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A New Class of Activation-Defective TATA-Binding Protein Mutants: Evidence for Two Steps of Transcriptional Activation In Vivo

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Using a genetic screen, we isolated four TATA-binding protein (TBP) mutants that are specifically defective in vivo for the response to acidic activators. In contrast to previously described activation-defective TBP mutants, these TBP derivatives are not specifically defective for interactions with TATA elements or TFIIA. Three of these derivatives interact normally with a TATA element, TFIIA, TFIIB, or an acidic activation domain; presumably, they affect another protein-protein interaction important for transcriptional activation. The remaining derivative (with F-237 replaced by D) binds a TATA element with wild-type affinity, but the TBP-TATA complex has an altered electrophoretic mobility and interacts poorly with TFIIA and TFIIB; this suggests that the conformation of the TBP-TATA element complex plays a role in transcriptional activation. To determine the step at which the TBP derivatives were unable to activate transcription, we utilized an artificial recruitment assay in which TBP is targeted to the promoter via fusion to the LexA DNA-binding domain. Consistent with previous evidence that acidic activators can increase recruitment of TBP to the promoter in vivo, the activation defect of some of these TBP derivatives can be corrected by artificial recruitment. In contrast, the activation defect of the other TBP derivatives is not bypassed by artificial recruitment. Thus, these TBP mutants define two steps in the process of transcriptional stimulation by acidic activators: efficient recruitment to the TATA element and a postrecruitment interaction with a component(s) of the initiation complex.

Acidic activator proteins stimulate transcription by RNA polymerase II (Pol II) by a mechanism that is conserved among eukaryotic organisms. Most models invoke protein-protein interactions between the acidic activation domain of an activator protein(s) bound at enhancer elements and general transcription factors assembled at the TATA and initiation elements (48). In vitro, acidic activation domains can interact directly with a number of components of the general transcription machinery. These include the TATA-binding protein (TBP) (16, 45), TBP-associated factors (TAFs) that are components of the TFIID complex (14, 15), TFIIA (38), TFIIB (35), TFIIF (18), and TFIIH (53). In stepwise assembly reactions, acidic activation domains can stimulate formation of a TFIID-TFIIA-TATA element complex (6, 33, 51), recruitment of TFIIB (34), and recruitment of later-acting components (7). The relative importance of the protein interactions and mechanistic steps involving acidic activation domains is difficult to assess, particularly in light of evidence suggesting that many of the basic transcription factors may be preassembled into a Pol II holoenzyme (25, 28, 37).

The mechanism of transcriptional activation under physiological conditions is less well understood. In yeast cells, kinetic analysis indicates that acidic activation domains can enhance recruitment of TBP to promoters (27). Thus, any interaction that influences the association of TBP with the TATA element could be critical to the mechanism of action of acidic activators. Conversely, destabilization of the TBP-TATA element interaction has the potential to be specifically deleterious to the process of activated transcription.

Several observations in vivo are consistent with this reasoning. First, we previously described a TBP mutant whose properties indicate that the TBP-TFIIA interaction is essential for activation by acidic activator proteins in vivo (44). The role of the TBP-TFIIA interaction in transcriptional activation may reflect the ability of TFIIA to stabilize the interaction of TBP on the TATA element (30). Second, activation deficiency can also arise by mutating the DNA binding surface of TBP and reducing the ability of TBP to bind DNA (1, 31). Although the mechanism is unknown, activation deficiency most likely involves perturbations of the TBP-TATA interface (31). Third, in wild-type strains, an efficient TBP-TATA element interaction is essential for high levels of activated transcription, because nonconsensus TATA elements reach saturation at low levels of transcription (17). Independent support for the importance of TBP recruitment comes from the observation that the requirement for an activation domain can be bypassed by artificially recruiting TBP to a promoter via physical connection of TBP to a heterologous DNA-binding domain (5, 26, 52).

Although the above results indicate that acidic activators stimulate TBP recruitment in vivo, they do not address whether activators influence TBP functions after recruitment to the TATA element. Here, we identify a new class of activation-defective TBP derivatives that contain mutations that do not reside on the DNA-binding surface or on the TFIIA interaction region of TBP. Further, we show that the activation defect of some, but not all, of these TBP derivatives can be corrected by artificial recruitment to the promoter via fusion to a heterologous DNA-binding domain. These results provide the first evidence that the response to acidic activators in vivo not only requires efficient recruitment of TBP to the TATA element but also involves TBP at a step that occurs after binding to the TATA element.

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MATERIALS AND METHODS

Strains and DNAs. *Saccharomyces cerevisiae* YLAS1 was derived from BYΔ2, which contains a deletion of the chromosomal copy of TBP (9) and a *URA3*

plasmid (pRS316) bearing the 2.4-kb *Eco*RI-*Bam*HI genomic fragment of the TBP gene encoding the F-155-to-S (F155S) substitution (12). LexA fusion constructs were tested in strain FT4, which contains a LexA operator 45 bp upstream of the *his3* TATA element and structural gene (49) and a *lacZ* reporter construct driven by the same promoter on a *URA3* high-copy-number vector (5).

To generate TBP-TFIIA fusion proteins, an *Eco*RI site was introduced upstream of the TBP mutants by subcloning the relevant fragments into the wild-type TBP construct already containing the *Eco*RI site (44). The coding sequence of *TOA2* (obtained by PCR using specific oligomers flanked with *Eco*RI sites) was cloned into the upstream *Eco*RI site to generate protein fusions between the TFIIA subunit and TBP separated by the linker peptide RIPFLI.

To generate molecules expressing the various LexA-TBP derivatives, the *CYC8* coding region of YCp91-LexA*CYC8* (49) was replaced with the structural gene of TBP. The resulting molecule contains a 1.5-kb fragment of the *ADH* promoter driving expression of the coding sequence for 202 amino acids of LexA, followed by an HA1 epitope and the simian virus 40 nuclear localization signal, fused to the open reading frame of TBP on a *TRP1*-marked plasmid. Mutant versions of TBP were generated in a similar manner.

