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Drosophila $TAF_{II}230$ and the transcriptional activator VP16 bind competitively to the TATA box-binding domain of the TATA box-binding protein

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ABSTRACT **The transcription initiation factor TFIID, consisting of the TATA box-binding protein (TBP) and many TBP-associated factors (TAFs), plays a central role in both basal and activated transcription. An intriguing finding is that the 80-residue N-terminal region of *Drosophila* $TAF_{II}230$ [$dTAF_{II}230$-(2–81)] can bind directly to TBP and inhibit its function. Here, studies with mutated forms of TBP demonstrate that $dTAF_{II}230$-(2–81) binds to the concave surface of TBP, which is important for TATA box binding. Previously, it was reported that a point mutation (L114K) on this concave surface destroys the ability of TBP to bind VP16 and to mediate VP16-dependent activation *in vitro*, but has no effect on basal transcription. Importantly, the same TBP mutation eliminates TBP binding to $dTAF_{II}230$-(2–81). Consistent with these effects of the L114K mutation, $dTAF_{II}230$-(2–81) and the VP16 activation domain compete for binding to wild-type TBP. These results indicate that transcriptional regulation may involve, in part, competitive interactions between transcriptional activators and TAFs on the TBP surface.**

Transcriptional initiation of eukaryotic protein-encoding genes requires at least six transcription initiation factors (TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH) in addition to RNA polymerase II, and biochemical analyses with separated factors have defined a sequence of steps which lead to the *in vitro* formation of a preinitiation complex on a TATA box-containing promoter (reviewed in ref. 1). The first step is TFIID binding, a process that may be facilitated by TFIIA. The subsequent binding of TFIIB, through specific TATA box-binding protein (TBP) and DNA contacts, creates a platform that is in turn recognized by a complex consisting of RNA polymerase II and TFIIF. Further incorporation of TFIIE and TFIIH completes preinitiation complex formation. More recent studies have revealed complexes of RNA polymerase II, general initiation factors, and cofactors that may enter the preinitiation complex as a preassembled unit (reviewed in refs. 2 and 3). Because variable compositions of general factors have been reported for these holoenzyme complexes, depending upon both the species and the preparation methods, the assembly pathway most relevant to the *in vivo* situation remains unclear. However, TFIID binding to the promoter could be a critical checkpoint for promoter activation in several different pathways, consistent with studies showing both qualitative and quantitative effects of activators on TFIID binding (4–11).

TFIID itself is a multimeric protein complex consisting of TBP and TBP-associated factors (TAFs) whose sizes range from $M_r \approx 10{,}000$ to $>200{,}000$ (for a review, see ref. 12). To date, cDNAs encoding nine TAF subunits of the *Drosophila* TFIID ($dTAF_{II}230$, -150, -110, -85, -62, -42, -28α, -28β, -22) have been cloned (reviewed in refs. 12–14). Although the detailed mechanisms by which TAFs contribute to transcriptional activation are unclear, several TAFs have been shown to provide interaction sites for distinct activators (reviewed in ref. 12). These interactions could serve either to facilitate TFIID recruitment *per se* (4, 7–9) or to induce conformational alterations that effect recruitment or function of downstream factors (ref. 6; reviewed in ref. 1), and in some cases their functional relevance has been supported by transcriptional studies with partially reconstituted TBP-TAF complexes (see, for example, refs. 10 and 11).

TAFs also play an important role in promoter selectivity in basal transcription. Our earlier experiments with partially purified TFIID demonstrated that TFIID binds stably to a specific core promoter in a manner that depends on both the TATA and the downstream initiator-like elements (15). Deletion of the downstream element significantly reduces TFIID binding not only at the initiator-like region but also at the TATA box (15), suggesting that TAFs may have either positive or negative effects on TFIID binding depending on the specific core promoter sequences. In agreement, more recent reports with a recombinant partial TFIID complex have suggested that $dTAF_{II}150$ acts in conjunction with $dTAF_{II}230$ to stabilize promoter binding of TBP when both TATA and downstream elements are present (16). In contrast, TAFs were found to destabilize promoter binding of TBP when the downstream sequences are absent (16), although the molecular basis for the destabilization has not been demonstrated.

