-mediated eviction of the SPT15-containing plasmid pDE28-6, transformants were replica plated to YPSuc medium. Plates were incubated at 30°C for 2 days prior to photography. (B and C) Strain KY787 was transformed with plasmid expressing TBP (pKA75) or TBP-A100P (pJVS49), and transformants were plated on 5-FOA medium to evict plasmid pDE28-6. The resulting strains were transformed with the reporter plasmid hoxΔUAS-lacZ (p740), cyc1ΔUAS-lacZ (pLGA-178), HO-lacZ (pL701), or CYC1-lacZ (pLG669-Z), as indicated. Cultures were grown, and β-galactosidase assays were performed as described in Materials and Methods. Mean values of β-galactosidase activity from three independent transformants assayed at two different extract concentrations are presented. Hatched bars, wild-type TBP; solid bars, TBP-A100P. Note the differences in scales. Standard errors were less than 15% of the mean and are depicted as error bars. (D) Mot1 disruption of TBP-DNA complexes. TBP-DNA complexes, containing wild-type TBP or TBP-A100P, were incubated with TATA recognition (13). However, using an in vitro protein cross-linking assay (15), we found that TBP-A100P and wild-type TBP formed dimers at equivalent levels (data not shown). Moreover, on the crystal structure of a TBP dimer, Ala100 does not participate directly in the TBP-TBP interaction (13). While these results lead us to favor models in which the A100P substitution directly influences TATA box binding or bending, we cannot rule out the possibility that this substitution also alters potential intramolecular interactions involving a domain of TBP, such as the amino-terminal domain, that represses DNA binding (40, 44) or interactions with other negative regulators in vivo.

Whereas the substitution of a proline for Ala100 significantly elevated the DNA binding affinity of TBP, substitution of an alanine for Pro191 had the opposite effect. TBP-P191A was severely impaired in its ability to bind the AdMLP TATA box in vitro and was unable to direct transcription from the RTATA reporter in vivo. Crystallographic studies of TBP-TATA complexes have shown that Pro191 makes direct contact with the first base pair of the TATA box (34). The reduced DNA binding affinity of TBP-P191A may simply arise from the elimination of this contact. Alternatively, because Pro191 is located immediately C terminal to Phe190, which together with Phe207 intercalates between the first two base pairs of the TATA box (34), the P191A substitution may significantly alter the ability of TBP to bend the TATA box. Strikingly, the severe DNA binding defect of TBP-P191A could be intragenically suppressed by changing Ala100 to a proline. This result indicates that TBP can bind a TATA box with near wild-type affinity if it contains a proline at either position 100 or 191 and an alanine at the other position; the naturally occurring configuration is not essential for DNA binding (Fig. 7), transcriptional activity in vivo (Fig. 3), or cell viability (data not shown).

Insights into directionality of TBP binding. Originally, we were interested in testing the hypothesis that Pro191 positions TBP on the TATA box in the proper orientation (30). Juo et al. (30) postulated a dominant role for Pro191 in the establishment of TBP binding orientation because of steric constraints imposed by the proline side chain on interacting bases. Because the side chain of proline would sterically clash with the functional groups of T, G, or C, the most energetically favorable situation places proline in contact with an A residue, as observed for Pro191 and base A1’. Replacement of this proline with an alanine would be expected to eliminate these restrictions and lead to altered recognition of reversed and perhaps other variant TATA elements. Surprisingly, the TBP-P191A mutant protein exhibited no preference for the RTATA sequence relative to wild-type TBP, nor for AT-rich TATA elements. These results indicate that TBP can bind TATA boxes with near wild-type affinity if it contains a proline at either position 100 or 191 and an alanine at the other position; the naturally occurring configuration is not essential for DNA binding (Fig. 7), transcriptional activity in vivo (Fig. 3), or cell viability (data not shown).
discrimination, Pro191, in the absence of a proline at position 100, is critical for promoting or stabilizing the TBP-TATA interaction. Further, we observed significant utilization of the RTATA reporter only when prolines, not alanines, occupied both positions 100 and 191. In effect, the A100P substitution changed the amino-terminal subdomain of TBP to resemble the carboxy-terminal subdomain in both sequence and function. Whether the proline introduced at position 100 also makes direct contact with the TATA sequence is not known. Moreover, since we do not know the exact positioning of TBP-A100P on the RTATA sequence in vivo, we cannot readily predict which base pair would be contacted by this proline or whether this interaction establishes the binding register.

The TBP of the archaeal species Pyrococcus woesei naturally contains two proline residues on its DNA binding surface at positions analogous to 100 and 191 of S. cerevisiae TBP. Interestingly, the first crystal structure of P. woesei TBP that was bound to its TATA-like sequence (box A) revealed a binding orientation that was reversed relative to all known eukaryotic TBP-TATA complex structures (38). Further analysis revealed that the archaeal TFIIB orthologue, TFB, could reverse the binding orientation of P. woesei TBP to that seen in the eukaryotic structures (51) and allow unidirectional transcription (6). This effect was dependent on the presence of a TFB recognition element, or BRE, on the DNA template immediately 5' to the TATA element. The BRE was originally identified as a recognition sequence for human TFIIB (42). Consistent with these observations, solution studies of TBP binding orientation demonstrated a significant involvement of TFII in the establishment of directionality (31). The sequence 5' to the RTATA box does not closely resemble the human or archaeal BREs, and a BRE has yet to be identified for S. cerevisiae RNA polymerase II promoters (R. Ebright, personal communication). Therefore, we propose that the promoters used in our analysis lacked a functional BRE and consequently allowed us to detect expression from the RTATA sequence. Interestingly, in a different study, TBP-A100P was identified in a genetic screen for suppressors of a yeast TFIIIB mutant defective in DNA binding (9a).

In summary, using a genetic approach, we investigated the contributions of two amino acids in yeast TBP to transcription directionality in vivo. Our results indicate that Pro191 and Ala100, which define a significant break in the symmetry of the TBP-TATA interface, play crucial roles in facilitating DNA binding and determining specificity. Consistent with the modest intrinsic preference of wild-type TBP for the transcriptionally relevant binding orientation, our results indicate that a single amino acid change on the DNA binding surface of yeast TBP can allow recognition of a reversed TATA sequence. Further genetic studies employing reporter constructs such as those described here may uncover additional regions of TBP that govern the directional assembly of the preinitiation complex.

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