Screen for TBP derivatives which complement the loss of Pol III function of the TBP F155S allele. YLAS1 was transformed with TBP mutant libraries generated by regional codon randomization, carried on a *TRP1*-marked centromeric plasmid (12). The screen involved mutant libraries spanning three regions of yeast TBP: N2, encompassing amino acids 135 to 147; N3, from residues 148 to 168; and N5, spanning amino acid 229 to 240. Five thousand primary transformants from libraries N2 and N3 were assayed for complementing alleles and 3,000 were assayed from library N5. Approximately 45% of the *TRP1* plasmids from each of the three libraries complemented the Pol III-defective F155S derivative of TBP at the restrictive temperature. After 4 days at the restrictive temperature, colonies were replica plated onto 5-fluoro-orotic acid (FOA) and incubated at 30 and 37°C, to select for loss of the *URA3*-marked Pol III-defective TBP allele. Colonies scored as unable to grow on FOA at 37°C but viable at 30°C occurred at a frequency of <0.3% and were selected for further analysis. Plasmids encoding the TBP derivatives were rescued from these candidates and retransformed into strain YLAS1 to confirm the Pol III-complementing and temperature-sensitive (ts) phenotypes. Fifteen derivatives were assayed and eight of them retained the original phenotypes. The remaining seven complemented the Pol III defect, but strains carrying them as the sole source of TBP grew slightly at the restrictive temperature (indicating that the alleles were not tight ts alleles).

Transcriptional analyses. In most cases, RNA levels were measured by quantitative S1 nuclease analysis using oligonucleotide probes (11). For temperature shift experiments, strains were shifted to the restrictive temperature (37°C) for 15 min, returned to the permissive temperature (30°C) for 1 h, and then incubated at the restrictive temperature for 1 h. RNAs were analyzed for *RPS4*, *TRP3*, *HIS3*, and *DED1* mRNAs generated by Pol II, rRNA transcribed by Pol I, and tryptophan tRNA ($tRNA^W$) synthesized by Pol III (44). For copper induction of *CUP1* transcription, strains were grown overnight in synthetic complete medium in the presence or absence of 100 μM copper sulfate. Parallel reactions were performed by using a probe for the ribosomal protein gene *RPS4*. Activation by Gcn4 was examined in *gcn4* deletion strains that lack or contain YCp88-*GCN4*, a plasmid constitutively expressing Gcn4, which were analyzed for *HIS3* and *DED1* RNAs. Repression by the Cyc8-Tup1 complex was analyzed using 10 μg of total RNA from the indicated TBP derivative and *tup1* deletion strains by blotting to nylon filters and hybridizing with *RNR2* or *SUC2* probes (50).

Biochemical analyses. TBP derivatives were cloned into Pet-15b for expression and purification of His-tagged proteins from *Escherichia coli* DE3/pLysS (Novagen). Proteins thus prepared were approximately 50% pure as assayed by Coomassie blue staining. For interaction with VP16, 20 μl of glutathione-agarose beads containing glutathione *S*-transferase (GST)-VP16 or GST (35) were incubated with equal amounts of His-tagged wild-type or mutant TBPs for 1 h in 100 μl of buffer containing 100 mM KCl, 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.9), 5 mM $MgCl_2$, 20 μg of bovine serum albumin per ml, 0.2 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 10% glycerol, and 0.03% Nonidet P-40. After the beads were washed five times with 200 μl of buffer, bound proteins were eluted by boiling in 40 μl of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer, separated by SDS–10% PAGE and blotted to nitrocellulose. Immunoblots were probed with an N-terminus-specific TBP antibody (kindly provided by S. Buratowski).

To assay TATA element binding, a 45-bp fragment (0.5 ng) containing the adenovirus early 1B TATA box (20) was incubated with 20 ng of the indicated TBP derivative in the presence of 200 ng of poly(dG-dC), 100 mM KCl, 40 mM HEPES (pH 7.9), 20 mM Tris (pH 7.5), 5 mM $MgCl_2$, 10% glycerol, 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride. TBP-DNA complexes were separated on a 5% acrylamide gel containing 0.5× Tris-borate-EDTA and 2 mM $MgCl_2$ in both the gel and the running buffer. Recombinant yeast TFIIA (40) and His-tagged human TFIIB were isolated and incubated with wild-type or mutant TBP and the TATA element probe and treated as described above, except that $MgCl_2$ was omitted from the gel and running buffer.

Assay for recruitment competency of TBP derivatives. YCp22 (the *TRP1* vector) and molecules expressing LexA and the various LexA-TBP derivatives were transformed into strain FT4. Growth phenotypes were assayed by spotting 10^4 cells on plates lacking histidine and containing either 0 or 20 mM aminotriazole, a competitive inhibitor of the *his3* gene product. *lacZ* assays were performed by growing cells to an optical density of 0.7 and treating them as described previously (42). LexA-TBP fusions were detected by immunoblot analyses of 100 μg of whole-cell extracts (11), using a polyclonal antibody to TBP.

RESULTS

Isolation of ts TBP mutants that complement a loss of TBP-Pol III function. Because TBP is required for transcription by all three RNA polymerases, it is possible to utilize intragenic complementation assays in which the combination of two inviable TBP derivatives with distinct molecular defects can support cell growth (10). Here, we utilize intragenic complementation in a genetic screen for TBP mutants that are functional for Pol III transcription at the restrictive temperature but specifically affect functions involved in Pol I or Pol II transcription. A ts strain that contains a TBP mutant (F155S) with a specific defect in Pol III transcription was transformed with TBP mutant libraries, and colonies able to grow at the restrictive temperature were selected (Fig. 1A). To eliminate wild-type TBP derivatives in the libraries that would complement the Pol III defect, we shuffled out the F155S allele and screened for strains with a ts growth phenotype. Thus, this genetic screen requires that the Pol III-complementing derivatives are ts alleles of TBP. The strength of this approach is that the mutant derivatives cannot be substantially structurally compromised (i.e., unfolded, degraded, or truncated), because they are functional for Pol III transcription at the restrictive temperature.

Eight such ts TBP mutants, which represented less than 0.1% of the TBP derivatives examined, were recovered from this screen. One of the TBP derivatives, termed N2-1, has been described (44). Four more of these Pol III-complementing mutant TBP derivatives are characterized here (Fig. 1B). As expected from the design of the screen, all four derivatives complement the molecular defect of F155S for Pol III transcription at the restrictive temperature (Fig. 1C). Furthermore, immunoblot analysis reveals wild-type levels of TBP in each of the mutant strains (Fig. 1D), indicating that the transcriptional phenotypes (see below) of these derivatives when present as the sole source of TBP are not the result of reduced expression or instability of the mutant TBPs.