We previously have reported that the 80 N-terminal amino acid residues of *Drosophila* $TAF_{II}230$ [$dTAF_{II}230$-(2–81)] bind directly to TBP and negatively regulate its TATA-box binding activity (17). To obtain further insights into the roles of the negative regulatory domain in activator-induced transcription and promoter utilization, we used mutant forms of TBP to determine the interaction sites of $dTAF_{II}230$-(2–81). We mapped an interaction site for $dTAF_{II}230$-(2–81) to the concave face, which binds to the TATA box. Importantly, the VP16 activation domain and $dTAF_{II}230$-(2–81) bind competitively to TBP, apparently through interactions with a common

Abbreviations: TBP, TATA box-binding protein; TAF, TBP-associated factor; y, yeast; GST, glutathione *S*-transferase.

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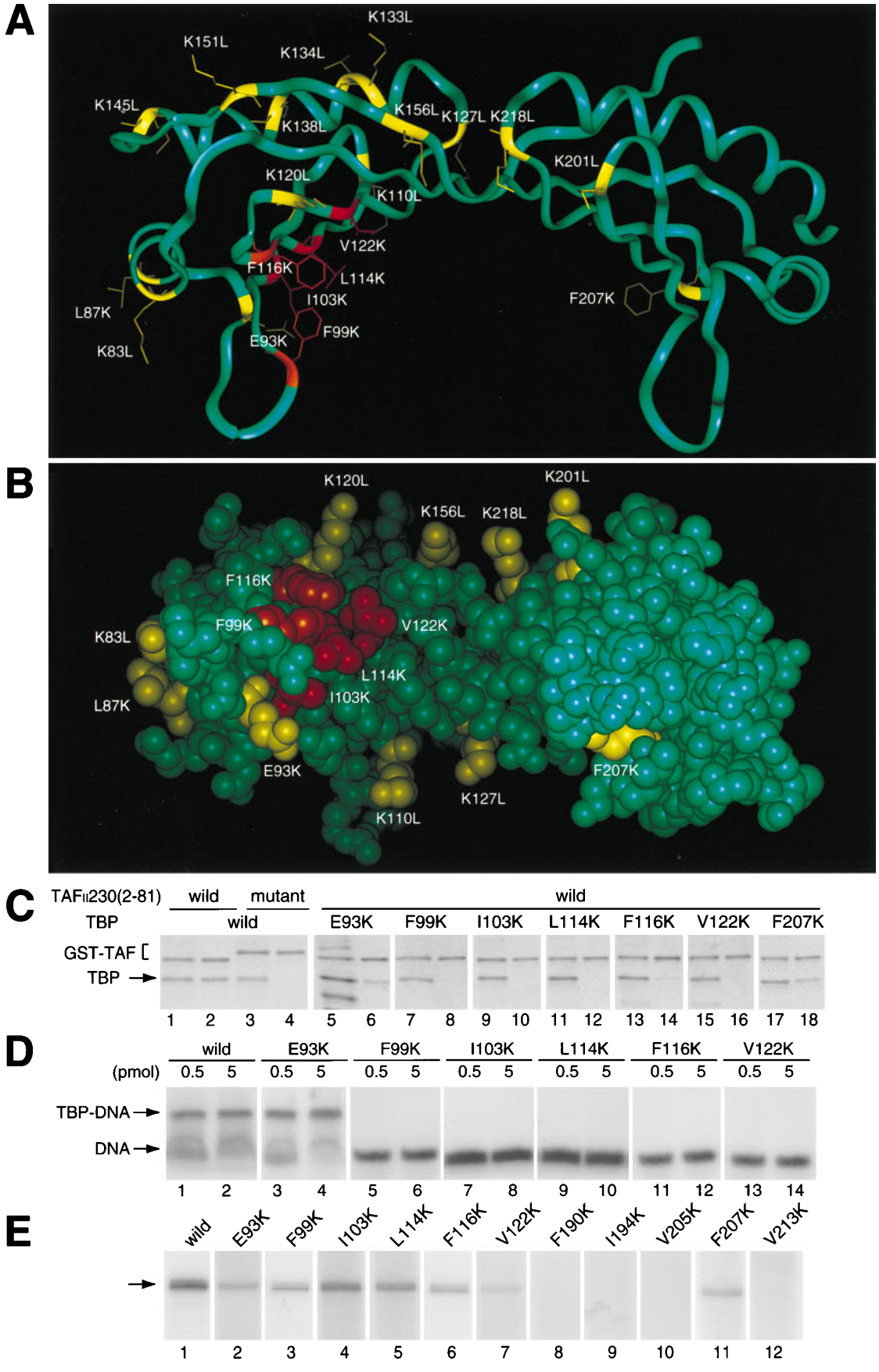

FIG. 1. dTAF$_{II}$230-(2–81) binds to the concave surface of TBP. (*A*) Ribbon drawing of TBP viewed perpendicular toward the internal pseudodyad axis and (*B*) space-filling drawing viewed to the concave surface. The binding capability of TBP mutants to dTAF$_{II}$230-(2–81) is indicated by colors. Mutations that cause a weak or no effect are shown by yellow (>50% activity of wild-type TBP), whereas mutations that cause serious defects are shown by orange (2–10%) or red (<2%). (*C*) TBP interaction with dTAF$_{II}$230-(2–81). GST–dTAF$_{II}$230-(2–81) was incubated

site, suggesting that this competition could play an important role in transcriptional activation.

MATERIALS AND METHODS

Preparation of TBP and dTAF$_{II}$230 Derivatives. To prepare histidine-tagged TBP mutants, the 0.7-kb *Nde*I–*Bam*HI fragment encoding yeast TBP (yTBP) (18) was subcloned into the *Escherichia coli* expression vector pET28a (Novagen). Site-directed mutagenesis was performed as described (19). To prepare histidine-tagged dTAF$_{II}$230-(1–81) and dTAF$_{II}$230-(1–158), DNA encoding the corresponding sequence was amplified by PCR as *Nde*I–*Eco*RI fragments and subcloned into pET28a. Histidine-tagged protein was expressed in *E. coli* BL21(DE3) (Novagen) and purified as described (20). ^{35}S-labeled yTBP was produced by an *in vitro* transcription–translation coupled system (Promega). dTAF$_{II}$230-(2–81) (ref. 17), VP16 (ref. 21), and VP16Δ456 (ref. 21) were expressed as glutathione *S*-transferase (GST) fusion proteins. *E. coli* was disrupted in buffer B (20 mM Tris·HCl, pH 7.9/12.5 mM $MgCl_2$/10% glycerol/0.1 M KCl, 1 mM phenylmethylsulfonyl fluoride/1 mM DTT) and crude extracts were used for interaction experiments. For gel shift experiments, GST–VP16 and GST–VP16Δ456 were purified by glutathione-Sepharose 4B affinity chromatography with buffer B according to the manufacturer's instructions (Pharmacia).

Protein–Protein Interactions. To study interactions between TBP and dTAF$_{II}$230, *E. coli* extracts containing GST–dTAF$_{II}$230-(2–81) (30 pmol) and yTBP (30 pmol) were mixed in 100 μl of buffer B containing 50 μg/ml of BSA. To study interactions between VP16 and TBP, an *E. coli* extract containing GST–VP16 (10 pmol) and 5 μl [^{35}S]yTBP were incubated in 50 μl of buffer B containing 50 μg/ml of BSA at 4°C for 30 min. Samples were further incubated with 10 μl of glutathione-Sepharose 4B beads (Pharmacia) at 4°C for 30 min with rotation. The matrix was washed three times with 500 μl of buffer B. Protein was eluted with 10 μl of SDS sample buffer, separated by SDS/PAGE, and visualized by Coomassie brilliant blue staining or autoradiography.

