Mutations in the TATA-binding Protein, Affecting Transcriptional Activation, Show Synthetic Lethality with the TAF145 Gene Lacking the TAF N-terminal Domain in Saccharomyces cerevisiae*

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The general transcription factor TFIID, which is composed of the TATA box-binding protein (TBP) and a set of TBP-associated factors (TAFs), is crucial for both basal and regulated transcription by RNA polymerase II. The N-terminal small segment of yeast TAF145 (yTAF145) binds to TBP and thereby inhibits TBP function. To understand the physiological role of this inhibitory domain, which is designated as TAND (TAF N-terminal domain), we screened mutations, synthetically lethal with the TAF145 gene lacking TAND (taf145ΔTAND), in Saccharomyces cerevisiae by exploiting a red/white colony-sectoring assay. Our screen yielded several recessive nsl (ΔTAND synthetic lethal) mutations, two of which, nsl1-1 and nsl1-2, define the same complementation group. The NSL1 gene was found to be identical to the SPT15 gene encoding TBP. Interestingly, both temperature-sensitive nsl1/spt15 alleles, which harbor the single amino acid substitutions, S118L and P65S, respectively, were defective in transcriptional activation in vivo. Several other previously characterized activation-deficient spt15 alleles also displayed synthetic lethal interactions with taf145ΔTAND, indicating that TAND and TBP carry an overlapping but as yet unidentified function that is specifically required for transcriptional regulation.

In eukaryotes, transcriptional initiation of protein-coding genes is precisely regulated by the concerted action of a large number of proteins, e.g. general transcription factors (TFIIA, TFIIIB, TFIIID, TFIIIE, TFIIF, and TFIIH), negative and positive cofactors, coactivators, and chromosome-modifying factors, in addition to RNA polymerase II (reviewed in Refs. 1–5). The general transcription factor TFIID, a multiprotein complex composed of the TATA-binding protein (TBP) and more than 10 TBP-associated factors (TAFs), can recognize specifically a number of core promoter elements such as the TATA box, initiator element, and downstream promoter element (reviewed in Refs. 6–9). It nucleates the assembly of the preinitiation complex around the transcriptional initiation site by recruiting several other general transcription factors and RNA polymerase II, either in a stepwise manner or as a preassembled unit (i.e. RNA polymerase II holoenzyme) (reviewed in Refs. 10–12). The importance of the binding of TFIID (or TBP) to the core promoter in transcriptional regulation has been extensively studied by various approaches (13–19). Biochemical studies have demonstrated that suboptimal core promoter recognition by TFIID might generate a substantial energetic barrier for initiating transcription, however, gene-specific activators should overcome this rate-limiting step by inducing conformational changes of TFIID (16–19). Activator-bypass experiments, mostly performed in yeast, in which TBP or TAFs were physically connected to the heterologous DNA binding domain of gene-specific activators, showed that binding of TFIID (or TBP) to the core promoter was indeed a rate-limiting step in vivo that could be alleviated by artificial recruitment of TFIID (reviewed in Refs. 12 and 20). The recently developed DNA-cross-linking chromatin immunoprecipitation assay has made it possible to test directly whether binding of TFIID (or TBP) to the core promoter could be a bona fide regulatory step for activators in living cells (14, 15, 21, 22). Results of assays for transcriptional activity using more than 30 promoters correlates well with the degree of TBP occupancy on the core promoter (14), arguing that TBP-TATA interactions should be the most critical step for gene regulation. These observations imply the presence of cofactors that modulate TBP-TATA interactions negatively and/or positively.

Several proteins have been reported to date that inhibit TBP-TATA interactions (reviewed in Refs. 23–25). TAND (TAF N-terminal domain), originally isolated from the N terminus of Dro sophila TAF230 (dTAF230), interacts with TBP directly so that TAND prevents TBP from binding to the TATA element (26–29). Functionally equivalent domains are conserved at the N terminus of orthologous TAFs among various species, suggesting that TAND should be involved in certain principal functions of TFIID (24, 32, 33). TAND consists of two subdomains, TAND1 and TAND2, each of which binds to the concave and convex surface of TBP in a competitive fashion.

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1 The abbreviations used are: TBP, TATA-binding protein; pol, polynucleotide; TAFs, TBP-associated factors; 5-FOA, 5-fluoroorotic acid; bp, base pair; aa, amino acid; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; kb, kilobase pair; HA, hemagglutinin; BSA, bovine serum albumin; TS, temperature-sensitive; AD, activation domain.

2 T. Ichimiya, M. Yuhki, K. Kasahara, M. Kawaichi, and T. Kokubo, unpublished observations.
with acidic activation domains and TFIIA, respectively (28, 30, 34). This structural configuration of the complex formed between TAND and TBP was recently confirmed by NMR spectroscopic studies (29, 35). Together with previous observations that activators and TFIIA are essential factors for inducing transcription of certain genes presumably by regulating the distribution of a limiting pool of TBP between promoter and nonpromoter sites (44, 45). Interestingly, it was demonstrated that TOA1, SPT3, and TOA1, which encodes the larger subunit of TFIIA, interacted genetically with each other, i.e. toa1 and spt3 are synthetically lethal with toa1 and the Δspt3 phenotype is partially suppressed by overexpression of Toa1 (45). These observations strongly indicate that TBP-TATA interactions are intricately regulated in vivo by a wide variety of factors such as TFIIID, SAGA, Mot1, and TFIIA.

Here, to identify factors that are functionally related to the TAF N-terminal domain (TAND) of TAF145, we have screened for nsl (ΔTAND synthetic lethal) mutations that cause lethality in combination with the TAF145 gene that lacks TAND (taf145ΔTAND) in S. cerevisiae. Our screen identified two distinct temperature-sensitive (TS) alleles of the NSL1 gene, as alleles of the SPT15 gene which encodes TBP. Further characterization suggests that activation-defective TBP mutants tend to display synthetic lethal interactions with the taf145ΔTAND gene. This is in accordance with a hypothetical role of TAND in transcriptional regulation that we have recently proposed. Taken together with previous observations that the TS phenotype of TAND-lacking strains can be suppressed by overexpressing TBP or TFIIA, TAND is likely to assist TBP function rather than simply inhibit it, at least in vivo.

### EXPERIMENTAL PROCEDURES

#### Yeast Strains, Media, and Genetic Analyses—Standard techniques were used for yeast growth and transformation (46–48). Yeast extract/potato/dextrose (YPEP) and selective media have been described (46). Transformation was done using the lithium acetate procedure (49). Yeast extract/peptone/dextrose (YEED) and selective media have been described (46). Transformation was done using the lithium acetate procedure (49). Yeast strains used in this study are listed in Table I.

#### TABLE I

| Strain | Genotype | Source |
|--------|-----------|--------|
| YK11   | MATa ura3–52 trp1–63 leu2–3,112 Δnsl145 pM11/TAF145 (y1-YY) | Katani et al. (28) |
| YTR271 | MATa ura3–52 trp1–63 leu2–3,112 his3–609 Δopt15 pYN11/TBP | This study |
| YAK289 | MATa ura3–52 trp1–63 leu2–3,112 his3–609 Δopt15 pTM85/TBP | This study |
| YAK293 | MATa ura3–52 trp1–63 leu2–3,112 his3–609 Δopt15 pM1228/TBP (P65S) | This study |
| YAK493 | MATa ura3–52 trp1–63 leu2–3,112 his3–609 Δopt15 pM1581/TBP (K1387Y139A) | This study |
| YAK495 | MATa ura3–52 trp1–63 leu2–3,112 his3–609 Δopt15 pM486/TBP (N159D) | This study |
| YAK582 | MATa ura3–52 trp1–63 leu2–3,112 his3–609 Δopt15 pM1868/TBP (F148H) | This study |
| YAK584 | MATa ura3–52 trp1–63 leu2–3,112 his3–609 Δopt15 pM1864/TBP (T1531) | This study |
| YAK586 | MATa ura3–52 trp1–63 leu2–3,112 his3–609 Δopt15 pM1865/TBP (E236P) | This study |
| YAK588 | MATa ura3–52 trp1–63 leu2–3,112 his3–609 Δopt15 pM1866/TBP (F237D) | This study |
| YAK620 | MATa ura3–52 trp1–63 leu2–3,112 his3–609 Δopt15 pM1868/TBP (R230I) | This study |
| YAK622 | MATa ura3–52 trp1–63 leu2–3,112 his3–609 Δopt15 pM1867/TBP (Y231A) | This study |
| YAK633 | MATa ura3–52 trp1–63 leu2–3,112 his3–609 Δopt15 pM1867/TBP (N159L) | This study |
| YAK636 | MATa ura3–52 trp1–63 leu2–3,112 his3–609 Δopt15 pM1866/TBP (V161A) | This study |
| YAK938 | MATa ura3–52 trp1–63 leu2–3,112 his3–609 Δopt15 pM2009/TBP (S118L) | This study |
| YAK939 | MATa ura3–52 trp1–63 leu2–3,112 Δopt15 pM11/TAF145 | This study |
| YAK940 | MATa ura3–52 trp1–63 leu2–3,112 Δopt15 pM486/TBP (N159D) | This study |
| YAK307 | MATa ura3–52 trp1–63 leu2–3,112 Δopt15 pM145/TAF145 | This study |
| CH1305 | MATa ade2 ade3 leu2 ura3 ts2 con1 | Kranz and Holm (50) |
| TMY4–2 | MATa ade2 ade3 leu2 ura3 ts2 con1 taf145ΔTAND | This study |
| TMY17–2c | MATa ade2 ade3 leu2 ura3 ts2 con1 taf145ΔTAND | This study |
| TMY16–2c | MATa ade2 ade3 leu2 ura3 ts2 con1 taf145ΔTAND | This study |
| C40 | MATa ade2 ade3 leu2 ura3 ts2 con1 taf145ΔTAND spt15–S118L pTM17/TAF145–ADE3–URA3 | This study |
| D7 | MATa ade2 ade3 leu2 ura3 ts2 con1 taf145ΔTAND spt15–P65S pTM17/TAF145–ADE3–URA3 | This study |
| YAK287 | MATa ade2 ade3 leu2 ura3 ts2 con1 spt15–P65S | This study |
| YAK978 | MATa ade2 ade3 leu2 ura3 ts2 con1 spt15–S118L | This study |
The YTK271 strain was generated from H2440 (kindly provided by Dr. A. G. Hinnebusch) (30) by targeted disruption of the \textit{SPT15} gene on the chromosome. The resulting Ura\(^{-}\) strain was linked to synthetic lethality. As both mutations are linked to the TS phenotype, mutant segregants derived from C40 and D7 were recovered, the sectoring phenotype and the 5-FOA lethality segregated 2:2, indicating that the synthetic lethality was presumably caused by mutation at a single locus. Crosses between mutant segregants from C40 and D7 assigned them to a complementation group.

**Construction of a Gene That Complements C40 and D7 nsl Mutants—** C40 and D7 mutants were crossed to TMY17-2c bearing the tar453\textit{A}TAND allele, and in both cases red to white sectors were observed, indicating that these mutations were recessive. Diploids were sporulated, and 18 tetrads were dissected and analyzed phenotypically. When four spores were recovered, the sectoring phenotype and the 5-FOA lethality segregated 2:2, indicating that the synthetic lethality was presumably caused by mutation at a single locus. Crosses between mutant segregants from C40 and D7 assigned them to a complementation group.

**Identification of Amino Acid Substitutions in \textit{SPT15} Gene of C40 and D7—** The mutations in the \textit{SPT15} gene of C40 and D7 strains responsible for synthetic lethality were identified by sequencing. The 1.2-kb DNA fragment, including the entire \textit{SPT15} gene, was amplified by PCR using the primer pairs TK127 and TK128 from the isolated genomic DNA of these mutants. Direct sequencing of amplified DNA fragments using TK222, TK223, TK224, and TK225 as sequence primers revealed single C \rightarrow T point mutations at 353 bp in C40 and at 193 bp in D7, which result in the amino acid substitutions S118L and P65S, respectively. Proof that these mutations conferred synthetic lethality was obtained by testing the \textit{nsl} phenotype of tar453 alleles bearing these mutations produced by site-specific mutagenesis (57) as described below.

**Cloning of a Gene That Complements C40 and D7 nsl Mutants—** C40 and D7 mutants were crossed to TMY17-2c bearing the tar453\textit{A}TAND allele, and in both cases red to white sectors were observed, indicating that these mutations were recessive. Diploids were sporulated, and 18 tetrads were dissected and analyzed phenotypically. When four spores were recovered, the sectoring phenotype and the 5-FOA lethality segregated 2:2, indicating that the synthetic lethality was presumably caused by mutation at a single locus. Crosses between mutant segregants from C40 and D7 assigned them to a complementation group.

**Construction of Plasmids Encoding Activation-defective TBP Mutants—** pTM8 was constructed by ligating the 2.4-kb EcoRI-BamHI fragment including the entire \textit{SPT15} gene, as described above, into the EcoRI-BamHI sites of pRS145 (56). pTM8 was subjected to site-specific mutagenesis (57) to create plasmids bearing the wild type \textit{TAND} gene. From a total of \textasciitilde 40000 colonies screened, 14 strains (A1, A6, A22, A38, B9, B16, C2, C40, C60, C72, D7, D16, E4, and E10) had the synthetic lethal phenotype with the tar453\textit{A}TAND gene (i.e. \textit{nsl} phenotype). C40 and D7 strains were further characterized in this study.
Synthetic Lethal Interaction between TANB and TBP

TFIIA was prepared as described previously (30). E. coli BL21 (DE3) (Novagen) was transformed with pRBS14-based plasmids encoding TBP derivatives (30 pmol) and TBP, in the cleared lysate or purified through the Ni2⁺-agarose column, in 100 μl of 0.2 M KCl/buffer D at 4 °C for 30 min, and washed three times with 500 μl of 0.2 M KCl/buffer D. The complexes on the beads were eluted by boiling in SDS sample buffer and analyzed by Western blotting with polyclonal antibodies to TBP. Interactions between GST-VP16 and TBP derivatives were examined similarly except that two different concentrations of KCl (0.1 M and 0.2 M) were used in the binding and washing buffers. Immunoprecipitation Assay—Immunoblot and coimmunoprecipitation analyses were performed as described previously (28, 58).

RESULTS

Screen for nsl Mutants That Are Synthetic Lethal with the Loss of TANB Function—Recently we proposed that TANB, located at the N terminus of TAF145, might be involved in transcriptional regulation by acidic activators (32). However, as target gene(s), whose expression depends on the TANB-TBP interactions, have not been identified despite our extensive efforts, it seems likely that some other regulatory systems may exist in yeast cells that compensate for the loss of TANB-TBP interactions. To identify genes involved in such a parallel pathway, we screened nsl genes displaying synthetic lethal interactions with the taf145 gene that lacks TANB (taf145ΔTANB), by exploiting an ade2/ade3-based red/white colony sectoring assay (50, 55). The principle of a synthetic lethal screen is that, although a single mutation is tolerable for the cell, a combination of mutations in functionally related pathways results in severe growth inhibition or cell death. This type of screen was often used for the identification of unknown component(s) regulating a common process in a wide variety of biological phenomena (60–62).

The host strain, TMY4-2, used for our screen was constructed to harbor a taf145ΔTANB allele at the original chromosomal locus by homologous recombination and was then transformed with pTM17, a centromere-based plasmid that contains the URA3 and ADE3 nutritional markers and the wild type TAF145 gene. This host strain, TMY4-2, is red on YPD media but forms white sectors when the plasmid pTM17 is lost under nonselective conditions. Thus, we can expect that nsl mutant strains should show no white sectors in colonies since the TANB function on pTM17 becomes essential for growth. TMY4-2 was mutagenized by UV irradiation, and of approximately 40000 surviving colonies screened, 30 failed to show red/white sectoring at 25 °C. Of these, 14 regained a sectoring phenotype when transformed with a plasmid carrying the wild type TAF145 gene, but not with the taf145ΔTANB gene, and failed to grow on 5-FOA media (i.e. pTM17 plasmid carrying the URA3 and ADE3 genes is essential for cell growth). These observations strongly suggest that they contain mutations that are synthetically lethal with the taf145ΔTANB gene. Two of these nsl mutants, C40 and D7, were further characterized in this study.

Two nsl Mutants Carry Different Amino Acid Substitutions in the SPT15 Gene Encoding TBP—C40 and D7 mutants segregated into the plasmid-dependent red/white sectoring phenotype 2:2 when backcrossed to an isogenic parent strain harboring the chromosomal taf145ΔTANB gene, indicating that their nsl phenotypes resulted from single gene mutations. A complementation test revealed that they were recessive and allelic, thus hereafter this gene is designated as NSL1 (ΔTANB syn-
thetic lethal 1). We also refer to mutant alleles of C40 and D7 as nsl1-1 and nsl1-2. Both nsl1 alleles seemed to exhibit TS growth phenotypes since all nsl1 spores obtained through backcross experiments with a parental strain harboring the chromosomal TAF145 or taf145ΔTAND gene were TS even in the presence of the plasmid, pTM17, expressing the wild type TAF145 gene (data not shown). Thus we tried to isolate the wild type NSL1 gene by complementing the TS phenotypes of these nsl1 mutants. nsl1-1 and nsl1-2 mutants were transformed with a partial Sau3A yeast genomic library, and several complementing colonies were isolated from both mutants. Retransformation analysis established that growth recovery at 35 °C was dependent on the presence of the plasmids that were isolated from the original colonies (data not shown). Consistent with the allelic test, restriction enzyme digestion and sequencing analysis revealed that genomic inserts included in plasmids, derived from either mutant (i.e. nsl1-1 or nsl1-2), overlapped with each other (data not shown). Importantly, only the SPT15 gene encoding TBP was found in all inserts. Thus we subcloned an ~2.4-kb EcoRI-BamHI fragment, from which only the SPT15 gene could be expressed, into a centromeric vector and tested it using the complementation assay. The plasmid carrying the subcloned fragment rescued the TS growth defects of both mutant strains (Fig. 1), suggesting that NSL1 might be allelic to SPT15.

To identify possible mutation(s) in the SPT15 gene of both mutants, we sequenced PCR-amplified genomic fragments encompassing the entire open reading frame plus 5'- and 3'- adjacent DNA regions (~500 bp each) of the SPT15 gene. We found the single amino acid substitutions, S118L and P665S, in the coding region of the SPT15 gene of C40 and D7 mutants, respectively. Thus, we next asked whether these TBP mutations were sufficient to reproduce the nsl phenotype and whether such a phenotype depended on a particular genetic background. To address these questions, we constructed yeast strains containing either the wild type TAF145 gene (YAK303) or taf145ΔTAND or taf145ΔTAND gene (YAK307) on a centromeric LEU2 plasmid as well as the wild type SPT15 gene on a centromeric URA3 plasmid in combination with double deletions of chromosomal TAF145 and SPT15 genes. These strains have different general genetic backgrounds from the one used in the original genetic screen for the nsf mutants. The spt15-S118L and spt15-P665S alleles were reconstructed on a centromeric TRP1 plasmid by site-directed mutagenesis to exclude any other possible mutations. These plasmids were transformed into YAK303 and YAK307 strains described above and tested for their growth on 5-FOA plates. We reasoned that if spt15-S118L and spt15-P665S are responsible for the nsl phenotype, only strains carrying the wild type TAF145 gene (i.e. derived from YAK303) would be viable on 5-FOA plates that select for cells that had lost the URA3 and SPT15 containing plasmid. Consistent with this expectation, yeast strains carrying the taf145ΔTAND gene (i.e. derived from YAK307) grew well on 5-FOA plates only when they expressed the wild type SPT15 gene but not when they expressed the spt15-P665S or spt15-S118L alleles (Fig. 3A). We also confirmed that these mutant alleles were recessive and TS in the genetic background of YAK303 strains as observed for the original mutant strains. These observations support the notion that these TBP mutations, S118L and P665S, are synthetically lethal with the taf145ΔTAND gene even under the different general genetic background. Similar conclusions were confirmed by genetic experiments in which spores harboring the combination of taf145ΔTAND and spt15-S118L or taf145ΔTAND and spt15-P665S alleles were never recovered, as more than 20 ascis were dissected for each diploid (data not shown).

Transcriptional Activation Is Impaired in spt15/nsl1 Mutants—The spt15-S118L allele was previously isolated as one of the TBP mutants that was specifically impaired in its response to acidic activators (63). Yeast strains containing the S118L mutant as the sole source of TBP are deficient for activation by Gcn4, Gal4, and Ace1, whereas transcription from pol I (rDNA), pol III (t-RNA-I), TATA-less pol II (TRP3), and constitutive pol II (DED1 and RPS4) promoters is not impaired (63). On the other hand, the P665S mutant was reported to be defective for transcription in vitro from pol II (CYC1) as well as pol III (5 S rDNA and t-RNA-L) promoters (64). These TBP mutants, S118L and P665S, showed poor growth at higher temperatures (63, 64), and the TS phenotype of the latter was exploited to isolate the BRF1 gene (65) (also called as TDS4 (66) and PCF4 (67)) encoding a component of the pol III-specific general transcription factor, TFIIIB. As the overexpression of BRF1 suppressed the TS phenotype of the P665S mutant, transcription by pol III should be more severely affected than that by pol II in this mutant (65).

We proposed that TAND may play an important role in transcriptional activation by acidic activators (32). In this regard, it is intriguing that the activation-defective TBP mutant, S118L, was isolated in our screen as one of the nsl alleles. We reasoned that the P665S mutant also might be deficient in the response to acidic activators. Thus, we examined activation efficiencies in the P665S mutant that had been backcrossed to an isogenic wild type strain more than three times so as to avoid the effect of other unrelated mutations. The α-galactosidase activity from the Gal4 upstream activating sequence-dependent reporter plasmid was measured when various activation domains fused to Gal4 DNA binding domain were coexpressed in the cell (Fig. 2A). As we expected, activation efficiencies were constantly lower in the P665S mutant than that in the wild type strain. TAND1, which binds the concave surface of TBP, can function as a strong activation domain when recruited onto the promoter by the Gal4 DNA binding domain (32). Interestingly, activation by TAND1 was most severely affected in the P665S mutant (4.2% of the wild type), whereas activation by TADIV of ADR1, which requires the presence of intact TFIIID for its function (68), was least affected (50.7% of the wild type). Weakened interactions of TBP, either with the TATA ele-
assays were 20 ng of TBP (upper panel) and TAND-TBP interactions (activity expressed from a reporter plasmid). Adenovirus major-late promoter (~119 to +61) was used as a probe. The amounts of protein used for assays were 20 ng of TBP (upper panel) and 10 ng of TBP and 12 ng of TFIIA (lower panel). The positions of the TBP-DNA complex and TFIIA-TBP-DNA complex are indicated by an asterisk on the right. C, interaction of TBP derivatives with GST-TAND and GST-VP16. GST fusion proteins were incubated with an equimolar amount of TBP. Complexes were trapped by glutathione-Sepharose beads, washed extensively with the buffer containing 0.2M KCl (TAND-TBP interactions) and 0.1 or 0.2 M KCl (VP16-TBP interactions), and then analyzed by SDS-PAGE followed by Western blotting.

Fig. 2. In vivo activation and biochemical properties of TBP-P65S and TBP-S118L mutants. A, GAL4-dependent transcriptional activation in the wild type (solid bars) and ns11-2 (sp15-P65S) mutant strains (open bars). Expression vectors encoding various activation domains fused to the GAL4 DNA binding domain were transformed into yeast, and transcription activity was determined by measuring the lacZ reporter activity expressed from a reporter plasmid. B, gel mobility shift analyses of the TBP-TATA element interactions (upper panel) and TFIIA-TBP-promoter DNA interactions (lower panel). Adenovirus major-late promoter (~119 to +61) was used as a probe. The amounts of protein used for assays were 20 ng of TBP (upper panel) and 10 ng of TBP and 12 ng of TFIIA (lower panel). The positions of the TBP-DNA complex and TFIIA-TBP-DNA complex are indicated by an asterisk on the right. C, interaction of TBP derivatives with GST-TAND and GST-VP16. GST fusion proteins were incubated with an equimolar amount of TBP. Complexes were trapped by glutathione-Sepharose beads, washed extensively with the buffer containing 0.2M KCl (TAND-TBP interactions) and 0.1 or 0.2 M KCl (VP16-TBP interactions), and then analyzed by SDS-PAGE followed by Western blotting.
should be impaired in binding to unknown factors that are important for activation (71).

To see the correlation between the activation defects and the nsl phenotype, we selected different types of TBP mutants like N159D, N159L, and V161A (defective for TATA binding) (63, 69), K138/139A and F237D (defective for TFIIA binding) (70, 71), F148H, T153I, and E236P (impaired interaction with unknown factors) (71), and R220H and Y231A (specifically impaired in pol III-driven transcription) (75), and we determined their nsl phenotypes (Fig. 3A). As described for S118L and P65S mutants, we transformed the yeast strains, YAK303 and YAK307, with a centromeric TRP1 plasmid expressing each TBP mutant, and we examined their growth on 5-FOA plates at 30 °C. Like the S118L and P65S mutants, other activation-defective TBP mutants also exhibited synthetic growth defects with the taf145ΔTAND gene but not with the wild type TAF145 gene. In contrast, the mutants, R220H and Y231A, which are specifically defective for transcription by pol III, did not cause any deleterious effect on growth with either alleles of the TAF145 gene. Despite all of the spt15/NSL1 alleles tested here that exhibit the TS phenotype on their own (data not shown), only the activation-defective TBP mutants had the nsl phenotype. Therefore, we assumed that the P65S mutant was isolated as a nsl1-2 mutant in our original screen due to its deficient activation by pol II rather than to its impairment in pol III transcription.

We next investigated how these TBP mutants interacted with the TATA element, TFIIA, TAND, and VP16 (Fig. 3B and C), although similar analyses had been conducted for some of these mutants previously (63, 69). We compared transcript levels for each TBP mutant with the wild type TBP. The resulting transformants were grown on 5-FOA plates at 30 °C for 5 days. B, TBP-TATA and TFIIA-TBP-DNA interactions were analyzed as described in Fig. 2. Note that TBP-K138T, Y139A binds weakly to the TATA element in the presence of 2 μg/μl BSA (lane 6), whereas it binds as well as the wild type in the absence of BSA (lanes 8 and 9). C, TBP-TAND and TBP-VP16 interactions were analyzed as described in Fig. 2.

**Fig. 3.** A, activation-defective TBP mutants show the nsl phenotype. The TRP1-marked plasmids encoding TBP derivatives, as indicated on the left, were individually introduced into the strains with double deletions of TAF145 and SPT15 genes but which instead contained either the LEU2-marked plasmid encoding the wild type gene or the taf145ΔTAND gene, as indicated at the top, in addition to the URA3-marked plasmid encoding wild type TBP. The resulting transformants were grown on 5-FOA plates at 30 °C for 5 days. B, TBP-TATA and TFIIA-TBP-DNA interactions were analyzed as described in Fig. 2. Note that TBP-K138T, Y139A binds weakly to the TATA element in the presence of 2 μg/μl BSA (lane 6), whereas it binds as well as the wild type in the absence of BSA (lanes 8 and 9). C, TBP-TAND and TBP-VP16 interactions were analyzed as described in Fig. 2.
R220H and Y231A that are supposed to be specifically impaired in pol III transcription, were found to be more or less affected in activation by these activators (Fig. 4A). Nonetheless, we did not see any distinct correlations among the degree of the nsl phenotypes and the activation defects or the sort of activation domains present and the molecular defects observed. It was noteworthy that F148H and T153I mutants were affected in activation to a similar level as other TBP mutants (Fig. 4A) although they showed a weaker nsl phenotype than the others (Fig. 3A).

Previous studies demonstrated that transcription could be activated in the absence of activators by artificial recruitment of TBP that is physically connected to a heterologous DNA binding domain (76–78). This simple in vivo recruitment assay could predict roughly which step(s) is impaired in each activation-defective TBP mutant (71). The rationale of how to interpret the result was originally provided by Stargell and Struhl (71), namely if an activation-defective TBP can activate transcription when it is recruited to the promoter, its defect must be involved in the step(s) before recruitment to the TATA element. Conversely, if the TBP mutant fails to activate transcription under the same conditions, it should lack the ability to proceed to post-recruitment steps. We transformed wild type strains with the reporter plasmid harboring the GAL1 promoter-driven lacZ gene as well as the effector plasmid expressing each TBP mutant fused to the GAL4 DNA binding domain (Fig. 4B). Consistent with previous studies, K138T/Y139A, E236P, and F237D mutants activated transcription less efficiently than the wild type gene (71, 79). Besides these mutants, P65S, S118L, N159D, N159L, and V161A mutants also showed lower activities than the wild type gene in this assay. In contrast, F148H, T153I, R220H, and Y231A mutants activated transcription at similar levels to the wild type gene. Thus the defects in the post-recruitment step tend to represent a stronger nsl phenotype.

Integrity of the TFII D Complex Containing TAF145 Lacking TAND and the Activation-defective TBP Mutant—We previously demonstrated that the same amount of TAF145 protein was coprecipitated with TBP from cell lysates prepared either from wild type or ΔTAND strains (28). The reason differences between these two strains were not observed was probably because other regions of TAF145 or other TAF components contributed to the stability of the TAF145-TBP interactions and/or supported the integrity of TFII D. Therefore, it is likely that the nsl phenotype may occur due to the instability of TFII D enhanced by combining two different mutations within the same complex. To explore this possibility, we constructed yeast strains in which the chromosomal SPT15 gene was deleted and instead carried two plasmids, one expressing the HA-tagged taf145ΔTAND gene (LEU2 marked plasmid) and the other expressing either wild type or activation-defective TBP (TRP1 marked plasmid). All strains were viable due to the presence of the wild type TAF145 gene on the chromosome. We tried to examine the integrity of TFII D in these strains by
measuring the amounts of TAF145ΔTAND and TAF61 proteins that were coimmunoprecipitated with TBP (Fig. 5). Precipitates were blotted onto a membrane and probed with anti-HA, TAF145, TAF61, and TBP antibodies, respectively. The signal detected by the anti-HA antibody represents the amount of TAF145ΔTAND proteins, whereas the signal detected by anti-TAF145 antibody is derived from the sum of wild type TAF145 and TAF145ΔTAND proteins. Note that nontagged wild type TAF145 proteins and HA-tagged TAF145ΔTAND proteins migrated at the same position on this gel. When the signals were normalized with the amount of precipitated TBP (lowest panel), it appeared that similar amounts of TAF145 and TAF61 proteins were included in the TFII D complex in all yeast strains. In contrast, smaller amounts of TAF145ΔTAND proteins were coimmunoprecipitated with TBP in the N159D mutant. Such differences were not observed for other TBP mutants. However, it is still possible that even the N159D mutant can make a stable TFII D complex with the TAF145ΔTAND protein if the wild type TAF145 protein is not present in the cell, although we cannot test this possibility directly due to its synthetic lethality. Collectively, the instability of the TFII D complex does not simply explain why these TBP mutants display the nsl phenotypes.

**FIG. 5.** Coimmunoprecipitation analysis to test the integrity of the TFII D complex containing both TAF145ΔTAND protein and TBP derivatives. Whole cell extracts were prepared, using glass beads, from yeast strains containing the wild type gene or the indicated TBP derivatives and the HA-tagged TAF145ΔTAND proteins. Note that these strains have the nontagged wild type TAF145 gene on the chromosome to support viability. Aliquots of whole cell extract proteins were immunoprecipitated with anti-TBP polyclonal antibodies (even-numbered lanes) or preimmune antibodies (odd-numbered lanes). Proteins coimmunoprecipitating with TBP were fractionated on SDS-PAGE, transferred to nitrocellulose membranes, and probed with the indicated antibodies for the good correlation between the defect in the post-recruitment step and the nsl phenotype (Table III).

We recently proposed that TAND might be involved in the initial stage of activation by acidic activators (32). Some functional similarities have been found between TAND1 (that is the N-terminal subdomain of TAND that binds to the concave surface of TBP) and acidic activation domains like VP16, GAL4, and EBNA2 (32). Such unexpected functional similarities prompted us to build a two-step hand off model in which TAND1, bound to the concave surface of TBP, could first be displaced by an acidic activation domain (AD) and the AD could be successively displaced by the TATA element (32). We believe that such transfer of TBP from TAND to the TATA element may trigger the isomerization process of TFII D that leads to increased stimulation of transcription. According to this model, TAND must play two different roles, i.e. when activators are absent near the promoter, TAND inhibits TBP-TATA interactions to prevent leaky transcription, whereas it must be able to release TBP once activators come close to the promoter. Taken that TFII D can recognize the core promoter, not only by TBP-TATA interactions but also by TAF-DNA interactions (reviewed in Refs. 8 and 7), TBP might be converted into an active form at a much closer position to the TATA element when it is liberated from TAND by the action of activators. If this is the case, TAND performs dual functions as a negative and positive regulator. Previous studies demonstrated that the TS phenotype of taf145ΔTAND strains could be suppressed by overexpression of TBP and TFIIA (30, 31). The assumption described above may explain why TFII D that lacks TAND needs higher concentrations of TBP or TFII A to perform its normal function.

Our studies demonstrate that activation-defective TBP mutants confer severe damage to yeast strains carrying the taf145ΔTAND gene. It is especially intriguing that TBP mutants defective in the post-recruitment step produce a stronger nsl phenotype (Table III). As described above, it is believed that TAND is involved in the initial step during activation, i.e. stable binding of TFII D to the promoter (pre-recruitment step). The combined defects of the pre- and post-recruitment steps can be expected to yield the lowest activation and thereby inhibit cell growth. In this regard, the results obtained here support our previous model that TAND is involved in pre-recruitment steps.

Another issue to be discussed is how these TBP mutants are impaired in the post-recruitment step. TBP may function together with several other transcription machineries besides TFII D, e.g. SAGA and RNA pol II holoenzyme (reviewed in Ref. 23). Thus, it is possible that these TBP mutants affect the function of other machineries involved in post-recruitment steps.
steps. Although we have not yet examined the integrity of those machineries, most of these TBP mutants appear to be normal at least in the integrity of TFIIID. However, it is still likely that TFIIID may be impaired in its interactions between TBP-TAF or TBP-TFIIB that are required specifically for the post-recruitment steps. To determine which complex is most severely damaged in these TBP mutants, we need to separate each complex and test its activity individually using in vitro transcription experiments.

Recently, TBP has been shown to exist, at least in part, as an independent form of TAFs (21, 22). TAFs are recruited in much smaller amounts to TAF-independent (TAFind) promoters when compared with TAF-dependent (TAFdep) promoters. Consistently, TBP is recruited, in a manner apparently independent of TAF function, to the TAFind promoters. Conversely, TAFs are recruited independently of TBP function to TAFdep promoters. These observations strongly suggest that there are at least two transcriptionally active forms of TBP, i.e. associated form and nonassociated form with TAFs (21, 22). Thus it might be possible that activation-defective TBP mutations affect the TAF-associated form of TBP predominantly whereas TAF145/TAND affects only the TAF-associated form of TBP, so that double mutants can decrease the expression of a much broader range of genes. It has yet to be determined whether these alternative forms of TBP, other than TFIIID, correspond to the free TBP molecule or any other known or unknown TBP-containing complexes.

It is still unclear whether TAND is involved in the activation of most genes or just a particular set of genes in vivo. Earlier studies demonstrated that activation may occur even in the absence of TAFs in vitro as well as in vivo (80, 81). More recent genome-wide expression analyses have shown that the requirements for various TAFs are not the same among different genes probably because some TAFs are shared by TFIIID and SAGA, which are functionally redundant (reviewed in Refs. 82–84). Consistently shared TAFs such as TAF17, TAF25, and TAF60 appear to be more generally required for gene expression than TFIIID-specific TAFs such as TAF145 and TAF150 (84). However, there are exceptions like TAF40, which is a TFIIID-specific TAF but nonetheless required by most promoters (85). Since our preliminary genome-wide expression analysis showed that TAF145/TAND produced much smaller effects, we believe that the possible detrimental effect of TAF145/TAND on gene expression might be concealed in vivo by compensatory interactions between TFIIID and other machineries like SAGA, as just recently observed for TAF145 and Gen5 (84). TAF145 and Gen5, both of which encode catalytic subunits of histone acetyltransferase in TFIIID and SAGA, appear to regulate the expression of a large fraction of genes through the function of either complex. In this regard, it would be intriguing to examine the redundancy between TAND and Spt3/Spt8 that are analogous subunits, which negatively regulate TBP function in each complex. Isolation of other NSL genes could solve the functional redundancies in vivo that are accomplished by multiple TBP-interacting factors.

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