The cocrystal structure of the conserved C-terminal 180 amino acids of TBP complexed with a TATA element indicates that the protein consists of a saddle-shaped molecule with DNA binding the concave undersurface (21, 22, 24). All four activation-defective substitutions (F148H, T153I, E236P, and F237D) reside on the exposed, convex upper surface of TBP in the conserved core domain (Fig. 2). The substitutions are not in residues that directly contact DNA, suggesting that the mutant TBPs are compromised for protein-protein interactions. Because of the relative positions of these residues in the crystal structure (two are located four amino acids apart near the N terminus while the other two are adjacent at the extreme C terminus), it is likely that these mutants represent at least two distinct classes of defects. Consistent with the anticipated outcome of the screen, the mutations map to amino acid residues that are not represented in the large collection of Pol III-specific derivatives (12).

The TBP derivatives are specifically defective in the response to acidic activators. To characterize the transcriptional properties of the mutant proteins, RNA levels for various genes were determined in strains carrying only the Pol III-complementing TBP mutants. Normal levels of Pol II transcription from the *DED1*, *HIS3*, *RPS4*, and *TRP3* genes, Pol I transcription (rRNA), and Pol III transcription ($tRNA^W$) were

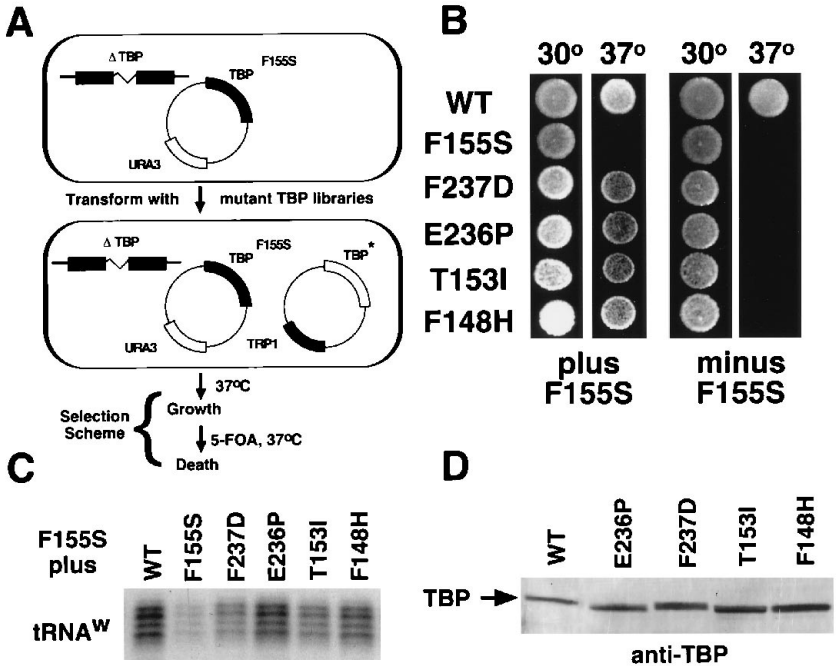

FIG. 1. Isolation of ts TBP mutants that complement a loss of Pol III function of the F155S TBP allele at the restrictive temperature. (A) Genetic selection. The starting strain, containing a chromosomal deletion of TBP and a centromeric *URA3* plasmid containing a ts, Pol III-specific derivative of TBP (F155S), was transformed with the mutant TBP libraries on a centromeric *TRP1* plasmid. Colonies were selected for growth at 37°C and then screened for the inability to grow on FOA at 37°C. (B) Growth of strains containing the indicated TBP derivatives at 30 and 37°C. Approximately 10^5 cells were spotted to plates deficient for the appropriate markers. (C) S1 analyses of $tRNA^W$ transcription using 10 μg of total RNA from strains containing the indicated TBP derivatives after shifting to 37°C for 1 h. (D) Immunoblots using N-terminus-specific yeast TBP antibody and 25 μg of whole-cell protein extract from the indicated strain grown at 30°C. WT, wild type.

observed in cells grown at the permissive (data not shown) or restrictive (Fig. 3A) temperature. Thus, these derivatives appear to be fully functional for transcription by Pol I and Pol III as well as constitutive transcription by Pol II at both the permissive and restrictive temperatures.

In contrast, strains containing the complementing derivatives are defective in the response to the acidic activators Gal4, Ace1, and Gcn4, even when grown at the permissive temperature (30°C). The response to the activator Gal4, which stimulates transcription in the presence of galactose, was assayed by using the *lacZ* reporter YCp86-Sc3801 (42). The wild-type strain exhibited 430 ± 30 U (mean ± standard deviation) of β-galactosidase activity. In contrast, mutant strains showed only 5 to 10% of this level of activity, as follows: F237D, 23 ± 4 U; E236P, 22 ± 2 U; T153I, 36 ± 2 U; and F148H, 42 ± 3 U. (Activities obtained with glucose were <1 U for all strains, including the wild type.) The response to Ace1, a copper-dependent activator (13), was determined by measuring *CUP1* RNA levels (Fig. 3B). In the presence of copper, the wild-type strain shows a 10- to 12-fold increase in *CUP1* transcription, whereas the mutant strains show diminished abilities to respond. Moreover, *CUP1* RNA levels in the mutant strains grown in the absence of added copper are also lower than those in the wild-type strain. *CUP1* transcription in the absence of added copper represents Ace1-dependent activation that is due to micromolar amounts of copper present in the media and activation by Ace2 and heat shock factor (4, 46). Finally, by using strains containing a plasmid expressing Gcn4 and assaying for *HIS3* transcription, the TBP mutants show reduced responsiveness to Gcn4 in comparison to wild-type TBP (Fig. 3C).