Gel Shift and *in Vitro* Transcription Assays. Gel shift was performed as described (20) with a probe containing adenovirus major late promoter sequences from −40 to +10. The ability of TBP mutants to support basal transcription was determined in a TBP-dependent transcription system (22).

RESULTS

dTAF$_{II}$230-(2–81) Interaction Site on TBP. The TBP core has a highly symmetric structure resembling a molecular "saddle" (23). The saddle's concave face plays an important role in TATA box binding, whereas the convex face of the saddle provides interaction sites for several initiation factors that include TFIIA, TFIIB, and dTAF$_{II}$42 (reviewed in refs. 24 and 25). To understand the mechanism by which dTAF$_{II}$230-(2–81) inhibits TBP function, we attempted to map the TBP residues important for its interaction with dTAF$_{II}$230-(2–81). First, we tested the specificity and strength of the interaction between wild-type TBP and either wild-type or mutant dTAF$_{II}$230-(2–81) proteins. The dTAF$_{II}$230-(2–81) proteins, expressed as GST fusions, were incubated with equimolar amounts of TBP and subsequently purified by glutathione-Sepharose affinity chromatography. A Coomassie blue-stained SDS/PAGE gel (Fig. 1*C*) of the bound proteins showed that TBP binds almost stoichiometrically to the wild-type GST–dTAF$_{II}$230-(2–81) (lane 2), whereas no binding is observed with the GST–dTAF$_{II}$230-(2–81) containing alanine substitutions in five contiguous residues between positions 24 to 28 (ref. 17) (lane 4).

To define the interaction face on TBP, we tested various TBP mutants having single amino acid substitutions of either leucine for lysine or vice versa (26). To exclude mutants that may be nonfunctional because of improper folding, we selected only those mutants which can support basal transcription in a TBP-dependent transcription system (26). These mutants include K83L, L87K, L114K, K133L, K134L, K138L, K145, K151L, K156L, K201L, and K218L (Fig. 1 *A* and *B*). Among these mutants only L114K, bearing a mutation on the concave surface, showed a complete failure to interact with dTAF$_{II}$230-(2–81) (Fig. 1*C* and data not shown).

For a more detailed analysis, mutations were introduced at residues surrounding L114. These included E93K, F99K, I103K, R105L, A113K, A113L, I115K, F116K, M121K, and V122K (Fig. 1 *A* and *B*). Consistent with the fact that this surface is important for DNA-binding (24, 27), none of these mutants other than E93K bound detectably to DNA in a gel shift assay (Fig. 1*D* and data not shown). However, of the 10 new mutants, 5 (E93K, F99K, I103K, F116K, V122K) behaved like L114K (26) and supported basal transcription (Fig. 1*E* and data not shown). This suggests that while the intrinsic DNA-binding activity of these mutants alone may be too weak to be detected by gel retardation analysis, they may still be functional as a result of stabilizing interactions with other initiation factors (see, for example, refs. 28 and 29). The ability of these five transcriptionally competent mutants to interact with dTAF$_{II}$230-(2–81) was determined (Fig. 1*C*). The mutant (E93K) that maintained DNA binding activity showed ≈50% loss of the dTAF$_{II}$230-(2–81) binding activity, whereas those mutants that failed to show DNA binding exhibited over a 90% reduction in dTAF$_{II}$230-(2–81) binding. Hence, four novel mutants with alterations in the N-terminal side of the concave face of TBP showed phenotypes identical to those of L114K—namely, loss of intrinsic TATA binding and dTAF$_{II}$230-(2–81) binding but retention of basal transcription function.

Given that TBP has intramolecular two-fold symmetry (Fig. 1*A*), we tested whether dTAF$_{II}$230-(2–81) also binds to the symmetric region in the C-terminal repeat of the concave face. The symmetric residues for F99, I103, L114, F116, and V122 are F190, I194, V205, F207, and V213, respectively. Of the five mutants, four (F190K, I194K, V205K, and V213K) did not support basal transcription (Fig. 1*E*). These TBP mutants also showed no detectable binding to dTAF$_{II}$230-(2–81) (data not shown). However, these results cannot be given a structural interpretation because the absence of basal transcription could mean the protein has not folded properly. On the other hand, F207K exhibited a reduced but significant level of basal transcription (Fig. 1*E*) and, in contrast to F116K, retained ≈50% of the dTAF$_{II}$230-(2–81) binding activity (Fig. 1*C*, compare lanes 14 and 18). This retention of significant binding activity following mutation of a symmetrically related amino acid suggests that dTAF$_{II}$230-(2–81) may not bind to the symmetric face, although additional functional mutants must be analyzed for this to be regarded as a firm conclusion.

dTAF$_{II}$230-(1–81) and the VP16 Activation Domain Bind Competitively to TBP. It has been shown previously that the

with wild-type (lanes 1–4) or mutant (lanes 5–18) TBP. GST–dTAF$_{II}$230-(2–81) with alanine substitutions in five contiguous residues (residues 24–28) (ref. 17) was used as a control (lanes 3 and 4). Input (odd lanes) and purified (even lanes) materials were analyzed by SDS/PAGE and visualized by Coomassie brilliant blue staining. (*D*) Analysis of TBP–DNA interactions by gel shift assay. Reaction mixtures contained 0.5 (odd lanes) or 5 (even lanes) pmol of wild-type (lanes 1 and 2) or mutant (lanes 3–14) TBP. (*E*) Analysis of basal transcription activity. Transcription activity was determined in a TBP-dependent reconstituted transcription system with 1 pmol of wild-type (lane 1) or mutant (lanes 2–12) TBP. The position of the accurately initiated transcript is indicated by an arrow.

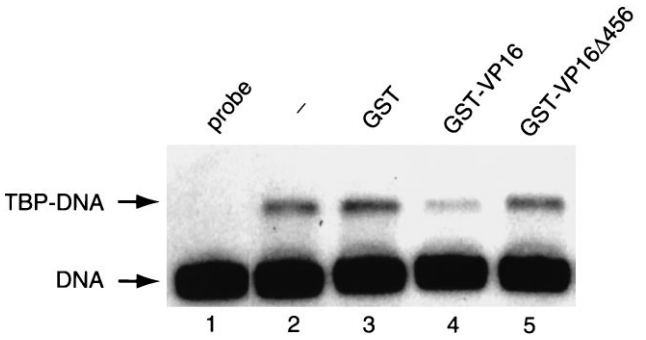

FIG. 2. The VP16 activation domain inhibits TBP binding to the TATA box. All lanes except lane 1 contained 0.125 pmol of yTBP. The reaction mixture contained 12.5 pmol of GST (lane 3), GST–VP16 (lane 4), or GST–VP16Δ456 (lane 5).

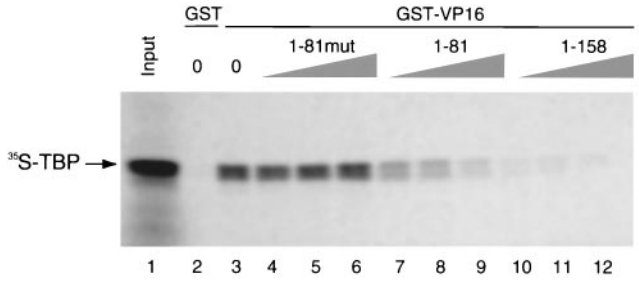

FIG. 3. $dTAF_{II}230$-(1–81) and the VP16 activation domain competitively bind to TBP. GST (lane 1) or GST–VP16 (lanes 3–12) were incubated with ^{35}S-labeled yTBP in the presence of histidine-tagged $dTAF_{II}230$-(1–81) with alanine substitutions in five contiguous residues (residues 24-28) (ref. 17) (lanes 4–6), $dTAF_{II}230$-(1–81) (lanes 7–9), or $dTAF_{II}230$-(1–158) (lanes 10–12). Input (lane 1) and purified materials were analyzed by SDS/PAGE and visualized by autoradiography.

L114K mutation in TBP eliminates a stable interaction with the VP16-activation domain (28). As described above, the L114K mutant also fails to interact with $dTAF_{II}230$-(2–81) (Fig. 1*C*, lane 12). These results suggest that both proteins bind to the concave face of TBP, but do not exclude the formal possibility that the L114K mutation alters the conformation of TBP in a way that destroys an interaction site.

If L114 is a target for the VP16 activation domain, excess amounts of the activation domain might be expected to affect TBP binding to the TATA box. To test this possibility TBP–DNA interactions were determined in the presence of the VP16 activation domain or a truncated version, VP16Δ456, which has much weaker TBP-binding activity in comparison to the intact domain (30) (Fig. 2). VP16 moderately inhibited the TBP–DNA interaction (lane 4), whereas VP16Δ456 showed no obvious effect (lane 5). These results were reproducible in multiple independent experiments.

For further confirmation that the VP16 activation domain and $dTAF_{II}230$-(1–81) both bind to the TBP concave surface, we tested whether their binding is competitive (Fig. 3). $dTAF_{II}230$-(1–81) drastically inhibited the interaction between VP16 and TBP in a dose-dependent manner (lanes 7–9), whereas the mutant $dTAF_{II}230$-(1–81), which does not bind to TBP, did not (lanes 4–6). From these results, we conclude that L114 is itself, or affects, a bona fide interaction site for both VP16 and $dTAF_{II}230$-(2–81).

$dTAF_{II}230$-(82–158) Contributes to Stable TBP Binding. Both the TBP binding and inhibitory activities of $dTAF_{II}230$ are conserved in the homologous yeast $TAF_{II}130$ ($yTAF_{II}130$) (T.K., J.-i.N., and Y.N., unpublished data). A detailed analysis indicates that the $yTAF_{II}130$ N-terminal 96 amino acid residues contain two functional subdomains, termed I and II (T.K., J.-i.N., and Y.N., unpublished data). Sequence alignment (Fig. 4*A*) suggests that $dTAF_{II}230$-(2–81) corresponds to subdomain I, although the similarity is low, and that $dTAF_{II}230$ (82–158)-corresponds to subdomain II. Unlike $dTAF_{II}230$-(2–81), $yTAF_{II}130$ subdomain I by itself binds poorly to TBP. When both $yTAF_{II}130$ subdomains I and II are present, $yTAF_{II}130$ binds to TBP strongly and inhibits TBP function (T.K., J.-i.N., and Y.N., unpublished data).

The function of the subdomain II-homologous region in $dTAF_{II}230$ was determined by a competition experiment (Fig. 4*B*). $dTAF_{II}230$-(1–81), which includes subdomain I, and $dTAF_{II}230$-(1–158), which includes both subdomains I and II, were used as competitors. The affinity of His-$dTAF_{II}230$-(1–158) for TBP (lanes 4 and 5) was obviously stronger than that of His–$dTAF_{II}230$-(1–81) (lanes 2 and 3), suggesting that amino acids 82–158 in $dTAF_{II}230$ indeed contribute to stable

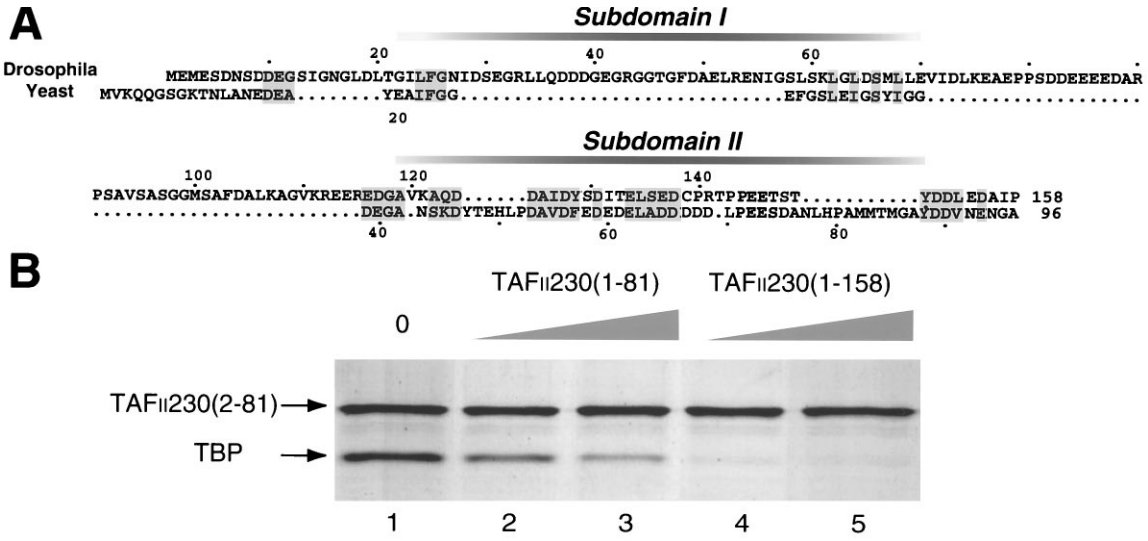

FIG. 4. $dTAF_{II}230$-(82–158) contributes to stable TBP binding. (*A*) Sequence alignment of N-terminal regions of $dTAF_{II}230$ and $yTAF_{II}130$. Dots indicate gaps introduced to maintain optimal alignment. Shading indicates residues identical or similar to each other. The positions of subdomains I and II are indicated. (*B*) TBP binding affinity of $dTAF_{II}230$ N termini. GST–$dTAF_{II}230$-(2–81) (40 pmol) was incubated with TBP (40 pmol) in the absence (lane 1) or in the presence of histidine-tagged $dTAF_{II}230$-(1–81) (lanes 2 and 3) or histidine-tagged $dTAF_{II}230$-(1–158) (lane 4 and 5) at 60 (lanes 2 and 4) and 200 (lanes 3 and 5) pmol. Binding experiments are represented as in Fig. 1*C*.

TBP binding. Consistent with this idea, and in support of the conclusion that VP16 and the $dTAF_{II}230$ N terminus compete for binding to TBP, $dTAF_{II}230$-(1–158) was more efficient than $dTAF_{II}230$-(1–81) in blocking VP16–TBP interactions (Fig. 3, lanes 7–9 vs. 10–12).

DISCUSSION

Several reports have suggested that the concave face of TBP might play an important role in transcriptional activation. TBP mutants (L114K and K211L) that selectively block activator (GAL4–VP16)-induced (but not basal) transcription have been identified by biochemical assays *in vitro* (28). Significantly, these mutations map to the concave face and affect DNA binding. Consistent with the *in vitro* results on VP16 interactions, these mutants are also impaired in their response to acidic activators *in vivo* but appear normal for basal RNA polymerase II transcription. Furthermore, yeast genetic screens have allowed the isolation of more TBP mutants (S118L, F148L, N159L, and V161A in ref. 31; P109A and N159D in ref. 32) that are defective both in activated (but not basal) transcription and in TATA box binding.

One of the TBP mutants (L114K) identified by the biochemical analysis impairs binding to VP16 (ref. 28). Although it was formally possible that loss of the interaction could be a result of a conformational change in the convex face of TBP caused by the mutation, there is now strong evidence to suggest that the deficiency is due to a direct effect. The L114K TBP mutation, which is on the concave face, blocks binding of both the VP16 activation domain and $dTAF_{II}230$, and these two proteins compete for binding to TBP. In addition, the VP16 activation domain inhibits partially the TBP–TATA interaction, which is mediated by the concave face of TBP. It should be noted that inhibition of the TBP–TATA interaction and/or TBP-mediated transcription by some activators has been reported by several groups (9, 33), but the possibility that this effect could be due to activator interactions with the concave face of TBP has not previously been recognized.

We have found that a second region of $dTAF_{II}230$, $dTAF_{II}230$-(82–158), also contributes to stable interactions with TBP (Fig. 4). Other studies have suggested that a corresponding region (subdomain II) in $yTAF_{II}130$ interacts with basic residues in α-helix-H2 on the convex surface on TBP (T.K., J.-i.N., and Y.N., unpublished data). This suggests that the extended $dTAF_{II}230$ N-terminal region (residues 1–158) binds to both concave and convex TBP surfaces to form a stable complex. We speculate that these negative interactions are dynamic and may play a role in transcriptional regulation. Indeed, binding studies have indicated that the strong $dTAF_{II}230$–TBP interaction is not necessary to hold $dTAF_{II}230$ in the TFIID complex, either because of tethering by other TAFs (e.g., $dTAF_{II}62$) (13, 17) or because of a second TBP interaction site (17, 34).

Given that TBP mutations in the basic repeats also affect TFIIA interactions (refs. 29, 35–37; T.K., J.-i.N., and Y.N., unpublished data), our data suggest that acidic activators and TFIIA might counteract, perhaps synergistically, interactions between the N terminus of $dTAF_{II}230$ and TBP. This competition might be related to the stimulation of TFIID binding to the promoter in an activator and TFIIA dependent manner (8, 9). However, in view of the close apposition of the TBP concave face and the TATA element in the cocrystal structure (24, 27), we do not predict that the activator–concave surface interactions persist, but rather than they are transient, during preinitiation complex formation and function.

Our earlier experiments with partially purified TFIID demonstrated that TFIID binds stably to the gfa promoter and gives footprints extending from sequences upstream of the TATA box through a downstream initiator-like element (15). In contrast, the same promoter with a mutated initiator-like element shows very weak footprints with TFIID not only at the downstream initiator regions but also at the TATA box (15). Consistent with these results TBP-dependent transcription of the gfa promoter in a reconstituted system was marginally affected by deletion of the initiator-like sequences, whereas TFIID-dependent transcription was almost completely dependent upon both TATA and initiator-like sequences. These results suggest that TAFs can stabilize or destabilize TFIID binding, depending on the sequence of the core promoter, and that these interactions have functional consequences. When the initiator-like element is present, the negative interaction between the N-terminus of $dTAF_{II}230$ and TBP may be counteracted through interactions between the initiator-like element and TAFs in a direct or indirect way. In contrast, when the initiator-like element is absent, TFIID binding may be destabilized because of the inhibition by the N terminus of $dTAF_{II}230$. In support of this view, experiments with highly purified TFIID indicated that a variety of TATA box-containing promoters that lack strong initiator elements bind poorly to TFIID (38). Moreover, recent reports have demonstrated that a recombinant TBP–$dTAF_{II}150$–$dTAF_{II}230$ complex binds more stably than TBP to a core promoter containing both TATA and downstream elements, reflecting the ability of TAFs (possibly $dTAF_{II}150$) to bind specific promoter sequences, whereas the same complex binds less stably than TBP when the downstream sequences are absent (16). In a similar analysis a TBP–$dTAF_{II}230$–$dTAF_{II}110$–$dTAF_{II}62$ complex that lacks $dTAF_{II}150$ and cannot bind to the the downstream sequences was reported to bind to the core promoter. However, this interaction was weak and observed in the presence of a 10,000-fold molar excess of the partial TFIID over the DNA probe, consistent with the possibility that the $dTAF_{II}230$ N terminus may have restricted TBP binding in the absence of certain other TAFs or interacting DNA sequences.

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