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The Yeast TAF145 Inhibitory Domain and TFIIA Competitively Bind to TATA-Binding Protein

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The *Drosophila* 230-kDa TFIID subunit (dTAF230) interacts with the DNA binding domain of TATA box-binding protein (TBP) which exists in the same complex. Here, we characterize the inhibitory domain in the yeast TAF145 (yTAF145), which is homologous to dTAF230. Mutation studies show that the N-terminal inhibitory region (residues 10 to 71) can be divided into two subdomains, I (residues 10 to 37) and II (residues 46 to 71). Mutations in either subdomain significantly impair function. Acidic residues in subdomain II are important for the interaction with TBP. In addition, yTAF145 interaction is impaired by mutating the basic residues on the convex surface of TBP, which are crucial for interaction with TFIIA. Consistently, TFIIA and yTAF145 bind competitively to TBP. A deletion of the inhibitory domain of yTAF145 leads to a temperature-sensitive growth phenotype. Importantly, this phenotype is suppressed by overexpression of the TFIIA subunits, indicating that the yTAF145 inhibitory domain is involved in TFIIA function.

Transcription factor TFIID is a multisubunit protein complex found in various organisms including *Drosophila melanogaster* (17, 40), human (12, 59, 61, 74), and more recently, the budding yeast *Saccharomyces cerevisiae* (23, 52–54). Holo-TFIID is composed of the highly conserved TATA box-binding polypeptide protein (TBP) and a number of associated polypeptides (TBP-associated factors [TAFs]). In vitro transcription studies revealed an important functional difference between holo-TFIID and TBP. Holo-TFIID mediates activator regulated transcription, whereas TBP itself mediates only basal levels of transcription. Thus, at least one or more TAFs included in holo-TFIID are essential for transmitting signals from various activators to the basal transcriptional machinery. Within the past 5 years, cDNAs encoding TAFs have been cloned from *Drosophila* (18, 21, 25, 36–39, 67, 70, 71, 73), human (4, 13, 16, 24, 26, 28, 42, 45, 62, 64), and yeast (23, 32, 33, 48, 54), and the sequences show that most of them are evolutionarily conserved while some of them are species specific (for a review, see reference 6).

Recently, Tjian and coworkers developed an in vitro assembly system for *Drosophila* TFIID with nine recombinant subunits (8, 9). Subcomplexes with different sets of TAFs are responsive to different activators (8, 55, 65), suggesting that different activation domains send signals through different pathways to the basal transcriptional machinery, presumably through a physical contact between an activation domain and an individual TAF. However, the mechanism of how such a physical contact can be decoded to modulate the polymerase activity remains unsolved.

The largest subunit of *Drosophila* TFIID (dTAF230) serves as a scaffold, providing specific interfaces for many other TAFs, and encodes two intriguing enzymatic activities, a protein kinase (15) and a histone acetyltransferase (46). In addition, we have identified an unusual activity in the N terminus (amino acid residues 2 to 81) of dTAF230 (35, 51). This particular region strongly interacts with TBP and inhibits TBP function, such as TATA box binding and TBP-mediated basal transcription. Importantly, this region binds to TBP in a competitive manner with the VP16 activation domain (51). Hence, we assume that this repressive activity might represent some functional aspect of holo-TFIID, and in fact, there are several observations that substantiate such an inhibitory activity in holo-TFIID.

First, holo-TFIID binds poorly to the TATA sequence if it is not near a strong initiator element, whereas TBP itself binds equally well with or without an initiator element (3). This argues that some of the TAFs can perceive the initiator element on the core promoter and function to modulate the binding activity of the TBP molecule to the TATA sequence. Indeed, a yeast homolog of dTAF230 has been shown to function as a core promoter selectivity factor, not as a general coactivator, at least on some specific genes (47, 56, 69).

Second, TBP loading onto the promoter appears to be the rate-limiting step in vivo and can be accelerated by transactivators such as GCN4 (34). Indeed, recruitment of TBP to the TATA box by fusing it to a heterologous DNA binding domain bypasses this rate-limiting step (7, 31). Conversely, some TBP mutants impaired in TATA binding do not respond to certain types of transactivators in vitro as well as in vivo (2, 30, 43), indicating that the integrity of the DNA binding domain of the TBP molecule is crucial for mediating regulated transcription.

Third, in a highly purified system in vitro, transactivators like Zta strongly stimulate TFIID-promoter complex formation in a TFIIA- and TAF-dependent manner, suggesting that the binding activity of TBP included in holo-TFIID can be potentiated by transactivators (11, 44). Taken together, these observations argue that holo-TFIID has an intrinsic activity down-regulating TBP function, and such a negative activity could be antagonized by transactivators and TFIIA to release the rate-limiting step.

Here we describe the TBP inhibitory domain of yTAF145

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(referred to as yTAF130 in reference 52) and dissect the structure-function relationship by detailed mutational analysis. Most importantly, the inhibitory domain and TFIIA bind to TBP competitively. Genetic experiments support the idea that the inhibitory domain is involved in TFIIA function. Implications for transcriptional regulation are also discussed.

MATERIALS AND METHODS

Preparation of TBP, TFIIB, and TFIIA. To prepare TBP and its derivatives, yeast TBP (wild type) was subcloned into pET-28a (Novagen). Site-directed mutagenesis was performed as described previously (41) to make the helix 2 TBP mutant K133,138,145E. Histidine-tagged proteins were expressed in *Escherichia coli* BL21(DE3) (Novagen) and purified on Ni^{2+}-nitrilotriacetic acid resin (Qiagen) as described previously (38). Wild-type and mutant TBP were also subcloned into pGEX-2T vector (Pharmacia) and expressed as glutathione *S*-transferase (GST) fusion proteins for the competition assay.

To prepare histidine-tagged yeast TFIIA, DNAs encoding TOA1 and TOA2 were amplified by PCR as *Nde*I-*Eco*RI fragments and subcloned in pET-15e (72). Each subunit was expressed and purified as described for TBP except with buffer containing 6 M guanidine-HCl. After renaturation by decreasing the guanidine concentration through dialysis, the complex was purified by gel filtration chromatography. For the competition analysis, both plasmids encoding the TOA1 and TOA2 genes (1 μg) were cotranslated to produce TFIIA in the reticulocyte lysate system (Promega) according to the manufacturer's protocol.

Preparation of *Drosophila* TFIIB was done as described previously (72).

Construction of yTAF145 derivatives. The full-length yTAF145 gene was expressed by the baculovirus system as described for dTAF230 previously (38). A *Sma*I site and a FLAG epitope were introduced 37-bp upstream from the initiation codon and just before the termination codon, respectively (41). The *Sma*I-*Pst*I fragment, including the entire FLAG-tagged yTAF145 gene, was transferred into the pVL1393 vector (Invitrogen) for expression.