The severity of the activation defect varies for the different TBP mutants and for the activator tested (Fig. 3D). Both F237D and E236P are severely defective for response to Gal4 (5% of wild-type activity), Ace1 and Gcn4. While T153I and F148H are nearly as severely compromised for Gal4 induction as the previous TBP alleles (down to around 10% of wild-type activity), these derivatives are less defective for the response to Ace1 and Gcn4. It is important to note that these activation defects are observed at 30°C, a condition in which the mutant strains grow indistinguishably from the wild-type strain. We

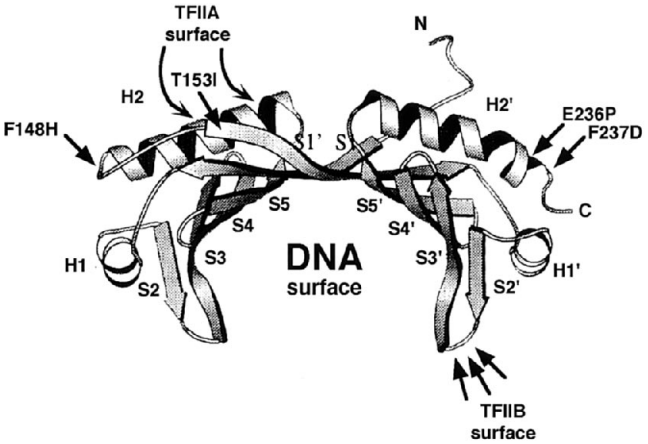

FIG. 2. Mapping of the four ts mutants on the TBP crystal structure. TBP is shown as a ribbon with four α helices (H1, H2, H1′, and H2′) and ten β strands (flat arrows S1 to S5 and S1′ to S5′). DNA interacts with β strands on the concave undersurface of TBP (DNA surface), whereas TFIIA interacts with H2 and TFIIB interacts with the stirrup between S2′ and S3′. The activation-defective TBP mutants are denoted by arrows indicating their relative positions (F148H, T153I, E236P, and F237D).

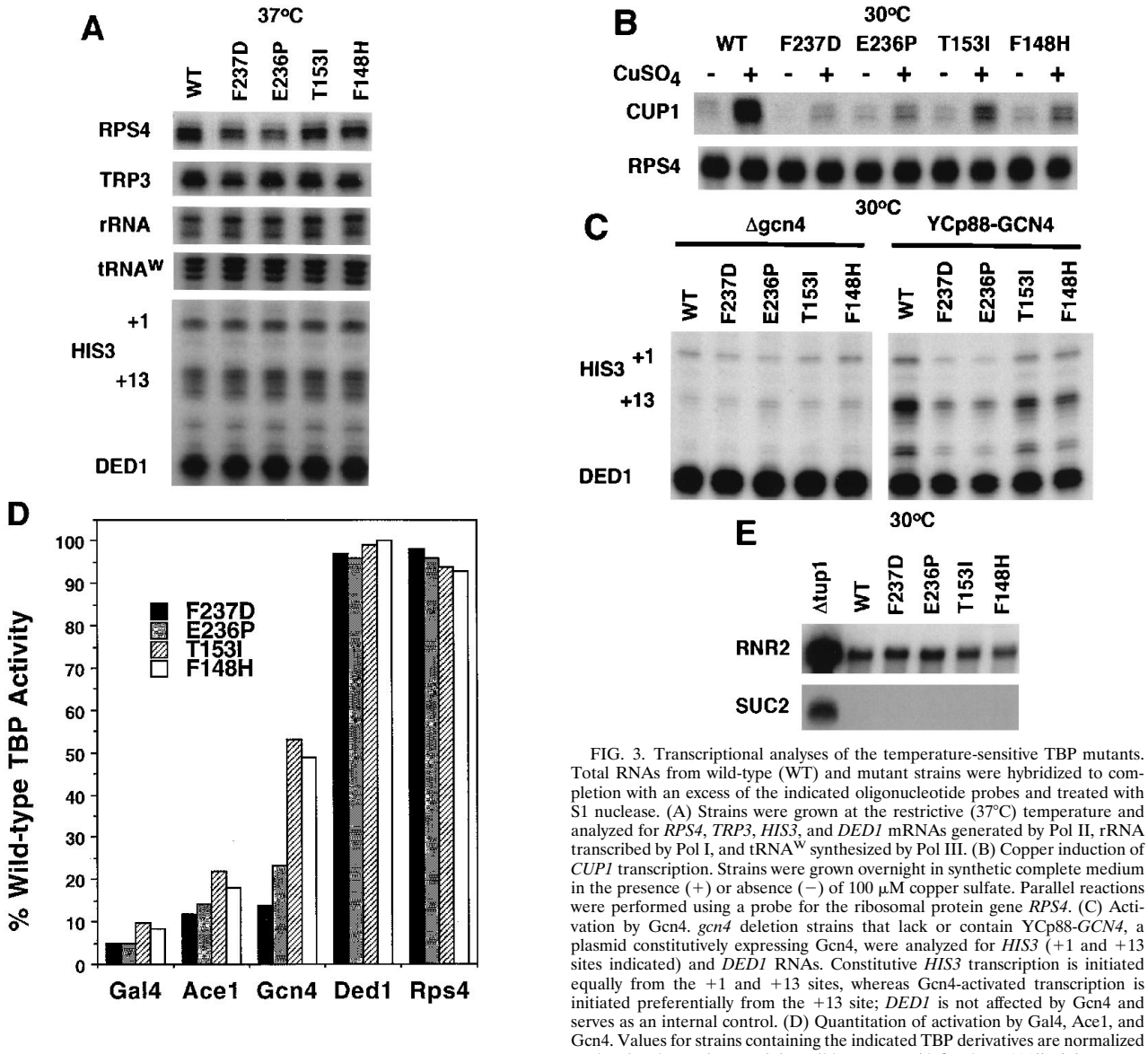

FIG. 3. Transcriptional analyses of the temperature-sensitive TBP mutants. Total RNAs from wild-type (WT) and mutant strains were hybridized to completion with an excess of the indicated oligonucleotide probes and treated with S1 nuclease. (A) Strains were grown at the restrictive (37°C) temperature and analyzed for *RPS4*, *TRP3*, *HIS3*, and *DED1* mRNAs generated by Pol II, rRNA transcribed by Pol I, and $tRNA^W$ synthesized by Pol III. (B) Copper induction of *CUP1* transcription. Strains were grown overnight in synthetic complete medium in the presence (+) or absence (−) of 100 μM copper sulfate. Parallel reactions were performed using a probe for the ribosomal protein gene *RPS4*. (C) Activation by Gcn4. *gcn4* deletion strains that lack or contain YCp88-*GCN4*, a plasmid constitutively expressing Gcn4, were analyzed for *HIS3* (+1 and +13 sites indicated) and *DED1* RNAs. Constitutive *HIS3* transcription is initiated equally from the +1 and +13 sites, whereas Gcn4-activated transcription is initiated preferentially from the +13 site; *DED1* is not affected by Gcn4 and serves as an internal control. (D) Quantitation of activation by Gal4, Ace1, and Gcn4. Values for strains containing the indicated TBP derivatives are normalized to that for the strain containing wild-type TBP (defined as 100%). (E) Repression by the Cyc8-Tup1 complex. Total RNA (10 μg) from the indicated TBP derivative and *tup1* deletion strains was blotted to nylon filters and hybridized with *RNR2* or *SUC2* probes.

suspect that the ts phenotype of the mutant strains reflects an exacerbation of this activation defect and/or the increased importance of transcriptional activation for growth at nonoptimal temperatures. Thus, all four TBP mutant strains display defects in the response to three acidic activators that induce the expression of physiologically unrelated sets of genes.

The mutant TBP derivatives are competent for repression by the Cyc8-Tup1 and NOT complexes. There is no difference in the levels of *SUC2* and *RNR2* RNAs between the activation-deficient mutant strains and those in the wild-type strain (Fig. 3E), indicating that repression by Cyc8-Tup1 is not defective. *NOT*-dependent repression is observed in the mutant strains because a preferential increase in *HIS3* transcription from the +1 initiation site is not observed (8). Instead, the ratio of +1 to +13 transcripts in the mutant strains is nearly 1:1 and comparable to wild-type levels (Fig. 3C). Taken together, these observations indicate that the mutant TBP derivatives are specifically defective in the response to acidic activators.

Biochemical characterization of the mutant TBP derivatives. To investigate the molecular basis of the activation defect, the mutant proteins were produced in *E. coli*, and their biochemical properties were compared with those of wild-type TBP. Interaction of these TBP derivatives with an acidic activation domain was assessed by coprecipitation with GST-VP16 (Fig. 4A), whereas mobility shift assays were used to determine TATA element binding (Fig. 4B) and the abilities to form TBP-TFIIA-DNA (Fig. 4C) and TBP-TFIIB-DNA (Fig. 4D) complexes. Three of the four TBP derivatives (E236P, T153I, and F148H) are indistinguishable from wild-type TBP by all of these biochemical assays. These results are not surprising because the mutations do not map to the surfaces that mediate interactions with TATA elements (21, 22, 24), TFIIA (3, 44, 47), or TFIIB (23, 36, 47).

The remaining TBP derivative, F237D, has unusual biochemical properties. This protein efficiently binds a TATA element, but the complex migrates with a lower electrophoretic mobility, suggestive of an altered conformation. In addition, the F237D derivative is unable to form TBP-TFIIA-DNA or

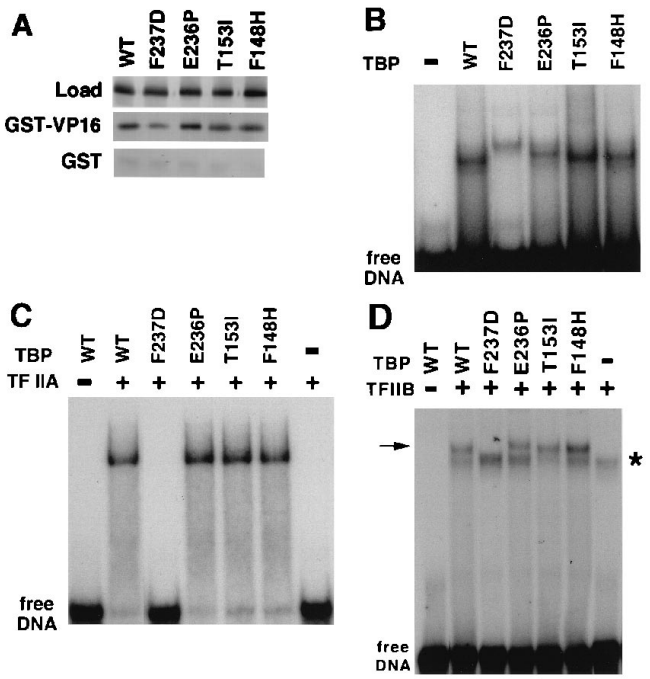

FIG. 4. Biochemical properties of the mutant TBP derivatives. (A) Interaction with the acidic activator VP16. Glutathione-agarose beads containing GST-VP16 or GST were incubated with equal amounts of wild-type or mutant TBPs and treated as described in the text. Immunoblots were probed with an N-terminus-specific TBP antibody. (B) TATA element binding. A 45-bp fragment containing the adenovirus early 1B TATA box was incubated with the indicated TBP derivative. TBP-DNA complexes were separated on an acrylamide gel containing 2 mM $MgCl_2$ in both the gel and running buffer. (C) Interaction with TFIIA. Recombinant yeast TFIIA was incubated with wild-type or mutant TBP and the TATA element probe and treated as described above except that $MgCl_2$ was omitted from the gel and running buffer. Under these conditions, only TBP-TFIIA-DNA complexes are stable. (D) Interaction with TFIIB. His-tagged human TFIIB was purified from *E. coli* and incubated with wild-type or mutant proteins and analyzed under the conditions used for panel C. The arrow indicates the position of the TBP-TFIIB-DNA complex (the E236P complex has a slightly reduced mobility). The asterisk denotes a nonspecific binding activity that copurifies with recombinant TFIIB from bacteria. This activity is observed in all reactions including that lacking TBP. It is very unlikely that F237D generates a TBP-TFIIB-TATA complex of abnormally rapid mobility that comigrates with the band representing the nonspecific activity; in fact, the F237D-TATA complex migrates more slowly than the wild-type TBP-TATA complex. WT, wild type.

TBP-TFIIB-DNA complexes, and it shows a slightly diminished ability to interact with the VP16 activation domain. As F237D does not map to the TFIIA or TFIIB interaction surface, these results are suggestive of an altered structure (see Discussion).

Fusion of the activation-defective TBP mutants to TFIIA does not restore the response to acidic activators. We have previously described an in vivo activation-defective TBP mutant (termed N2-1) that is severely defective for interaction with TFIIA in vitro (44). Strains supported solely by N2-1 grow slowly at 30°C and are unable to grow at 37°C. Fusion of the small subunit of TFIIA (Toa2) to the N2-1 derivative restores the ability to grow at 37°C as well as the ability to respond to acidic activators, demonstrating that an efficient interaction between TBP and TFIIA is required for transcriptional activation in vivo.

We tested the hypothesis that loss of TFIIA interaction causes the activation deficiency of these new TBP derivatives by constructing Toa2 fusions and assaying them for their growth phenotypes (Fig. 5). Recovery of normal growth properties is specific to the N2-1 derivative. In contrast, fusion of Toa2 to wild-type TBP is deleterious to its function, resulting in a ts phenotype. The TFIIA fusion context is lethal for a ts, Pol III-specific derivative of TBP derivative (F155S) and the activation-defective derivative F237D. This loss of viability probably represents an exacerbation of the ts phenotype displayed with the wild-type TBP-TFIIA fusion. Fusion to Toa2 does not restore function to the remaining three activation-defective TBP alleles because they retain their ts phenotypes. These observations indicate that, unlike the deficiencies in N2-1, the activation deficiencies of the TBP derivatives described here are not due to a defect in the TFIIA interaction.

Activation deficiency of some TBP derivatives can be overcome by artificial recruitment to the promoter. When TBP is artificially brought to a promoter by being physically connected to a heterologous DNA-binding domain bound upstream of a TATA element, transcriptional activation occurs in the absence of an activator (5, 26, 52). This is not due to a fortuitous activation domain on TBP but instead occurs because the heterologous DNA-binding domain recruits TBP to the TATA element, where it can then bind and nucleate the assembly of the remaining factors. Under these circumstances, interactions that occur prior to TBP recruitment to the TATA element can be bypassed.

This artificial recruitment assay was used to determine the step at which the TBP derivatives were unable to activate transcription (Fig. 6). Specifically, the heterologous DNA-binding domain LexA was fused to the activation-defective TBP derivatives, and the resulting LexA-TBP fusions were tested for their ability to activate transcription of an appropriate promoter. If an activation-defective TBP mutant can activate transcription when artificially recruited by LexA, then the TBP mutant must be defective for recruitment to the TATA element because the defect can be bypassed. Conversely, if the mutant TBP fails to activate transcription even when artificially recruited to the promoter, then the mutant must be impaired for interactions that occur after recruitment to the TATA element.

Transcriptional activation by the LexA-TBP derivatives was determined in a strain containing a *his3* allele with a single

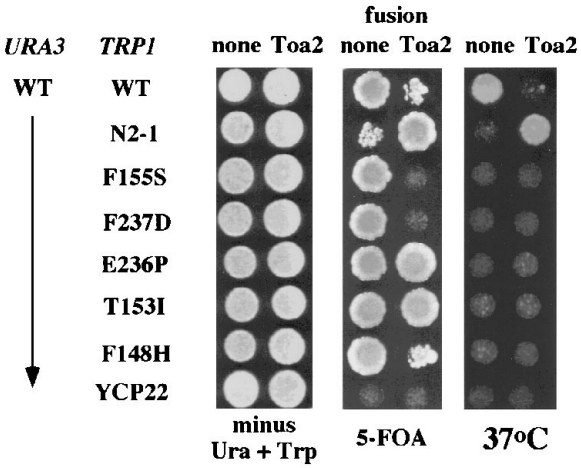

FIG. 5. Fusion to TFIIA does not rescue the growth phenotype of the activation-defective TBP mutants. The *TOA2* coding sequence was fused to the N terminus of the indicated TBP derivatives. The growth of strains containing the indicated TBP derivatives (or vector), with or without the Toa2 fusion, was analyzed. Approximately 10^5 cells were spotted to plates deficient for tryptophan or containing FOA. After shuffling of the wild-type TBP (carried on a *URA3* plasmid) on FOA, strains were printed to YPD plates and assayed for growth at 37°C. WT, wild type.

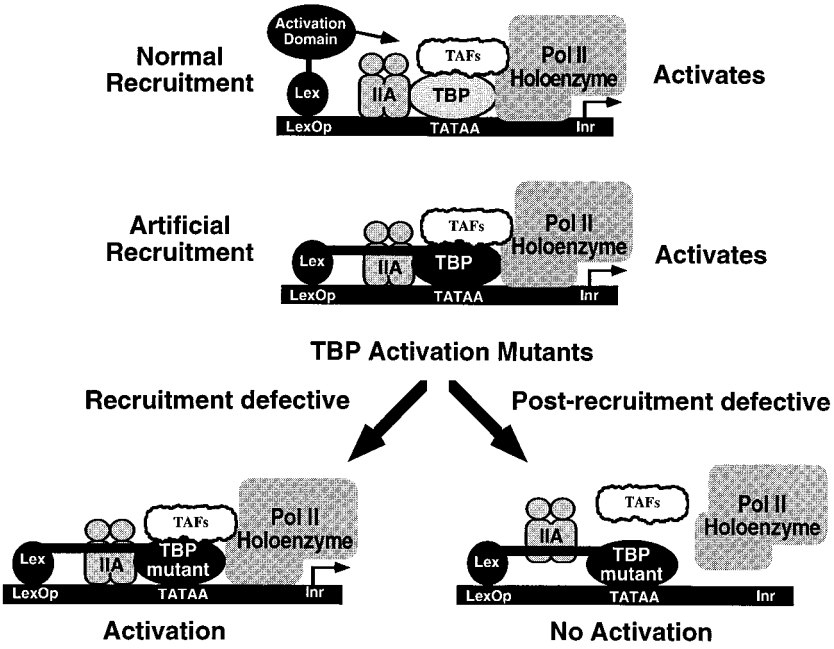

FIG. 6. Recruitment assay for determining whether the activation-defective TBP mutants are competent for targeting to the TATA element in vivo. Under normal recruitment conditions, an upstream activator interacts with some component of the initiation complex and recruits (directly or indirectly) TBP to the promoter. Under artificial recruitment conditions, binding of LexA brings the connected TBP to the promoter. In either case, TBP interacts with the TATA element and directs recruitment of the remainder of the initiation complex, including the Pol II holoenzyme and perhaps the TAFs. If a TBP with an activation-defective allele has a recruitment defect, it should activate transcription when artificially recruited to the promoter. In contrast, if a TBP mutant is postrecruitment defective because of loss of interaction with TAFs or holoenzyme, then it should remain activation defective even when artificially recruited. LexOp, Lex operator.

LexA operator 45 bp upstream of the TATA element (5). When grown in the presence of 20 mM aminotriazole, a competitive inhibitor of the *HIS3* gene product, a strain containing LexA-TBP (wild type) grows very well, whereas control strains containing LexA or the *TRP1* vector fail to grow (Fig. 7A). LexA-T153I behaves indistinguishably from LexA-TBP, while LexA-F148H confers an intermediate growth phenotype. In contrast, strains containing LexA-F237D and LexA-E236P fail to grow under these conditions and are comparable to the control strains.

Expression levels were directly measured from a *lacZ* derivative of the promoter (Fig. 7B), and the results were in good accord with the growth phenotypes. LexA-T153I exhibited a slightly higher level of activation than did LexA-TBP, LexA-F148H activated transcription at an intermediate level, and LexA-E236P activated transcription only twofold over background levels of transcription produced by LexA alone. Unexpectedly, the transcription level in the strain containing LexA-F237D was nearly six times lower than the level in the strain containing LexA alone. Immunoblot analysis (Fig. 7C) indicated that LexA-T153I, LexA-F148H, and LexA-E236P were all present at levels equivalent to that of LexA-TBP; the amount of LexA-F237D was reduced to approximately 30% of the LexA-TBP level.

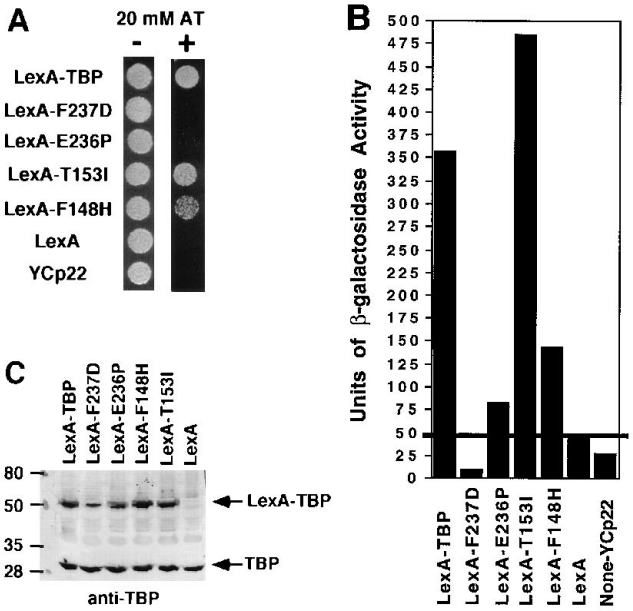

FIG. 7. Artificial recruitment assay of LexA-TBP and mutant derivatives. (A) Strains containing the indicated LexA-TBP derivative, LexA or vector (YCp22), and a promoter with a LexA operator 45 bp upstream of the *his3* TATA element and structural gene were tested for growth on 20 mM aminotriazole (AT). Plates were photographed after four days at 30°C. The degree of AT resistance is directly related to the level of *his3* transcription. (B) β-Galactosidase assays of the same promoter fused to the *lacZ* gene. Each value represents the average from two independent assays on two different isolates from each strain. (C) Immunoblot analyses of 100 μg of whole-cell extract from each of the indicated strains probed with anti-TBP antibody. Molecular weight markers (in thousands) and the positions of endogenous TBP and LexA-TBP fusions are indicated.

DISCUSSION

A new class of TBP mutants defective for the response to acidic activators in vivo. Previous work has identified two classes of TBP derivatives that are specifically defective in the response to acidic activators in vivo. One class of activation-defective derivatives contains mutations on the DNA-binding surface of TBP and is defective in binding a TATA element in

vitro (1, 31). The second class, exemplified by the N2-1 derivative, is specifically defective for the interaction with TFIIA in vitro, and this biochemical defect is responsible for the activation defect in vivo (44).

The four activation-defective TBP derivatives described in this paper are biochemically distinct from the two previously characterized classes. First, the four TBP mutations do not map to residues that make direct contacts with DNA, and all four derivatives efficiently bind the TATA element in vitro (Fig. 4B). Second, the TBP mutations described here do not map to the surface involved in the TBP-TFIIA interaction (3, 29, 44, 47), and three of the four mutant proteins are comparable to wild-type TBP in forming TBP-TFIIA complexes (Fig. 4C). The remaining derivative, F237D, is not specifically defective for the TFIIA interaction but instead is impaired for all protein-protein interactions tested (see below). Furthermore, unlike the N2-1 derivative, whose growth and activation defects are restored by fusion to the small subunit of TFIIA (44), the growth defects of the four TBP derivatives described here are either unaffected or exacerbated by the TFIIA fusion (Fig. 5).

The categorizing of activation-defective TBP mutants into at least three discrete mechanistic classes indicates a multiplicity of TBP surfaces and functions involved in the process of activated transcription. For the three derivatives that are indistinguishable from wild-type TBP with respect to interaction with the TATA element, an acidic activation domain, TFIIA, or TFIIB, the biochemical defect that causes activation deficiency in vivo is unknown. It is likely that the biochemical defects reflect an impaired protein-protein interaction, with TBP-associated factors (TAFs) or components of the Pol II holoenzyme being the most likely candidates.

Properties of the F237D derivative that suggest a role for the conformation of the TBP-TATA element complex in transcriptional activation. The F237D derivative is unusual in that it is defective for all protein-protein interactions assayed in vitro (Fig. 4). This general biochemical defect does not simply reflect protein unfolding, because the F237D derivative binds a TATA element with an affinity comparable to that of wild-type TBP (Fig. 4B). However, the F237D-TATA complex has reduced electrophoretic mobility (Fig. 4B), suggestive of an altered conformation. An altered TBP-TATA conformation could explain the instability of higher-order complexes containing either TFIIA or TFIIB. Although TFIIA and TFIIB bind to different regions of TBP (3, 23, 29, 36, 44, 47), both of them also interact with DNA (19, 32, 36). Biochemical and crystallographic evidence indicates that TFIIB recognizes the TBP-TATA complex (32, 36), and it is likely that the same is true for TFIIA.

Two observations are consistent with the hypothesis that the altered TBP-DNA complex of F237D is relevant to the activation defect in vivo. First, although F237D is defective for interaction with TFIIA in vitro, fusion of TFIIA to F237D does not restore functional competency in vivo. Although the fusion may force the TFIIA-TBP interaction, TFIIB (and probably other proteins) may still not recognize the F237D-TATA complex. Second, artificial recruitment of F237D reduces expression levels below the background level observed in the strain containing the *TRP1* vector; this background level probably reflects a low level of nonactivated transcription mediated by endogenous TBP. The ability of LexA-F237D to "repress" this background transcription might reflect F237D binding the TATA element in an inactive conformation that distorts the promoter and blocks endogenous TBP from driving basal levels of transcription.

The F237 position is also interesting because it is the only instance in which various amino acid substitutions cause differential defects in Pol II and Pol III transcription. Unlike the F237D derivative, which is specifically defective in activated Pol II transcription, the F237R derivative is ts for uninduced (and presumably activated) Pol II transcription (12), whereas the F237P and F237L derivatives are specifically defective for Pol III transcription (12). Further, this phenylalanine residue is highly conserved in a wide variety of eukaryotic organisms (43).

Activation-defective TBP mutants impaired in recruitment to the promoter. In vivo, acidic activators can increase recruitment of TBP to the promoter (27), and the requirement for an activation domain can be bypassed by artificially recruiting TBP to the promoter via physical connection to a protein bound to the promoter (5, 26, 52). These observations suggest that the inherent ability of TBP to bind TATA elements is insufficient for efficient recruitment of TBP to promoters in vivo and that additional protein-protein interactions are required. This hypothesis predicts a class of activation-defective TBP mutants that are impaired for recruitment even though they have an inherent wild-type affinity for TATA elements.

The activation defect of the T153I derivative is completely bypassed by artificial recruitment to the promoter via the LexA DNA-binding domain (Fig. 7). This indicates that, in the normal situation (unfused to LexA), the activation defect of the T153I protein reflects an inability to be recruited effectively to the TATA element. Presumably, the T153I derivative is impaired for an activation-specific interaction(s) with another protein. In vitro, T153I interacts normally with TFIIA, TFIIB, and the VP16 acidic activation domain (Fig. 4), suggesting that another protein mediates the activation-specific interaction. In the case of the F148H derivative, fusion to LexA results in activation, although to a lower level than that observed for LexA-TBP. This intermediate phenotype is difficult to interpret, but it suggests that inefficient recruitment of the F148H derivative may partly account for activation deficiency. Thus, T153I, and to a lesser extent F148H, provides independent evidence that efficient recruitment of TBP to the TATA element is an important first step in the mechanism of activated transcription (Fig. 8).

Activation-defective TBP alleles impaired for a step(s) after recruitment to the TATA element. In contrast to the behavior of the T153I and F148H derivatives, the E236P and F237D proteins do not appreciably activate transcription when artificially recruited to the TATA element (Fig. 7). LexA-E236P only slightly stimulates transcription above the level of the LexA control, and LexA-F237D actually reduces transcription below background levels. Other LexA-TBP derivatives fail to activate transcription in this assay, because of mutations on the DNA-binding surface of TBP that prevent interaction with the TATA element (5). Importantly, this is not the case for E236P and F237D, because both TBP derivatives efficiently bind TATA elements (Fig. 4B). Thus, targeting of E236P and F237D to the promoter should be comparable to targeting of wild-type TBP, yet these derivatives fail to activate.

The observation that the activation defects of the E236P and F237D derivatives cannot be bypassed upon artificial recruitment to the promoter indicates that these TBP mutants are defective for interactions that occur after TBP is recruited to the promoter. These results provide the first evidence for a second step in the mechanism by which TBP responds to acidic activators in vivo (Fig. 8). Moreover, they demonstrate that recruitment of TBP to the promoter is not sufficient for transcriptional activation in vivo. The interactions involving TBP that define the postrecruitment step are specific to activated transcription, because the TBP mutants are fully functional for constitutive Pol II transcription.

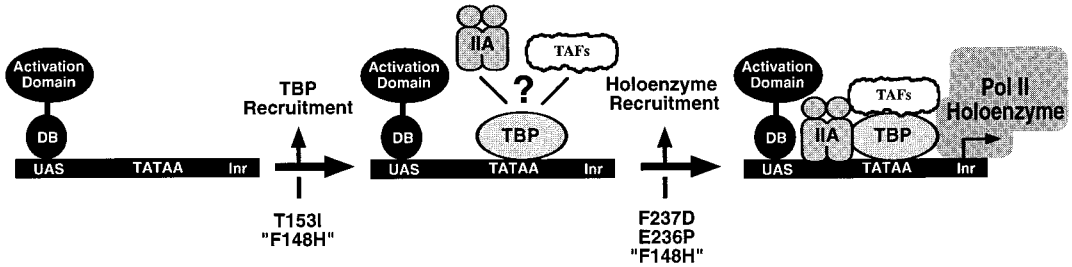

FIG. 8. Two-step model for transcriptional activation in vivo. TBP and holoenzyme recruitment steps (horizontal arrows) are defined by the indicated TBP mutations that block the activation process at different stages (vertical arrows). The quotation marks around F148H indicate the uncertainty about whether the intermediate phenotype in the artificial recruitment assay reflects partial defects at one or both steps. TFIIA and TAFs may be involved in the TBP recruitment step, although this remains to be determined in vivo. The Pol II holoenzyme could be recruited either as a preassembled complex or by sequential assembly of individual components or partially assembled subcomplexes. See the text for details and limitations of this model. UAS, upstream activation sequence.

Molecular implications of the two-step model for transcriptional activation in vivo. Steps in a complex biological process are often defined by mutations or inhibitors that block the process at distinct stages. In this study, we have used the properties of activation-defective TBP mutants to define two steps in the process of transcriptional activation in vivo (Fig. 8). The existence of mutations that block distinct stages of the process indicates that the steps occur under physiological conditions in wild-type yeast cells. However, because mutations perturb the natural process, they are unable to provide information about which steps are rate limiting in wild-type cells.

Our conclusion that there are at least two steps for transcriptional activation in vivo is in broad agreement with biochemical experiments. