Identification of Highly Conserved Amino-terminal Segments of dTAF\(_{II}\)230 and yTAF\(_{II}\)145 That Are Functionally Interchangeable for Inhibiting TBP-DNA Interactions in Vitro and in Promoting Yeast Cell Growth in Vivo* 

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TFIID is a multiprotein complex composed of TBP and several TAF\(_{I}\)s. Small amino-terminal segments (TAF N-terminal domain (TAND)) of Drosophila TAF\(_{II}\)230 (dTAF\(_{II}\)230) and yeast TAF\(_{II}\)145 (yTAF\(_{II}\)145) bind strongly to TBP and inhibit TBP-DNA interactions. yTAF\(_{II}\)145 TAND (yTAND) was divided into two subdomains, yTAND\(_{10-37}\) and yTAND\(_{48-71}\), that function cooperatively. Here, we identify dTANDII within the amino terminus of dTAF\(_{II}\)230 at 118–143 amino acids in addition to dTANDI\(_{18-77}\) reported previously. dTANDII exhibits pronounced sequence similarity to yTANDII, and the two were shown to be functionally equivalent in binding to TBP and inhibiting TBP-DNA interactions in vitro. Alanine scanning mutation analysis demonstrated that Phe-57 (yTANDII) and Tyr-129 (dTANDII) are critically required for the function of this segment in promoting normal cell growth at 37 °C. In these respects, the impact of yTANDII mutations on cell growth paralleled their effects on TBP binding in vitro, strongly suggesting that the yTAF\(_{II}\)145-DNA interaction and its negative effects on TFIID binding to core promoters are physiologically important. 

Transcription of protein coding genes in eukaryotes is carried out by RNA polymerase II and a set of auxiliary initiation factors (1, 2). These factors, including TFIID, TFIIB, TFIIF, and TFIIE, can be assembled in a combinatorial fashion in vitro to form a preinitiation complex. Recently, it was proposed that most of these factors are preassembled in vivo in the form of holoenzyme and recruited as a single complex to the core promoter to initiate transcription (3, 4). 

Barberis et al. (5) reported that recruitment of holoenzyme via a fortuitous interaction between GAL4 DNA binding domain and GAL11 or by fusing lexA to GAL11 would suffice for gene activation in Saccharomyces cerevisiae. A similar result was obtained for SRB2, another component of holoenzyme (6). On the other hand, there is evidence that TBP binding to the TATA box is also a rate-limiting step for transcriptional activation that can be accelerated by gene-specific activators (7). In fact, a physical connection of TBP to a DNA binding module bypasses the requirement for activators (8–10). Given that TBP is a subunit of TFIID and not a component of holoenzyme (11), it appears that recruitment of either TFIID or holoenzyme will suffice for gene activation in yeast (12). However, it is notable that TFIID is required for both cases because mutation of the TATA sequence greatly decreased activation even by holoenzyme recruitment (5). 

In higher eukaryotes, the question of how activators stimulate transcription has been addressed mostly by biochemical approaches. Particular attention has focused on TFIID, a multiprotein complex composed of TBP and a series of TBP-associated factors (TAF\(_{I}\)s), because TAF\(_{I}\)s were shown to be indispensable for activated transcription in vitro (13, 14). We and others cloned cDNAs encoding TAF\(_{I}\)s from various organisms to decipher the molecular basis of transcriptional regulation (15). It is currently known that TAF\(_{I}\)s possess some intriguing structural motifs and/or enzymatic activities. For instance, dTAF\(_{I}\)62/dTAF\(_{I}\)42 forms a histone octamer-like heterotetrameric structure (16). dTAF\(_{II}\)230 has multiple enzymatic activities, including a protein serine kinase activity that selectively phosphorylates RAP74 (17) and a histone acetyltransferase activity specific for histones H3 and H4 (18). Furthermore, several TAF\(_{I}\)s were shown to provide interaction sites for distinct activators and general transcription factors (19). Despite these observations, little is known about how each TAF\(_{II}\) is involved in decoding the information sent from activators and how the components of the basal transcriptional machinery communicate with each other to determine the final level of transcription. 

We previously identified a surprising activity within the amino terminus of dTAF\(_{II}\)230/yTAF\(_{II}\)145 that strongly inhibits TBP-DNA interactions (20–23). Cumulative evidence plausibly supports the notion that some of the TAF\(_{I}\)s might bear such an inhibitory activity critical for promoter recognition and transcription (24–27). First, TBP-TAF\(_{II}\)250-TAF\(_{II}\)150 subcomplex discriminates core promoter structures so that it can bind more stably to the DNA that has both TATA sequence and downstream core promoter elements (e.g. initiator) but less stably to the TATA sequence alone (25). Given that TBP binds equally to the DNA with or without downstream elements, TAF\(_{II}\)250 or
TAF$_{150}$ must negatively regulate TBP binding to the TATA sequence in the absence of downstream elements. In another experiment, the inhibitory effect of TAF$_{250}$ on TBP-mediated basal activity observed for TBP-TAF$_{250}$ subcomplex on the HIV or E1B core promoter was specifically antagonized by transcriptional activators (26). Second, gel shift and DNase I footprinting studies demonstrated that activators such as GAL4VP16 and Zta facilitate the formation of the TFID-TFIID complex on the promoter (28–30). Importantly, TFIIDA and TAF$_{145}$ are essential for this enhancement. These findings could be interpreted to indicate that the inhibition of TBP binding to the promoter by TAF$_{145}$ (possibly by TAF$_{250}$) creates a rate-limiting step during the course of transcription that would be overcome with the aid of both activators and TFIIDA (27). This idea is further supported by our observation that TFIIDA can compete with the amino terminus of yTAF$_{145}$ for TBP interaction (23).

Here we designate the aforementioned amino-terminal small portion of TAF$_{145}$ that inhibit TBP-DNA interactions specifically as TAF N-terminal domain (TAND) because the corresponding activities seem to be conserved among the species (23, 31).

We previously demonstrated that yTAFII$_{145}$ TAND (yTAND) was further divided into two subdomains, yTANDI (10–37 aa) and yTANDII (46–71 aa), that function cooperatively (23), whereas dTAFII$_{230}$ TAND (dTAND) functioned as a single entity (21). In this report, we identify dTANDI at 118–143 aa, neighboring the previously identified dTAND (hereafter called dTANDI; 18–77 aa). dTANDII is highly homologous to yTANDII. Intriguingly, however, neither corresponding subdomains of dTAND can function equivalently in vivo to the yeast TANDs, even though they are entirely interchangeable in vitro. Finally, we identify the highly conserved segments within TANDII that are functionally interchangeable in vitro and in vivo. These results have important implications for the molecular interaction between TBP and TAND and its role in transcriptional regulation.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains and Genetic Analysis**—Standard yeast genetic techniques were used for the growth and transformation of the yeast strains (22, 33).

Table I lists the S. cerevisiae strains used in this study. All yeast strains are derived from Y22.4 containing a deletion of the chromosomal yTAF$_{145}$ coding region and the wild-type yTAF$_{145}$ gene on a URA3-based low copy number vector (23). To determine the abilities of the yTAF$_{145}$ mutants to support yeast cell growth at elevated temperatures, a plasmid shuffle technique was used (34). Strain Y22.4 was transformed with plasmids derived from pRS314 (32, 33).

**TABLE I**

| Strain | Genotype |
|--------|----------|
| Y22.1  | MATa ura3–52 trp1–63 leu2,3–112 Δof145 pYN1/TAF145 |
| YKII   | MATa ura3–52 trp1–63 leu2,3–112 Δof145 pM11/TAF145 (y1-YY) |
| YKII2  | MATa ura3–52 trp1–63 leu2,3–112 Δof145 pM756/TAF145 (dy-YY) |
| YKII3  | MATa ura3–52 trp1–63 leu2,3–112 Δof145 pM757/TAF145 (dls-YY) |
| YKII4  | MATa ura3–52 trp1–63 leu2,3–112 Δof145 pM758/TAF145 (y1-YY) |

For experiments using the GAL4VP16 and ZTA proteins, a plasmid shuffle technique was used (34). Strain Y22.4 was transformed with a plasmid containing the GAL4VP16 or ZTA gene in a pRS314-based low copy number vector (23). To determine the abilities of the yTAF$_{145}$ mutants to support yeast cell growth at elevated temperatures, we used a plasmid shuffle technique (34). Strain Y22.4 was transformed with plasmids derived from pRS314 (32, 33).
Chimeric Analysis of yTAF145 and dTAF230 Amino Termini

AGGGATCCC GCCGGA AATCTCA AACAGG TCTAC3-'). To prepare N-CORE/CORE/CORE chimera, yI-YD was first constructed from yI-YY by using oligonucleotide TK42 (5'-AGCAGTTTGGCGCTGTA- TAGATTATCGATATAACTGATCTTGAAGCTGAGATTGCTTGG-3') and yI-YD by using TK68 (5'-GCGGGTTC GGCAGGGAGGCGCTGTAAG-3'). yI-YD was thus cloned into pBlueScript II to introduce alani ne sequence mutagenesis into the yTAND CORE region. Oligonucleotides used for this purpose were as follows: TK45 for D53A, 5'-TACCGAAGATCTGGGCCTGCTGTAATTGTAAG-3'; TK46 for V55A, 5'-GCATTCCGCTGCCTGGATTTTGAAGATG-3'; TK47 for D56A, 5'-TTTCGCGATGTCGCTGTAAG-3'; TK48 for F57A, 5'-TGCCGAGTCGTGAGATTCTGGAAGATGATGA-3'; TK49 for E58A, 5'-GGACGTGCTGAGGAGGTGAATTCTGGAAGATG-3'; TK50 for D59A, 5'-TGTGCTGAGGAGGTGAATTCTGGAAGATG-3'; TK51 for E60A, 5'-GAGCTGCTGAGGAGGTGAATTCTGGAAGATG-3'; TK52 for D61A, 5'-AGATTGCTGCTGAGGAGGTGAATTCTGGAAGATG-3'; TK53 for E62A, 5'-TTTGGAGATTCTGGAAGATGATGA-3'; TK54 for D65A, 5'-TGGCGAGTCGTGAGATTCTGGAAGATG-3'; and TK55 for D66A, 5'-AGATGCTGCTGAGGAGGTGAATTCTGGAAGATG-3'.

Preparation of GST-TAND Derivatives and TBP, and Protein-Protein Interaction Assays—TAND derivatives for the protein-protein interaction assay were expressed in Escherichia coli (DH5a) as GST fusion proteins. The cell pellet was suspended in 5/6 culture volume of Buffer D (20 mM Tris-HCl at pH 7.9, 12.5 mM MgCl2, 0.2 mM EDTA, 10% (v/v) glycerol, 1 mM dithiothreitol, 350 ng/ml bestatin, 2 mM benzamidine, 400 ng/ml pepstatin A, 500 ng/ml leupeptin), 1 mM phenylmethylsulfonyl fluoride) containing 0.2 M KCl and sonicated several times to prepare cell lysates. D (20 mM Tris-HCl at pH 7.9, 12.5 mM MgCl2, 0.2 mM EDTA, 10% (v/v) glycerol, 1 mM dithiothreitol, 350 ng/ml bestatin, 2 mM benzamidine, 400 ng/ml pepstatin A, 500 ng/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride). Precipitates were immunoblotted as described above.

Co-immunoprecipitation Analysis—Co-immunoprecipitation analyses was conducted as described previously (31) except using BA/200 extraction buffer (20 mM Hepes-KOH (pH 7.6), 2 mM EDTA, 2 mM EGTA, 0.25% Nonidet P-40, 200 mM potassium acetate, 1 mM dithiothreitol, 350 ng/ml bestatin, 2 mM benzamidine, 400 ng/ml pepstatin A, 500 ng/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride). Yeast cells at the log phase were harvested by centrifugation, washed with ice-cold water, and resuspended in cracking buffer (40 mM Tris-HCl (pH 6.8), 0.1 mM EDTA, 8 mM urea, 5% (w/v) SDS, 5% β-mercaptoethanol, 350 ng/ml bestatin, 2 mM benzamidine, 400 ng/ml pepstatin A, 500 ng/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 0.4 mg/ml BSA) (36). The cells were sonicated several times and boiled at 95 °C for 3 min. For Western blotting, cell extracts were separated on 6% SDS-PAGE and transferred to nitrocellulose membranes, and probed with anti-yTAF145 antibody (37). Detection of the immune signal was done with the Amersham Pharmacia Biotech ECL Western detection kit.

RESULTS
dTAF230 TAND Has Two Subdomains—We previously demonstrated that yTAND can be divided into two subdomains, yTANDI (10–37 aa) and yTANDII (46–71 aa), both of which are required for full activity (23). In contrast, we originally identified dTAND (18–77 aa) as a single functional unit (21) and noted several other differences between these two TANDs. First, the yTAND-TBP complex is highly salt-sensitive, being disrupted in buffer containing ~0.3 M KCl, whereas the dTAND-TBP complex is resistant to salt concentration up to ~1.0 M. Second, a TBP mutant (K133E/K138E/K145E), in which three lysines at positions 133, 138, 145 on helix 2 were changed to oppositely charged glutamates, could not interact with TFIID and TFIIE simultaneously. In contrast, it could still bind to dTAND, suggesting that only yTAND shares the interaction surface with TFIID. The latter conclusion was verified by biochemical competition analysis (23).

Given that the interactions between TBP, TAND, and TFIID are expected to play a significant role in transcriptional regulation, we presumed that they would be conserved across the species. However, obvious sequence similarities were not found between yTAND (10–76 aa) and dTAND (18–77 aa). Thus, we examined these elements more extensively for sequence conservation (Fig. 1, A and B). The region from 125 to 138 aa (DADYSDITELESD) outside the originally identified dTAND was found to be significantly similar to the region from 53 to 66 aa of yTANDII (DAVDFEDEELADD, see Fig. 1A) (22, 23). Intriguingly, the corresponding region is also conserved in human TAF250 (Fig. 1B) (DAVYDSDINEVEAD, the region from 106 to 119 aa). Therefore, we hypothesized that dTAND also consists of two subdomains, the minimally conserved dTANDI(18–77 aa; originally identified as dTAND) and more highly conserved dTANDII (118–143 aa) (Fig. 1A), both of which would be required for full activity. Indeed, the additional stabilizing effect of dTANDII on TBP binding was observed by the biochemical competition analysis (22). We designated the most conserved region in TANDII, composed of 14 amino acid residues, as the core region (CORE; Fig. 1B).

To test this hypothesis more directly, we asked whether dTANDII could substitute for yTANDII in forming a stable TBP-yTAND protein complex in vitro. Toward this end, we produced chimerical GST-TAND fusion proteins in bacteria and used them for GST pull-down experiments with purified recombinant TBP, GST fusions containing only yTAND (y1), yTAND (y1I), or dTANDII (d1II) showed little or no TBP binding activity (Fig. 1D, lanes 2, 3, and 5). However, the chimerical construct containing yTAND in TANDII (y1II) bound TBP nearly as strongly as did the wild type construct (y1y1I) (Fig. 1D, lanes 1 and 4). Thus we conclude that dTANDII is functionally equivalent to yTANDII for TBP binding in vitro and that a bipartite subdomain structure is conserved in both TANDs. The Highly Conserved Core Regions of TANDII from Yeast and Drosophila Function Independently of Flanking Nonconserved Regions—In our previous studies, mutations in the highly conserved core region (CORE) of yTANDII (e.g., Δ54–57 aa) affected the TBP binding activity most severely (23). Mutations close to the CORE (e.g., Δ46–49 aa) also impaired the activity but to a lesser extent. Sequence similarities between...
the species were confined to the CORE (Fig. 1A), suggesting that it is primarily responsible for TBP binding and that other regions of TANDII simply facilitate interaction between the CORE and TBP. To explore this possibility further, we divided TANDII into three subregions, namely, amino-terminal side of the CORE (N-CORE), the CORE itself, and carboxyl-terminal side of the CORE (C-CORE) and constructed the chimeric GST fusion proteins shown in Fig. 2A. We reasoned that if the N-CORE and C-CORE subregions of TANDII interact specifically with the CORE to stabilize a conformation of TANDII competent for TBP binding, we might find that certain combinations of heterologous segments would be unable to interact effectively with TBP. In contrast, if the N-CORE and C-CORE segments serve a less specific function, e.g. providing the proper spacing between the TANDII CORE and TANDI, then all of the chimerical molecules should bind TBP with similar efficiencies. As shown in Fig. 2B, the latter possibility was observed, indicating that the CORE regions from yeast and Drosophila function independently of the flanking nonconserved regions present in the two TANDII elements.