For the competition analysis, yTAF145 (6-96) was expressed as a histidine-tagged protein (see Fig. 9C). All other mutants were expressed as GST fusion proteins. For deletion analysis, DNAs encoding the corresponding regions were amplified by PCR as *Bam*HI-*Eco*RI fragments and subcloned into pGEX-2T (Pharmacia). Internal deletion and site-specific changes were introduced by site-directed mutagenesis (41).

Protein-protein interaction analysis. To study interactions between TBP and the yTAF145 N-terminal region, purified TBP (30 pmol) was incubated with bacterial lysate expressing yTAF145N-GST fusion derivatives or GST (30 pmol; quantitated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis [SDS-PAGE] and Coomassie staining) in 100 μl of 0.1 M KCl–buffer N (20 mM Tris-HCl [pH 7.5], 12.5 mM $MgCl_2$, 10% [vol/vol] glycerol, bovine serum albumin [50 μg/ml], 1 mM dithiothreitol) at 4°C for 30 min, incubated with 10 μl of glutathione-Sepharose 4B (Pharmacia) for another 30 min, and washed three times with 500 μl of buffer N containing the indicated amounts of potassium chloride or potassium acetate. The beads were boiled in SDS sample buffer to elute TBP bound on yTAF145N derivatives. Eluates were subjected to SDS-PAGE, and the gel was stained with Coomassie brilliant blue R-250.

Competition assays. Bacterial lysates containing wild-type or mutated TBP (K133,138,145E)-GST fusions (100 ng) were mixed with purified yTAF (6-96) (5 μg) or no protein (as a control) in 100 μl of 0.1 M KCl–buffer N and incubated at 4°C for 30 min. ^{35}S-labeled TFIIA (5 μl; 10% of total translated products) was then added to the mixture, and incubation was continued at 4°C for another 30 min. The complex was analyzed as described above except that the proteins were visualized by autoradiography.

Gel retardation assays. Gel retardation assays were performed as described previously (38) with affinity-purified yTAF145N derivatives and TBP. TFIIA was added to the reaction mixtures as indicated. Shifted bands were quantified by PhosphorImager (Molecular Dynamics) analysis.

Genetic analysis. Standard yeast genetic techniques were used for the growth and transformation of the yeast strains (22).

Strain H2440 was constructed by crossing yeast strain H2450 (*MAT***a** *ura3-52 trp1-63 leu2-3,112 ade2*) with H2451 (*MAT*α *ura3-52 trp1-63 leu2-3,112 his3-609*). The wild-type TAF145 gene was disrupted in the diploid strain H2440 by using a marker cassette that has a *URA3* gene between duplicated copies of a *Salmonella hisG* gene segment (1). The cassette plasmid has the 5′-flanking sequence (~500 bp upstream of the initiation codon) and 3′-flanking sequence (~500 bp downstream of the termination codon) of the TAF145 gene at each side of *URA3* marker. These flanking sequences were amplified by PCR with primers creating *Eco*RI-*Bgl*II and *Sal*I-*Bam*HI sites, respectively. The linear fragment digested with *Eco*RI and *Sal*I was used to transform H2440. The structures of the disrupted gene were confirmed by Southern blot analysis.

Tetrad analysis showed that the TAF145 gene is essential (data not shown). Thus, the heterozygously disrupted diploid strain YTK1 was transformed with pYN2 to make TAF145 deletion strains viable after tetrad dissection. Plasmid pYN2 was constructed by ligating the 5.2-kb *Sma*I-*Pst*I TAF145 gene fragment into plasmid pRS314 (57). Ura^+ Trp^+ haploid strains obtained from tetrad analysis were grown on 5-fluoroorotic acid (5-FOA) plates to excise *URA3* marker by homologous recombination between the two *hisG* sequences. The resulting Ura^-Trp^+ strains were subsequently transformed with pYN1 containing TAF145 gene (*URA3* marker plasmid; originally isolated from the genomic library) to replace pYN2 by segregation.

Through several steps described above, we constructed three parent strains, Y13.2 (*MAT*α *ura3-52 trp1-63 leu2,3-112 his3-609* Δ*taf145* pYN1/*TAF145*), Y15.3 (*MAT*α *ura3-52 trp1-63 leu2,3-112 his3-609* Δ*taf145* pYN1/*TAF145*), and Y22.1 (*MAT*α *ura3-52 trp1-63 leu2,3-112* Δ*taf145* pYN1/*TAF145*), for plasmid shuffle experiments.

The yeast strains used in this report were derivatives of Y13.2. The N-terminal deletion or double point mutation (F23K D66K) was introduced into pYN2 by site-directed mutagenesis (41). Both the wild-type and mutant plasmids were transformed into Y13.2, and the pYN1/*TAF145* plasmid was shuffled out on medium containing 5-FOA. *TOA1* was subcloned from pSH363 (29) as a *Bam*HI-*Xho*I fragment into pRS426 (14) cut with *Bam*HI and *Xho*I. *TOA2* was subcloned from pSH343 (29) as a *Pst*I fragment into *Pst*I-digested YEplac181 (20).

RESULTS

yTAF145 forms a complex with TBP which inhibits TATA box binding. We previously demonstrated that dTAF230 can form a stable complex with TBP when the two polypeptides were mixed in vitro and that such a complex was significantly impaired in TATA box binding activity compared with TBP alone (38). Mutational analyses indicated that the N-terminal 81 residues of dTAF230 interact with TBP and inhibit TBP-binding to the TATA box (35). To analyze the physiological role of this inhibitory activity, we isolated yeast genomic DNA encoding the dTAF230 homolog. During the course of this work, the Weil and Green laboratories independently isolated identical clones as the gene for the 130- or 145-kDa (by SDS-PAGE) TFIID subunit, respectively (52, 54). In the present work, we refer to this gene and the protein it encodes as the yTAF145 gene and protein.

First, we tested whether yTAF145 forms a complex with TBP. The full-length yTAF145 expressed as a FLAG epitope-tagged protein was immobilized on M2 anti-FLAG antibody agarose and then incubated with TBP. After extensive washing, bound polypeptides were eluted with FLAG peptide for SDS-PAGE analysis. A Coomassie blue-stained SDS-PAGE gel showed that yTAF145 forms a stoichiometric complex with TBP (Fig. 1A). Next, we tested the ability of the yTAF145-TBP complex to bind the TATA box (Fig. 1B). Increasing equimolar amounts of TBP (lanes 1 to 3) or the yTAF145-TBP complex (lanes 4 to 6) were tested for TATA-binding activity by a gel retardation assay. Although the ternary complex containing yTAF145, TBP, and DNA was not detected, a small amount of the TBP-DNA complex was detected in the system containing the yTAF145-TBP complex (lanes 4 to 6). This suggests that free TBP dissociated from the complex binds to the TATA box or that the yTAF145-TBP complex binds more poorly than TBP alone.

The N-terminal region of yTAF145 contains the inhibitory activity. We mapped the yTAF145 region important for the inhibition of TBP binding to the TATA box. Analyses of various yTAF145 segments expressed in *E. coli* (data not shown) showed that the N-terminal region of yTAF145 (residues 10 to 88) [yTAF145 (10-88)] is sufficient for inhibition of both TATA-binding activity (Fig. 1C) and TBP binding (data not shown). To map further the inhibitory domain of yTAF145, truncations of yTAF145 (10-88) were generated from the C-terminal end (Fig. 2A) and then tested for interaction with TBP (Fig. 2B) and inhibition of TBP binding to the TATA box (Fig. 2C). While yTAF145 (10-64) retained these activities at levels comparable to the parental construct, further deletion drastically reduced both activities. However, a weak inhibitory activity was still detected even in the shortest mutant, yTAF145 (10-58), in a sensitive system with 30-fold excess of the mutant TAF (Fig. 2C).

Similarly, truncations from the N-terminal end were ana-

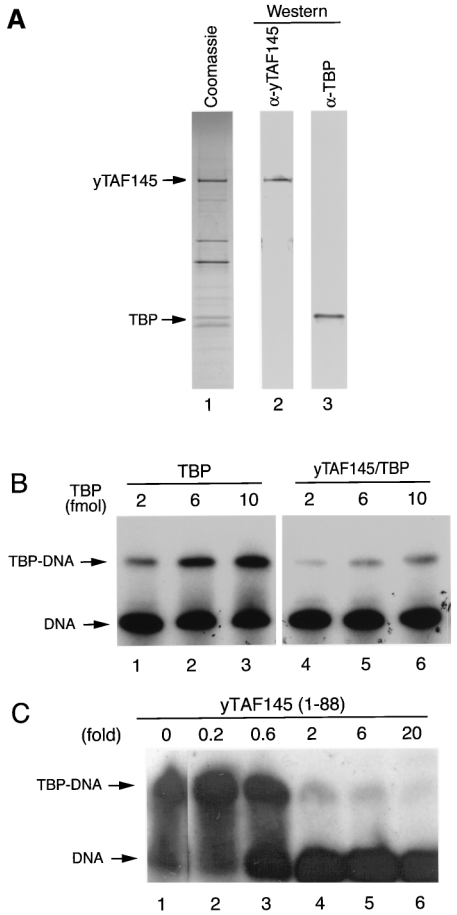

FIG. 1. yTAF145 forms a complex with TBP and inhibits TATA box binding. (A) Full-length yTAF145 forms a stoichiometric complex with TBP. Full-length yTAF145 was expressed in Sf9 cells as a FLAG epitope-tagged protein and purified on anti-FLAG antibody-immobilized agarose. Immobilized yTAF145 was incubated with yeast TBP and eluted with FLAG peptide after extensive washing. The complex was analyzed by Coomassie blue staining (lane 1) and immunoblotting with anti-yTAF145 antibody (lane 2) and anti-TBP antibody (lane 3). (B) TATA box binding of yTAF145-TBP complex. A gel retardation assay was performed with TBP (lanes 1 to 3) and the yTAF145-TBP complex described in panel A (lanes 4 to 6). Adenovirus major-late promoter (−40 to +10) was used as a probe. The positions of TBP-DNA complex and free probe (DNA) are indicated on the left. (C) Inhibition of TBP binding to the TATA box by yTAF145 (1-88). TBP (0.4 pmol) was incubated with DNA and increasing amounts of yTAF145 fragments containing residues 1 to 88 as indicated.

lyzed (Fig. 3). While yTAF145 (14-71) retained almost wild-type levels of TBP binding and TATA box binding inhibition, further deletion gradually reduced these activities. Like the C-terminal deletions, even the shortest construct, yTAF145 (28-71), still showed a weak inhibitory activity. These data suggest that multiple subdomains within the yTAF145 N terminus might be involved in these activities. This possibility is also supported by the results of the experiment shown in Fig. 4. Although both yTAF145 (10-66) and yTAF145 (10-71) have indistinguishable activities (Fig. 2), these constructs showed quite different activities when the N-terminal residues were further deleted. Note that these experiments were performed in the presence of a 100-fold excess of mutant TAF to detect any weak inhibitory activity. The activity was significantly impaired by deleting to residue 25 in mutants with residue 66 at the very C terminus (Fig. 4A). In contrast, deletion to residue 45 still retained the weak inhibitory activity in mutants with residue 71 as the most C terminal (Fig. 4B). We regard these data as suggesting that the integrity of each segment becomes important when the other segment is impaired.

There are two subdomains within the yTAF145 inhibitory domain. To determine more precisely the regions required for the inhibitory function, blocks of eight contiguous residues were converted to all alanine residues (Fig. 5A). These mutants were tested for interaction with TBP (Fig. 5B) and inhibition of TBP binding to the TATA box (Fig. 5C) as described above. Importantly, mutations in two discontinuous regions (residues 10 to 25 and 50 to 65; Fig. 5B, lanes 2, 3, 7, and 8) severely impaired TBP binding, while mutations in the intervening region (residues 26 to 49; lanes 4 to 6) had little or no effect. Similar results were obtained with TATA box binding inhibition (Fig. 5C), except that the mutations in residues 66 to 73 were also able to impair the inhibitory activity.

In a similar fashion, internal deletions were constructed and analyzed (Fig. 6). Important residues for the binding and inhibitory activities were mapped within two discontinuous regions (residues 14 to 25 and 50 to 65). Importantly, these regions overlap with the regions determined to be important for activity by the alanine substitution experiment (residues 10 to 25 and 50 to 73). Thus, we conclude that there are two discontinuous regions important for TBP binding and inhibition of TBP binding to the TATA box. We refer to these as subdomains I and II (residues 10 to 25 and 50 to 73, respectively, although the exact boundaries of these subdomains have not been determined).

Both hydrophobic and acidic residues are critical for TBP binding. The dTAF230 N-terminal 81 amino acids, which bind stably to TBP and inhibit TATA binding, align with yeast subdomain I with exceptionally low conservation and significant gaps (Fig. 7A). Based on this alignment, we previously tested whether the dTAF230 residues corresponding to yeast subdomain II are functional (51). Competition experiments indicated that dTAF230 (1-156) can bind to TBP more stably than dTAF230 (1-81). Thus, we concluded that subdomain II in dTAF230 is functional, further stabilizing the interaction between TBP and subdomain I.

In addition, important residues for the activities in subdomains I and II had been determined. Mutation experiments of the dTAF230 subdomain I demonstrated that hydrophobic residues, especially F25, were crucial. Thus, we mutated the conserved F23 in yTAF145 (Fig. 7A) and tested for TBP binding activity (Fig. 7B). While mutations that changed F23 into other hydrophobic residues, such as tryptophan, tyrosine, or leucine, retained almost full activity (Fig. 7B, lanes 5 to 7), mutation into alanine weakened the binding (lane 2). Moreover, mutations into charged residues, lysine or glutamate, severely weakened binding (lanes 3 and 4). Thus, we conclude that the conserved residue F23 plays an important role via its hydrophobic character in the interaction with TBP as observed in dTAF230.

Though subdomain I is not highly conserved, three contiguous acidic residues are conserved between the subdomain I of dTAF230 and that of yTAF145 (residues 9 to 11 and 15 to 17, respectively). Although mutations of these residues in dTAF230 did not affect the interaction with TBP (35), it is highly possible that the effect of the mutations is not detectable given that the dTAF230 subdomain I has a greater number of hydrophobic residues which, per se, might be sufficient for a stable interaction with TBP. Thus, we tested the contribution of the corresponding acidic residues in yTAF145 subdomain I, which contains fewer hydrophobic residues. Mutations of residues 15 to 17 in yTAF145 into all lysines drastically impaired the interaction with TBP (Fig. 7B, lane 8). In contrast, a mu-

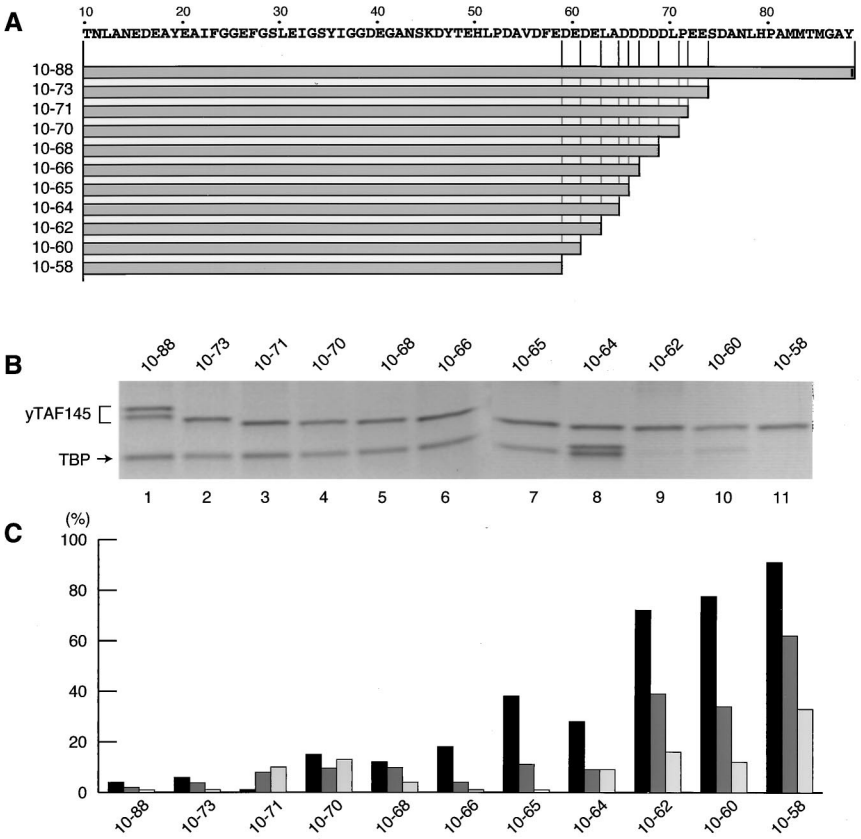

FIG. 2. C-terminal deletion analysis of yTAF145 (10-88). (A) Scheme of C-terminal deletions. The numbers on the left indicate the actual amino acid residues included and are used to denote each mutant. (B) Interaction of deletion mutants and TBP. All mutants were expressed as GST fusion proteins, and each was incubated with an equimolar amount of TBP. GST fusions were purified with glutathione agarose and analyzed by SDS-PAGE followed by Coomassie blue staining. The lower band in lane 1 represents a cut form due to the protease-hypersensitive sites near the C terminus. (C) Inhibition of TBP binding to the TATA box by the yTAF145 deletion mutants. Gel retardation assays were performed with a 3-, 10-, or 30-fold (left, middle, and right columns, respectively) excess of yTAF145 mutant proteins. The intensity of the bands representing the TBP-DNA complex was quantified by a PhosphorImager (Molecular Dynamics). The values are presented as percentages of the values obtained in the system without the yTAF145 protein.

tation in the nonconserved glutamate at position 20 had no effect (lane 9). These results indicate that both acidic and hydrophobic residues in the yTAF145 subdomain I contribute to its interaction with TBP.

Next, we mapped important residues within subdomain II. Subdomain II is more conserved than subdomain I between yeast and *Drosophila* and is rich in acidic residues (Fig. 7A). Thus, we mutated some of these acidic residues into lysines (Fig. 7B). Mutations of the contiguous acidic residues 58 to 62 severely decreased the TBP binding activity (lane 10). To test the individual contributions of residues 58 to 62, each residue was mutated individually to lysine (lanes 11 to 15). A single mutation at residue 60 severely reduced the TBP binding activity, although this residue is not conserved between yeast and *Drosophila*. In contrast, a single mutation in residue 66 or a triple mutation in the residues 67 to 69 had no effect on the interaction (lanes 16 and 17). These results indicate that specific acidic residues in subdomain II could contribute to the binding to TBP.

Stability of the complex under high ionic concentration. Mutational studies of the inhibitory domain in yTAF145 indicate that charged residues in subdomains I and II are functionally important, although hydrophobic residues determined to be crucial in dTAF230 (35) are also conserved in yTAF145 (Fig. 7). To confirm these results, we tested the salt sensitivity of the interaction between yTAF145 and TBP (Fig. 8). We employed potassium acetate and potassium chloride since the yeast TFIID complex was reported to be more sensitive to chloride ions than acetate ions (52, 54, 68). This is in contrast to the *Drosophila* TFIID complex, which is stable in buffer containing 0.5 M potassium chloride (40). The yTAF145 (6-96)–TBP complex was almost completely disrupted in buffer containing 0.3 M potassium chloride (Fig. 8A). The complex was slightly stabilized in buffer containing potassium acetate (Fig. 8B). In contrast, the dTAF230 (2-81)–TBP complex was stable even in buffer containing 1.0 M KCl (Fig. 8A, lanes 7 and 8). These observations support the view that ionic interactions mainly sustain the yTAF145–TBP complex.

TFIIA and yTAF145 bind competitively to TBP in vitro. We previously demonstrated that the dTAF230 subdomain I binds to the concave surface of TBP. Here, we attempted to determine TBP sites important for interaction with the yTAF145 subdomain II. Given that acidic residues in the subdomain II are important for TBP binding, we considered that the basic repeats within helix 2 on the TBP convex surface might be targets (50). This surface is also known to be important for TFIIA interaction both in vitro and in vivo (5, 58, 63). To test the contribution of this surface of TBP, we mutated three lysines at 133, 138, and 145 to all glutamate residues. The resulting mutant, TBP(K133,138,145E), retained full activities for TATA box binding (Fig. 9A, lane 2), TFIIB binding on DNA (lane 8), and transcription in vitro (data not shown).

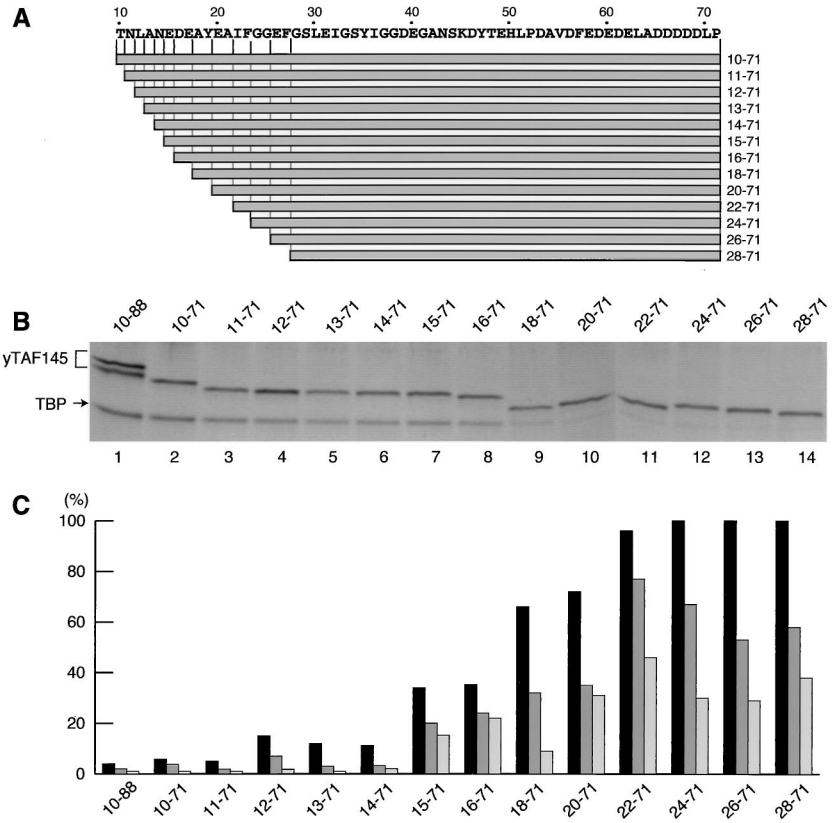

FIG. 3. N-terminal deletion analysis of yTAF145 (10-71). Structures of N-terminal deletion mutants (A), interaction with TBP (B), and inhibition of TBP binding to the TATA box (C) are presented as described in the legend to Fig. 2.

However, this mutant completely lacked TFIIA-binding activity on DNA (lane 6). Note that the TBP-DNA complex is not stable enough to be detected in the gel system which is suitable for the TBP-TFIIA-DNA and TBP-TFIIB-DNA complexes (lanes 3 and 4). The triple mutant of TBP was tested for interaction with the TAFs (Fig. 9B). The dTAF230 subdomain I, which binds to the concave surface of TBP (51), interacted almost equally well with the wild-type and mutant TBP, supporting the view that subdomain I binds to the concave surface of TBP. Importantly, binding of yTAF145 (6-96), including both subdomain I and II, to the mutant TBP was significantly weaker than that to wild-type TBP. These results, albeit indirect, indicate that the basic repeats of TBP might be targets for subdomain II.

Given that the basic repeats of TBP are also important for interaction with TFIIA, we tested whether TFIIA and yTAF 145 (6-96) bind competitively to TBP. We tested the interaction between TFIIA and TBP without DNA (Fig. 9C). As

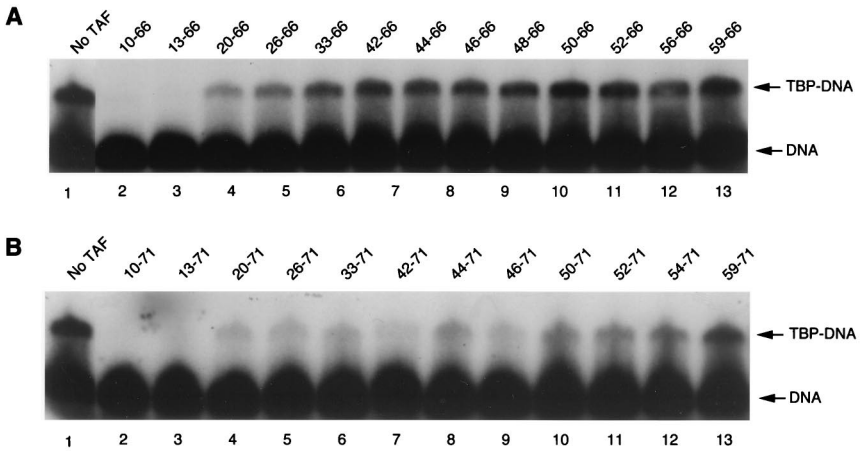

FIG. 4. Comparison of the N-terminal deletion effects of yTAF145 fragments that have distinct C-terminal ends. Mutants are referred to by the positions of residues remaining. Mutants have residue 66 as the C-terminal end (A) or end at residue 71 (B). A gel retardation assay was performed with 100-fold molar excess amounts of the yTAF145 fragments over TBP to examine weak inhibitory activities.

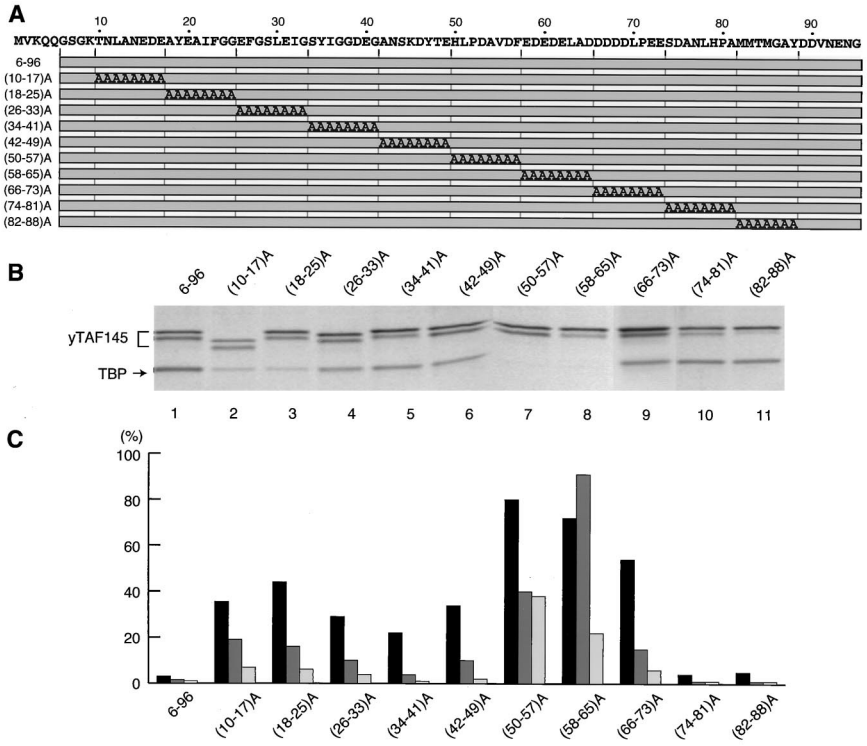

FIG. 5. Alanine substitution mutants of yTAF145 (6-96). Contiguous segments of eight amino acid residues were changed to all alanines as indicated. Structures of alanine substitution mutants (A), interaction with TBP (B), and inhibition of TBP binding to the TATA box (C) are presented as described in the legend to Fig. 2.

observed in the system with DNA (Fig. 9A), wild-type TBP bound to TFIIA while the mutant TBP did not (Fig. 9C, lanes 1 and 2). Importantly, the interaction between TBP and TFIIA was almost completely disrupted by adding yTAF145 (6-96), supporting the view that TFIIA and the yTAF145 subdomain II might share binding sites on the convex surface of TBP.

The yTAF145 N terminus is important for cell growth. In order to determine the in vivo relevance of the inhibitory

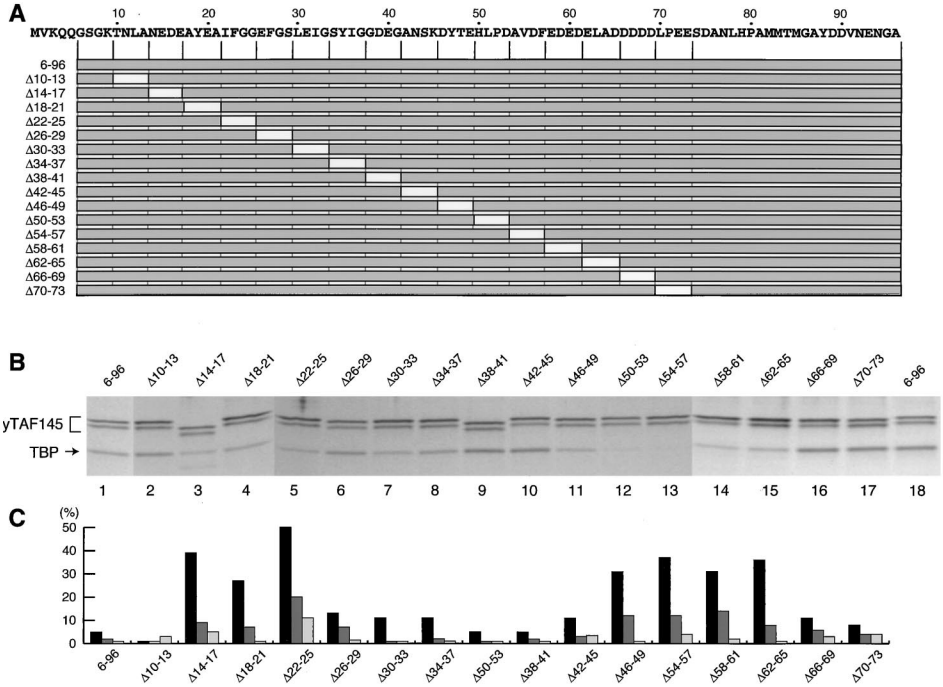

FIG. 6. Internal deletion mutants of yTAF145 (6-96). Four contiguous residues were deleted as indicated. Structures of N-terminal deletion mutants (A), interaction with TBP (B), and inhibition of TBP binding to the TATA box (C) are presented as described in the legend to Fig. 2.

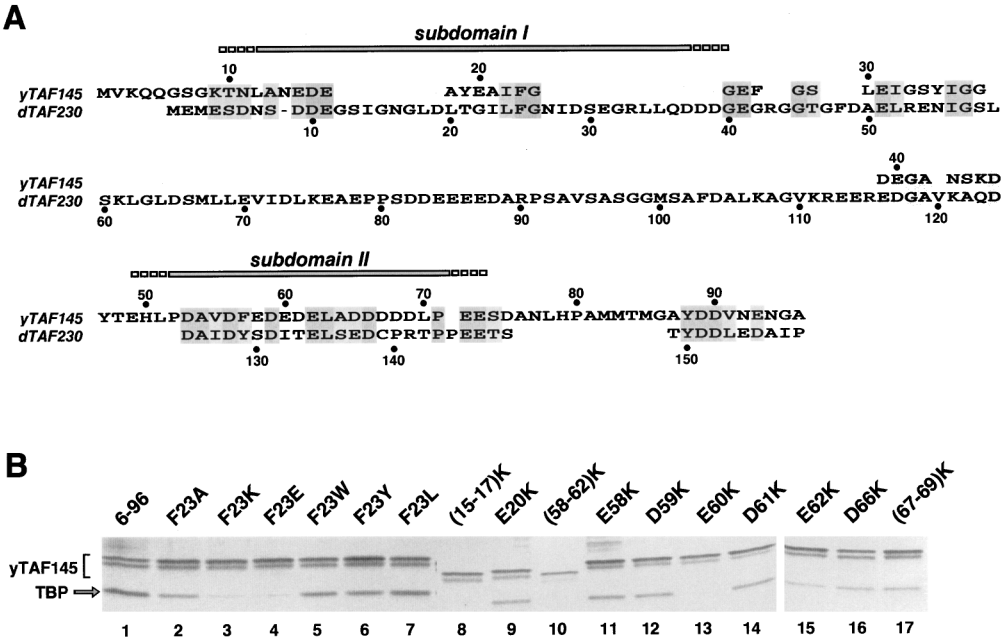

FIG. 7. (A) Alignment of the N-terminal regions of yTAF145 and dTAF230. The positions of subdomains I and II determined by analyses of yTAF145 are indicated. (B) Effect of point mutations in subdomains I and II of yTAF145. Various point mutations were introduced into the parental construct yTAF145 (6-96). Interaction of yTAF145 mutants with TBP is presented as described in the legend to Fig. 2B.

domain, we tested the phenotype of a yTAF145 N-terminal deletion (residues 6-96) or a double point mutation, F23K D66K, which eliminates the in vitro activity of this domain (data not shown). The constructs harboring these mutations were transformed into a haploid strain containing a *TAF145* deletion at the normal chromosomal locus and a *URA3*-marked plasmid carrying the wild-type *yTAF145* gene. The wild-type gene was shuffled out of the strain on medium containing 5-FOA in order to test the mutant phenotype. The mutant strains show slower growth than the wild-type strain at 30°C, and at 37°C their growth is severely restricted (Fig. 10A).

The results described above show that TFIIA competes with the yTAF145 N-terminal inhibitory domain for TBP binding. Therefore, we tested the genetic interaction between the inhibitory domain and TFIIA. Overexpression of both the yeast TFIIA subunits, *TOA1* and *TOA2*, on high-copy-number plasmids can suppress the growth defect of the N-terminal deletion of yTAF145 (Fig. 10B). Interestingly, overexpression of *TOA1* alone partially suppresses the growth defect, whereas overexpression of *TOA1* and *TOA2* simultaneously conferred nearly complete suppression. It cannot be simply ascribed to the change in the steady-state level of yTAF145 polypeptide since the N-terminal domain mutant is stably expressed at 37°C (data not shown). The suppression by TFIIA overexpression seems to be specific for the yTAF145 N-terminal domain mutant, since it cannot suppress the temperature-sensitive growth phenotype of C-terminal yTAF145 point mutations (data not shown).

DISCUSSION

We previously demonstrated that the N-terminal region of dTAF230 inhibits TATA box binding by interacting directly with the DNA binding surface of TBP. Importantly, the VP16 activation domain interacts with the same or overlapping surface and competes for TBP binding (51). Here we demonstrate that this inhibitory activity is evolutionarily conserved in *S. cerevisiae*, underscoring the physiological significance of this activity. The small, N-terminal region including residues 10 to 71 suffices for both TBP binding and inhibition of TBP binding to the TATA box. We observed a general correlation between the binding and inhibitory activities. Taken together with the result that the dTAF230 inhibitory domain binds to the DNA binding domain of TBP, it is very likely that yTAF145-dTAF230 inhibits TATA box binding by covering the TBP surface important for TATA box binding. The yTAF145 inhibitory domain contains two subdomains, I (residues 10 to 37) and II (residues 46 to 71). While the dTAF230 subdomain I per se forms a stable complex with TBP, yTAF145 requires both subdomains for a stable interaction. Subdomain I is barely

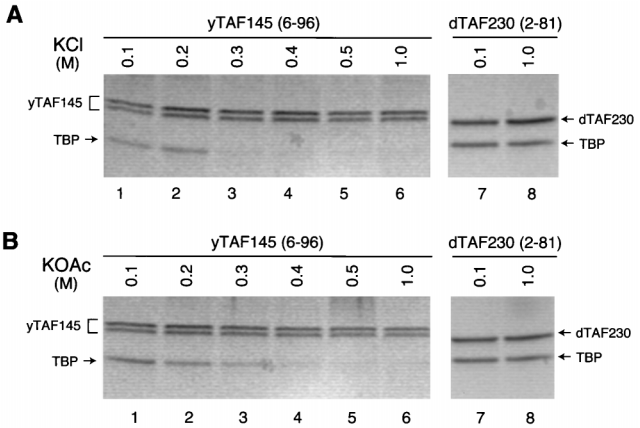

FIG. 8. The complex of TBP and yTAF145 (6-96) is salt sensitive. GST-TAF145 (6-96) (lanes 1 to 6) or GST-dTAF230 (2-81) (lanes 7 and 8) was incubated with yeast TBP and then immobilized on glutathione-coupled beads. The beads were washed with buffer containing the indicated concentrations of potassium chloride (A) or potassium acetate (B). Proteins remaining on the beads were separated by SDS-PAGE followed by Coomassie blue staining.

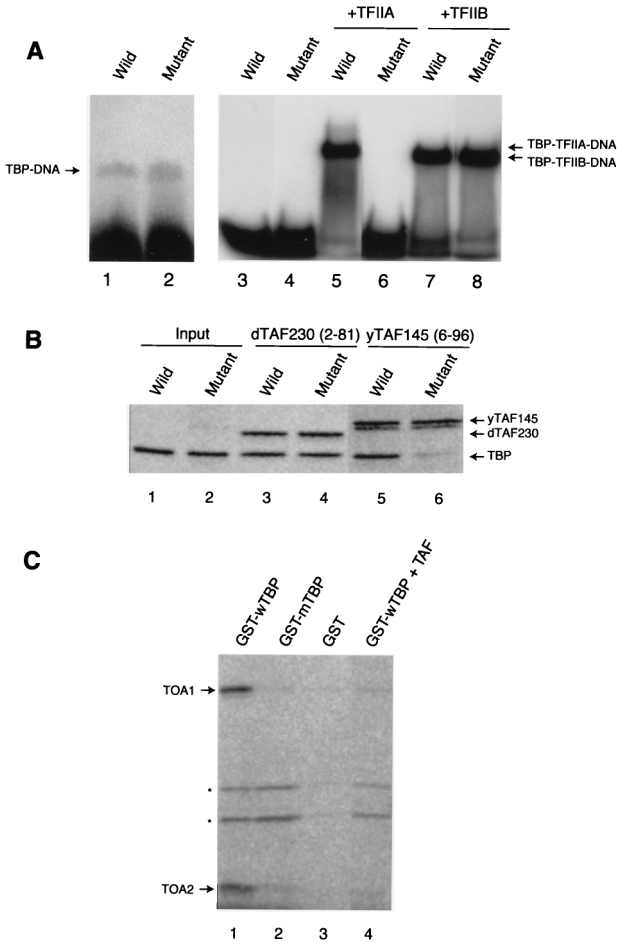

FIG. 9. A cluster of positive charges on TBP is important for the interaction with both TFIIA and yTAF145. (A) The TBP carrying oppositely charged mutations (K133,138,145E) on helix 2 lacks TFIIA-binding activity. No protein (lanes 1 to 4), recombinant yeast TFIIA (2 pmol; lanes 5 and 6), and recombinant *Drosophila* TFIIB (2 pmol; lanes 7 and 8) were incubated with DNA and wild-type yeast TBP (2 pmol; odd-numbered lanes) or mutant yeast TBP (K133,138, 145E) (2 pmol; even-numbered lanes). Tris-glycine-$MgCl_2$ buffer was used to detect the DNA-TBP complex (lanes 1 and 2), whereas Tris-borate-EDTA buffer was used to detect the DNA-TBP-TFIIA or DNA-TBP-TFIIB complex (lanes 3 to 8). (B) The mutant TBP interacts weakly with yTAF145. GST-dTAF230 (2-81) (lanes 3 and 4) or yTAF145 (6-96) (lanes 5 and 6) was mixed with wild-type yeast TBP (lanes 3 and 5) or mutant yeast TBP (K133,138,145E) (lanes 4 and 6). GST fusions were purified with glutathione agarose and analyzed by SDS-PAGE followed by Coomassie blue staining. (C) TFIIA and yTAF145 (6-96) bind competitively to TBP. ^{35}S-labeled yeast TFIIA, consisting of TOA1 and TOA2 subunits, was incubated with GST-TBP (lanes 1 and 4), GST-TBP (K133,138, 145E) (lane 2), or GST (lane 3) in the absence (lanes 1 to 3) or presence (lane 4) of yTAF145 (6-96). GST fusions were purified with glutathione agarose and analyzed by SDS-PAGE followed by autoradiography. Asterisks indicate in vitro-translated products which bound nonspecifically to TBP.

conserved between yeast and *Drosophila*, and the alignment contains significant gaps. Although some residues are conserved, the lack of a hydrophobic cluster in the yTAF145 subdomain I may reflect an unstable TBP interaction by the subdomain I per se. Nevertheless, yTAF145 (6-96), which contains both subdomains I and II, competes with the VP16 activation domain for TBP binding (48a). This suggests that the yTAF145 subdomain I binds to the same or closely spaced surface of TBP that is recognized by the dTAF230 subdomain I. Further analysis will be required to address this point more clearly. yTAF145 and TFIIA share residues on TBP which are important for their respective interactions. Consistently, yTAF145 and TFIIA compete for TBP binding.

Deletion of the yTAF145 inhibitory domain causes a temperature-sensitive growth phenotype. Surprisingly, overexpression of the yeast TFIIA subunits, TOA1 and TOA2, suppresses the growth defect of this mutant. This indicates that the yTAF145 inhibitory domain is also involved in TFIIA function. Since TBP overexpression also rescues the growth defect (data not shown), TFIIA might recover TBP function, which is somehow impaired in this particular mutant. We propose that the yTAF145 N-terminal domain can interfere both with TBP binding to DNA and with binding of TFIIA to TBP and that both of these inhibitory influences on the formation of a TBP-TFIIA-promoter complex can be overcome in vivo by the action of transcriptional activators. It is possible that deletion of the N-terminal domain of yTAF145 has the added effect of weakening the association of TBP with other components of TFIID and thereby impairing TBP binding to the promoter. This defect could be corrected by overexpression of TBP or TFIIA, which in each case would promote TBP-TFIIA-promoter complex formation.

In summary, subdomain I and the VP16 activation domain competitively bind to the concave surface of TBP, which is important for TATA box binding. On the other hand, subdomain II and TFIIA seem to bind competitively to the convex surface of TBP. These competitions between the negative and positive interactions could be important for transcriptional regulation.

Recently, the crystal structure of the TFIIA-TBP-DNA complex was determined (19, 60). Surprisingly, there is no contact between helix 2 of TBP and TFIIA. However, it is necessary to interpret these data carefully since proteins containing large deletions, which impair stable complex formation, were used for structural analysis. Moreover, the region (amino acids 217 to 240) of the large TFIIA subunit, which is necessary for stable complex formation, was not defined in the X-ray analysis, presumably due to its flexible structure. On the other

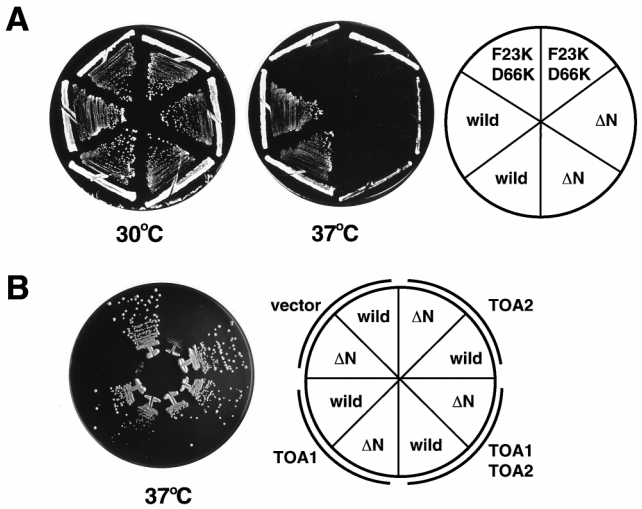

FIG. 10. (A) Elimination of inhibitory activity by deleting the yTAF145 N-terminal domain or double point mutation, F23K D66K, causes a temperature-sensitive growth phenotype. Strains carrying wild-type or the N-terminal mutants of yTAF145 were grown on yeast-peptone-dextrose medium at 30 and 37°C. (B) Multicopy TFIIA suppresses the temperature-sensitive phenotype of the N-terminal deletion. Wild-type and N-terminal deletion strains of yTAF145 were transformed with multicopy plasmids expressing TOA1, TOA2, both TFIIA subunits, or neither subunit. Cells were streaked on media selecting for the presence of the plasmids. Incubation was done at 37°C.

hand, the significance of helix 2 of TBP for interaction with TFIIA has been demonstrated not only in biochemical experiments in vitro but also by genetic experiments in vivo (5, 58, 63). Therefore, it is still likely that helix 2 of TBP is one of the crucial sites for interaction with TFIIA.

While mechanisms of how the inhibitory domain contributes to transcriptional regulation are still unclear, there are several reports that support such an inhibitory activity in TFIID. We previously demonstrated that TFIID binds stably to the *gfa* promoter and yields footprints extending from sequences upstream of the TATA box through a downstream initiator-like element (49). In contrast, a mutation in an initiator-like element significantly weakens the interaction not only at the downstream initiator regions but also at the TATA box. In contrast, the same mutation marginally reduces the TBP interaction, suggesting that TAFs destabilize TFIID binding when the downstream element is absent. In support of this view, a variety of TATA box-containing promoters which lack strong initiator elements bind poorly to TFIID (3). Moreover, the TFIID subcomplex reconstituted by recombinant TBP, dTAF150, and dTAF250 (corresponding to our dTAF230) binds less stably than TBP when the downstream sequences are absent (66). However, the same complex binds more stably when the downstream sequences are present. It is important that the interaction of TFIID with the downstream sequences is dependent on activators in some weak core promoters lacking strong initiator elements (27). While only the TATA region is protected by TFIID when no activator is present, activators in conjunction with TFIIA induce a downstream interaction, providing stable TFIID interaction with core promoters (10). It is reasonable to speculate that this phenomenon might result from counteracting the yTAF145-dTAF230 inhibitory domain by TFIIA and the activation domain.

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ADDENDUM IN PROOF

While this paper was being revised, Bai et al. published results describing a high-affinity TATA-binding protein interaction domain in the N terminus of yTAF145 (Y. Bai, B. M. Perez, J. M. Beechem, and P. A. Weil, Mol. Cell. Biol. **17:** 3081–3093, 1997). Yeast strains lacking this domain exhibit a slower growth phenotype at higher temperatures, as do our strains.

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