Thus, the fact that truncation mutation Δ46–49 aa diminished yTAND activity in previous studies (23) can probably be attributed to the fact that it reduced the spacing between yTANDI and the CORE of yTANDII.

dTANDII Can Inhibit TBP-DNA Interactions When Connected to yTANDI—To determine whether dTANDII can inhibit TBP-DNA interactions, we performed electrophoretic mobility shift assays with recombinant TBP and the GST-TAND fusion proteins described in Fig. 2 (Fig. 3). Like the original yTAND (i.e. yI-YYY; Fig. 3, lanes 2–4), chimeric polypeptides, such as yI-DDD (lanes 14–16) and yI-YDY (lanes 17–19), were able to inhibit TBP binding to the TATA box when present at stoichiometric amounts with TBP. Control polypeptides that bind to TBP quite unstably, i.e. yI (lanes 5–7), yII (lanes 8–10), and dII (lanes 11–13), did not inhibit the TATA binding of TBP as expected. We conclude that dTANDII is functional in inhibiting TBP-DNA interactions. The fact that dTANDII confers both activities of TANDs, TBP binding and inhibition of TBP-

FIG. 1. TANDII is highly conserved structurally and functionally among the species. A. proposed subdomain structures of yTAF145N and dTAF230N. Previously we mapped yTANDI (10–37 aa), yTANDII (46–71 aa) and dTANDI (18–77 aa) as shown by rectangles above the sequences. dTANDII (118–143 aa) was tentatively assigned based on sequence similarities and biochemical competition analysis (22, 23). Highly conserved region composed of 14 amino acid residues within TANDII is especially designated as the CORE. Note that the exact boundaries of each subdomain indicated above the sequence are not yet finally proven. B. comparison of yTAF145N CORE with corresponding regions of dTAF230 and hTAF250. Similar or identical amino acids are shaded according to the following groupings: (E, D), (K, R, H), (N, Q), (A, I, L, M, V), (F, W, Y), (S, T), (C), (G), and (P). C. schematic diagram of GST-TAND fusions that we tested in D. BssHII and MluI sites were introduced at the indicated positions to make chimeras. The region from 116 aa to 145 aa of dTAF230 including dTANDII was fused to the carboxyl terminus of yTANDI to construct yIdII. yI, yII, dI, and dII denote yTANDI, yTANDII, dTANDI, and dTANDII, respectively. D. interaction of GST-TAND and yeast TBP. TAND fusion proteins were incubated with an equimolar amount of TBP. Complexes were purified by glutathione-Sepharose and analyzed by SDS-PAGE followed by CBB staining. The lower band of GST-TAFs in all lanes represents a cut form due to the protease-hypersensitive sites near the carboxyl terminus.
Fig. 2. The CORE region is primarily responsible for TBP binding. A, schematic diagram of TANDII chimeras that we tested in B. TANDII was divided into three subregions, N-CORE, CORE, and C-CORE, as indicated. The origin of each subregion was denoted as a single letter, Y (yTAF145) or D (dTAF230). Note that yl-YYY and yl-DDD are the same polypeptide as ylII and yldII, respectively, in Fig. 1. All constructs were expressed as GST-fusion proteins. B, interaction of GST-TANDII chimeras and yeast TBP are presented as in Fig. 1D.

Fig. 3. tTANDII can inhibit TBP-TATA interactions when fused with yTANDI. Inhibition of TBP binding to the TATA box by GST-TAND chimeras. Gel retardation assays were performed with a 3-fold (lanes 2, 5, 8, 11, 14, and 17), 10-fold (lanes 3, 6, 9, 12, 15, and 18), or 30-fold (lanes 4, 7, 10, 13, 16, and 19) excess of yl (lanes 5–7), YYY (lanes 8–10), DDD (lanes 11–13), yl-DDD (lanes 14–16), yl-YDY (lanes 17–19), and wild type yl-YYY (lanes 2–4) over the amount of yeast TBP (0.5 pmol). Single and double asterisks denote the positions of TBP-DNA complex and free probe, respectively.

TATA interactions is in accordance with previous results using TAND mutants, in which reductions in the two activities were closely correlated (21) (23).

Do Drosophila and Saccharomyces TANDII CORE Regions Recognize the Same TFIIA Interaction Sites on TBP?

—We previously demonstrated that the interaction between yTAND and TBP is highly salt-sensitive, suggesting that ionic interactions greatly contribute to stability of the yTAND-TBP complex. The yCORE is highly acidic (9 of 14 residues are Asp or Glu; Fig. 1B), and substitution of glutamate at position 60 in the yCORE with oppositely charged lysine nearly destroyed TAND activity (23). Moreover, the cluster of positively charged lysine residues at positions 133, 138, 145 on helix 2 of TBP was shown to be important for yTAND interaction. These results strongly suggest that yCORE interacts directly with a surface on TBP containing lysines at positions 133, 138, and 145. To address whether the less negatively charged dCORE (6 of 14 residues are Asp or Glu; Fig. 1B) can recognize the same site on TBP as yCORE, we tested the interaction between the TANDII chimeras and the mutant TBP (K133E/K138E/K145E) (Fig. 4A, right panel). Neither of the chimeras containing dCORE that we tested (yl-DDD (lanes 2 and 7) and yl-YDY (lanes 3 and 8)) could make a stable complex with TBP (K133E/K138E/K145E); this was the same result obtained for yl-YYY (Fig. 4A, lanes 1 and 6) and yl-DYD (lanes 4 and 9). In addition, they all formed complexes with wild type TBP that dissociated in buffer containing 0.5 M KCl (data not shown). Taken together, these findings suggest that the manner in which the TANDII CORE interacts with TBP is conserved between the species. As expected, Drosophila TAND (di) bound equally well to wild type and mutant TBP (Fig. 4A, lanes 5 and 10), indicating that it interacts with a different surface on TBP (23).

Side Chain of Phe-57 in yTANDII CORE Is Important for Interaction with TBP

—To obtain additional evidence that the CORE directly interacts with TBP, alanine scanning mutagenesis was employed to identify yCORE residues critically required for TBP binding. This approach yields side chain substitutions that eliminate all interactions made by side chain atoms beyond Cβ (38). We reasoned that if yCORE binds to TBP directly, then side chain substitutions at certain CORE positions should destroy TBP binding activity. Each residue of yCORE (except the two alanines) was individually substituted with alanine, and the mutant GST-TAND proteins were tested for TBP binding in the GST pull-down assay (Fig. 4B). The side chain of phenylalanine 57 was the most critical for TBP binding because the complex was barely detectable with the F57A-substituted GST-TAND protein. Importantly, the aromatic side chain seems to be conserved at this position in TANDII as tyrosines are found there in dTAFII230 and hTAF II250 (Fig. 3). Substitution of phenylalanine 57 with alanine in yI-YYY decreased TBP binding activity dramatically (Fig. 4C). These observations strengthen the argument that both CORE domains are functionally equivalent.

 Unexpectedly, we observed no effect on TBP binding in response to the side chain elimination of acidic residues despite the fact that simultaneous replacements of multiple acidic residues weakened the yTAND-TBP interaction (23). Presumably
they are functionally redundant such that loss of a single acidic side chain can be compensated by interaction via neighboring acidic residues. Collectively, our results suggest that the yCORE directly interacts with TBP by making a critical contact via Phe-57, and multiple redundant interactions with the surrounding acidic residues.

Only the dCORE Region Is Functional in Yeast Cells—As a first step toward characterizing the in vivo function of the TAND subdomains, we examined the growth phenotype of yeast strains expressing yTAFII145 proteins, which lack either one or both subdomain(s) or harbor various TAND chimeras, using the technique of plasmid shuffling (Fig. 5, A–C, summarized in Table II). As described previously, yeast strains lacking both yTANDI and yTANDII (ΔN; deleted for 10–73 aa) almost ceased growth at elevated temperature (37 °C) (23). Deletion of either subdomain leads to a more modest growth defect at 37 °C (Fig. 5A). By careful inspection we found that the order of growth rate at 37 °C is as follows: wild type > yI (lacking yTANDII) > yI-YYY (lacking yTANDI) > ΔN (lacking both yTANDI and II), suggesting that yTANDI is more important than yTANDII for growth at 37 °C.

Because dTANDII seems to function equivalently to yTANDII in in vitro assays, we next tested whether the presence of dTANDII in yTAFII145 can complement the growth defects of
Strains lacking yTANDII. Unexpectedly, strains harboring the chimeric yI-DDD version of yTAF\(_{145}\) grew more poorly at 37 °C than strains harboring only yTANDII (yI) (Fig. 5A), suggesting that the dTANDII interferes with the function of neighboring yTANDII in vivo. Fig. 5B shows the phenotypes of strains expressing yTAF\(_{145}\) proteins lacking yTANDII and containing various TANDII chimeras. The strain containing dTANDII (DDD) grew more poorly at 37 °C than that harboring yTANDII (YY) and was nearly indistinguishable from the ΔN strain. In contrast to the effect of replacing the entire yTANDII domain, yCORE was replaced successfully with dCORE, because the yI-YDY and YDY strains grew at the same rate as the yI-YYY and YYY strains, respectively (Fig. 5, A and B). Strains expressing yTAF\(_{145}\) proteins that lacked TANDII and contained two of the three subregions of TANDII of Drosophila origin (i.e. YDD, DDY, and YDY) grew at a much slower rate than did YYY or YDY strains (Fig. 5B).

Next, we tested whether dTANDII can complement the lack of yTANDII (Fig. 5C). Strains having only dTANDII (dI) grew more poorly than strains having yTANDII (yI). In addition, strains having dTANDII and yTANDII chimeras (dI-YYY) grew at a much slower rate than did strains having only yTANDII (YYY). Thus, it appears that dTANDII interferes with the function of neighboring yTANDII, similar to the negative interaction observed between yTANDII and dTANDII. Growth rates were not improved even when the spacer region between dTANDII and II in dTAF\(_{230}\) (81–113 aa in Fig. 1A) was included (designated as dIs-YYY or dI in Fig. 5C), excluding the possibility that a function indispensable for growth at 37 °C resides in the spacer region.

To analyze the expression of these yTAF\(_{145}\) proteins containing different TAND sequences, Western blotting was performed on whole cell lysates from each strain with polyclonal antibodies against residues 288–489 of yTAF\(_{145}\) (Fig. 5D, summarized in Table II). To minimize degradation of proteins, we lysed yeast cells in the electrophoresis loading buffer containing 8 M urea and protease inhibitors (38). yTAF\(_{145}\) derivatives yI, YYY, ΔN, and yI-YDY were expressed at the same level as the wild type yI-YYY protein at both growth temperatures (25 and 37 °C). Unexpectedly, however, yTAF\(_{145}\) derivatives dI, dl-YYY, yI-DDD, yI-DYPD, and DDD seemed to be more rapidly degraded, even at lower temperature (25 °C), judging from the presence of cross-reacting species with higher than expected mobilities. The decreased growth rate at 37 °C of these strains may not simply result from the reduced steady state level of yTAF\(_{145}\) because degradation was not enhanced at 37 °C relative to that seen at 25 °C (Fig. 5D). Accordingly, the growth defects at 37 °C may reflect a loss of function in these heterologous derivatives. Alternatively, it is possible that the reduced amounts of these yTAF\(_{145}\) derivatives have a greater impact on cell division when the metabolic rate is elevated by growth at higher temperatures.

Side Chain of Phenylalanine at Position 57 Is Important for Cell Growth—Residue Phe-57 in yTANDII was found to be indispensable for TBP binding by GST-TAND in vitro (Fig. 4B). To explore the importance of TBP binding to yTANDII via the yTANDII CORE in vivo, we examined the effects of alanine substitutions of different CORE residue on the growth rates at 37 °C. When the mutations were tested in the presence of yTANDI, they had little or no effect on growth (data not shown). This result is not surprising because strains lacking the whole yTANDI region can grow at 37 °C (Fig. 5, A and C, yI). Therefore, we tested the same set of CORE mutations in the absence of yTANDI (Fig. 6A). As expected, growth of the strain harboring the F57A mutation was most severely affected. Side chain truncations of residues Asp-56, Asp-59, Glu-60, and Leu-63 produced much smaller effects. (Note that growth of these mutants should be compared with that of their parental strain YYY.) For unknown reasons, the E58A mutation improved the poor growth phenotype of the YYY construct. Expression of these yTAF\(_{145}\) derivatives at 37 °C was found to be similar to that of wild type YYY (Fig. 6B). The fact that among the CORE point mutations, F57A had the most severe effect on both TBP binding to yTANDII in vitro (Fig. 4B) and cell growth at 37 °C (Fig. 6A) implies that the CORE-mediated interaction between TAND and TBP is physiologically important, at least in the absence of TANDI and during growth at elevated temperatures. Similar results were obtained by comparing the growth rate of YDY with that of YDY (Y129A) (Table II).

Next, we tested whether the F57A mutation may affect the amount of yTAF\(_{145}\)-TBP complex in vivo. Whole cell extracts (WCEs) were prepared from five strains cultured at 25 °C having yI-YYY, YYY, yI-YYY (F57A), YYY (F57A), and ΔN forms of yTAF\(_{145}\). Immunoprecipitates of these WCEs with anti-TBP antibody were fractionated by SDS-PAGE, blotted, and probed for the amount of yTAF\(_{145}\) derivatives that were complexed with TBP (Fig. 6C). Unexpectedly, the same amounts of yTAF\(_{145}\) proteins were coprecipitated with TBP in all strains we tested, indicating that the yTAF\(_{145}\)-TBP interaction cannot be disrupted in vivo even by deleting the whole region of TAND. Probably, other regions of yTAF\(_{145}\) or other TAFII components contribute to the stability of the yTAF\(_{145}\)-TBP interaction (or the integrity of TFIIID complex).

Our observations are inconsistent with previous study by Bai...
et al. (31) showing that there is less of the yTAFII145-TBP complex in cells harboring the ΔN allele of yTAFII145. Although the reason for this discrepancy is still unknown, it might be due to the difference of strains used in these two studies. Bai et al. (31) used pseudodiploid strains expressing both untagged wild type and HA-tagged ΔN forms of yTAFII145. In contrast, we used a haploid strain expressing only the untagged ΔN form of yTAFII145. Supposing that wild type protein is preferentially incorporated into the complex during the in vivo TFIID assembly, the amount of HA-tagged ΔN protein in the immunoprecipitates with anti-TBP antibody could be much smaller than that of HA-tagged wild type protein. In any case, further experiments are necessary to clarify this issue.

Finally, we tested whether TBP overexpression can rescue the temperature-sensitive growth of YYY (F57A) strain as it does that of ΔN strain (23, 31). When TBP was overexpressed from either a single-copy CEN/ARS vector or a multicopy 2-μm vector in the YYY (F57A) strain, it corrected the slow growth phenotype of this strain at higher temperatures (data not shown). This result strongly supports the in vivo relevance of the in vitro TAND-TBP interactions described above.

Heterologous Interactions between TANDII and TBP Induce Degradation of yTAFII145 Derivatives—As summarized in Table II, the presence of TANDII segments derived from dTAF230 (except the CORE region) was closely correlated with the reduced expression of yTAFII145 derivatives. Thus, we made an assumption that heterologous interactions between TAND and TBP might hamper certain unknown but physiologically important regulation (e.g. isomerization of TFIID complex), as they cannot be dissociated even in case of necessity. The amino-terminal portion of chimeric yTAFII145 proteins might be specifically removed to avoid such a deleterious interaction. To explore this possibility, we tested the effects of F57A and D61A mutations on the steady state level of the yI-DYD chimera. We reasoned that if the primary sequence itself derived from dTAF230 induce degradation of yTAFII145 proteins in vivo, yI-DYD (F57A) and yI-DYD (D61A) derivatives should be de-

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**FIG. 6.** Growth phenotype of yeast strains carrying yTANDII CORE alanine scanning mutants of yTAFII145 and their expression in vivo. A, strains carrying wild type yI-YYY (YKII11), YYY (YKII61), ΔN (YKII466), or yTANDII CORE alanine scanning mutations lacking yTANDI, D53A (YKII158), V55A (YKII159), D56A (YKII160), F57A (YKII161), E58A (YKII162), D59A (YKII163), E60A (YKII164), D61A (YKII165), E62A (YKII166), L63A (YKII167), D65A (YKII168), or D66A (YKII169) of yTAFII145 derivatives were grown on YPD at 37 °C. Photographs were taken after 3 days. These strains grew normally at 30 °C (data not shown). B, expression and stability of yTANDII CORE alanine scanning mutants. Cells were harvested before (data not shown) and at 24 h (lanes 1–13) after the temperature shift from 25 to 37 °C. Western blot analysis was done as in Fig. 5C. An asterisk denotes the position of yTAFII145 derivatives. C, the amounts of yTAFII145 derivatives complexed with TBP in the WCEs were compared by co-immunoprecipitation analysis. Cells were cultured at 25 °C until reaching the logarithmic growth phase and then disrupted by glass beads to prepare WCEs. WCEs were processed with anti-TBP antibody (odd-numbered lanes) or preimmune antibody (even-numbered lanes) as a control. The resulting precipitated proteins were immunoblotted as described in B for the presence of yTAFII145 derivatives (positions are marked by an asterisk). Strains used were yI-YYY (YKII11), YYY (YKII61), yI-YYY (F57A) (YKII152), YYY (F57A) (YKII161), and ΔN (YKII466). D, effects of CORE mutations on steady state level of yI-DYD derivatives. Strains carrying yI-DYD (YKII33), yI-DYD (F57A) (YKII342) and yI-DYD (D61A) (YKII345) were cultured at 25 °C to compare the expression level of yTAFII145 derivatives by Western blotting. A single asterisk denotes the position of intact yTAFII145 derivatives, and a double asterisk represents a strong cross-reacting material in yeast extracts (a nonspecific band). The position of degraded products of yTAFII145 derivatives is marked by a rectangle between these two asterisks.
graded at the same rate as y1-DYD. In contrast, if heterologous TAND-TBP interaction is responsible for the degradation, the F57A mutation weakening the TANDII CORE-TBP interaction might specifically prevent the degradation of y1-DYD derivatives. As shown in Fig. 6D, the latter possibility was observed, indicating that TAND-TBP interaction need to be properly regulated (association to dissociation and/or vice versa) in vivo to perform the entire TFIIID function. Our preliminary experiments using amino- or carboxyl-termionally epitope-tagged yTAF145 proteins suggest that heterologously chimeric derivatives are degraded from the amino termini.3 We carefully inspected the effects of the F57A and D61A mutations on the growth rates at elevated temperatures, but the difference was too small to be detected among the strains having y1-DYD derivatives (Table II), probably because the F57A mutation destroys the TANDII function even as it prevents degradation.

**DISCUSSION**

We and others have shown that the amino-terminal region of dTAF230 and yTAF145 is capable of binding to TBP and preventing its stable interaction with the TATA element (21, 23, 31). Here we termed the region responsible for this TBP-antagonizing activity TAND. Importantly, we found that yTAND can compete with TFIIA and the activation domain of VP16 for interaction with TBP (22, 23). Assuming that the interaction between TBP and either TFIIA or an activation domain plays an important role in transcriptional regulation, it seemed likely that the ability of yTAND to interfere with these interactions would be evolutionarily conserved. However, previous studies demonstrated that the TAND from Drosophila (dTAND) spanning residues 18–77 and TFIIA did not share interactions but TANDII and TBP does via both ionic and hydrophobic interactions (see details in the text).

3 K. Banno, unpublished observations.

4 Y. Nakatani, unpublished observations.
copy of the other subdomain, e.g. yTANDI-yTANDI (yIyI) or yTANDII-yTANDII (yIIyII). They displayed detectable TBP binding to the glutathione-Sepharose beads, whereas the corresponding single subdomain fusions (GST-yI or yII) did not. Interestingly, wild type TBP and mutated TBP (K133E/K138E/K145E) bound equally to yIyI but only wild type TBP could bind to yIIyII. Furthermore, the yIIyII-TBP complex was salt-sensitive but the yIyI-TBP complex was not. These results are consistent with the model shown in Fig. 7 in which TANDI recognizes the concave surface of TBP (via salt-resistant hydrophobic interaction) and TANDII (i.e. CORE) makes a salt-sensitive ionic interaction with three positive charges on the convex side of TBP. Given the critical requirement for Phe-57 in yTANDII (or Tyr-129 in dTANDII), it appears that TANDII also makes a hydrophobic contact with this surface of TBP.

Evidence That the TAND-TBP Interaction Is Physiologically Important—Yeast strains lacking either yTANDI or II in otherwise wild type yTAF1145 exhibit a temperature-sensitive growth phenotype, and removal of both TAND elements has an additive deleterious effect on growth at elevated temperatures. The complete TAND deletion (ΔN) also leads to a small growth defect at lower temperatures. These findings are consistent with the idea that the yTAF1145-TBP interaction is important for cell growth and division, particularly at elevated temperatures. In this view, yTANDI and II make additive contributions to the yTAF1145-TBP interaction in vivo, as observed in vitro (Fig. 7). In contrast to the situation in vitro, however, each alone confers a degree of yTAF1145-TBP interaction that is sufficient for wild type growth at reduced temperatures. At elevated temperatures, at which the metabolic rate is much greater, the reduced yTAF1145-TBP interaction conferred by TANDI or TANDII alone is insufficient for wild type cell growth. Alternatively, such a reduced interaction might destabilize the protein structure and impair TFIID function due to altered folding at higher temperatures. Perhaps yTANDI or yTANDII is sufficient for yTAF1145-TBP interaction in vivo at least at lower temperatures, but not for GST-TAND-TBP interaction in vitro, because other regions of yTAF1145, or other yTAFII components, contribute to the stability of the yTAF1145-TBP interaction. Indeed, the amounts of yTAF1145 protein immunoprecipitated with anti-TBP antibody was not affected by deleting yTANDI and/or yTANDII (Fig. 6C). The yTAF1145 construct lacking yTANDI and containing the CORE region of dTANDII (YDY) had the same growth phenotype as the corresponding construct containing wild type yTANDII (YY) (Fig. 5B), indicating that the dCORE functions interchangeably with the yCORE in yTAF1145 in vivo, just as observed for GST-TAND-TBP binding in vitro. In addition, the Phe-57 substitution had a greater effect on the in vivo function of the YYY construct than did any of the other CORE substitutions examined (Fig. 6A), again paralleling the effects of yCORE substitutions on GST-TAND-TBP interactions in vitro. These correlations strongly support the idea that yTAF1145-TBP interaction mediated by the TAND element is crucial for cell growth and division.

Although the Drosophila and yeast CORE sequences of TANDI appeared to be interchangeable in vivo, replacement of the entire yTANDII or yTANDI with the Drosophila elements yielded yTAF1145 constructs which conferred slower growth rates at elevated temperature (37 °C) than did the corresponding constructs bearing endogenous yeast subdomains. These findings differ from those obtained in vitro with chimeric GST-TAND constructs wherein Drosophila TAND and TANDII sequences substituted completely for the yeast sequences in TBP binding assays. These discrepancies could be interpreted to indicate that the yeast and Drosophila TAND sequences are not functionally interchangeable in vivo, perhaps reflecting interactions between the TAND sequences and unknown factors that modulate the yTAF1145-TBP interaction. The poorer growth phenotypes of the chimeric yTAF1145 constructs could be explained if the Drosophila TAND sequences do not support the functions of additional factors, e.g. TFIIA or other yTAFII proteins, required to dissociate the yTAF1145-TBP complex and permit TFIID binding to TATA elements in the manner required for normal growth at 37 °C.

An alternative explanation for the reduced in vivo function of the chimeric yTAF1145 constructs was suggested by the results of Western blot analysis indicating reduced steady-state levels of chimeric TAND derivatives Y1Y1-DD, Y1Y1-DD, y1Y1-YY, and y1DYD compared with the corresponding proteins containing endogenous yeast sequences. According to this explanation, the chimeric and wild type yTAF1145 proteins are capable of interacting with TBP and other transcription factors with the same efficiency, but the reduced amounts of chimeric yTAF1145 proteins are insufficient to regulate TFIID function to the extent required for normal growth at 37 °C. Although the reductions in chimeric yTAF1145 expression were observed at both 25 and 37 °C, whereas the growth defects were seen only at 37 °C, it is possible that the role of yTAF1145 in regulating TFIID function is more critical at the higher metabolic rates in cells growing at 37 versus 25 °C. These two possible mechanisms might not be mutually exclusive because the result that the point mutation weakening TANDII CORE-TBP interaction specifically prevents the partial degradation of yTAF1145 protein (Fig. 6C) suggests that the interaction should be properly regulated in vivo by some unknown factors.

Implications for Transcriptional Regulation—Recently, the Green and Struhl laboratories reported independently that yTAF1145 proteins are not required for transcription of many genes in yeast cells (39, 40). Surprisingly, transactivation by several transcription factors, such as Gcn4, Ace1, Gal4, and Haf, was not severely affected after depletion of the yTAF1145 polypeptide (39, 40). At present, there are two possible explanations for these results: first, another regulatory system possessing redundant functions with TAFs might compensate for the loss of TAF(s)-mediated transcriptional activation of these genes. Indeed, the SBP complex associated with holoenzyme (known as “mediator”) was shown to mediate transactivation in the absence of TAFs in in vitro transcription experiments (3). Recently, we showed that the GCN4 activation domain can bind specifically in vitro to both TFIID and mediator, consistent with the utilization of redundant activation mechanisms by GCN4 (41). Second, TAFs might be specialized inherently for stimulating transcription of only a subset of genes in vivo. In either case, it would be difficult to detect the effects of TAF mutations on bulk gene expression. Indeed, TAND mutations appeared to have no effects on the transcription of most of the genes we have tested thus far.1

More recently, Shen and Green (42) identified a small subset of genes under the control of yTAF1145. Intriguingly, their results showed that the core promoter region, not the upstream enhancer region, is a determinant of yTAF1145-dependent transcription, suggesting that yTAF1145 functions as a core promoter selectivity factor. This is consistent with previous results indicating that some TAFs are involved in promoter recognition in higher eukaryotes. Verrijzer et al. (25) reported that holo-TFIID preferentially transcribes templates having downstream core promoter elements in addition to the TATA sequence. Importantly, the TBP-TAF1250-TAF1150 subcomplex also discriminates core promoter structures, indicating that TAF1250 (homologous to yTAF1145) or TAF1150 mediates the function of the downstream core promoter elements.
Interestingly, these two TAFs destabilize the interaction between TBP and the template lacking downstream elements. We assume that TAND of TAFII250 could be responsible for such negative regulation. Further experiments should be focused on clarifying whether or not TAND is involved in core promoter selectivity.

We observed strong conservation in the way in which TAN-DII interacts with TBP. Most interestingly, TFIIA competes with TANDII for TBP interaction (23). TFIID and TAF proteins are both crucial factors for transcriptional stimulation by the Zta or GAL4VP16 activation domains in HeLa extracts (27–30). Recent studies demonstrated that isomerization of TFIID with Zta or GAL4VP16 activation domains in HeLa extracts (27–30).

TFIID subcomplex was specifically relieved by transcriptional rate-limiting step in transcriptional initiation (43). We propose that TAND could be responsible for maintaining the TFIID complex in the preactivated (preisomerized) state and that alteration of the TAND-TBP interaction by TFIID and the activation domain would be integral to the process of transactivation. It is strongly supported by the fact that a repressive effect of TAFII145 is now in progress. The proper transcription is strongly dependent on the TAND-TBP interaction established in this study is of great importance as a first step toward understanding the physiological role of TAND. Systematic isolation of target genes of which the transcription is strongly dependent on the TAND region of yTAFII145 is now in progress.

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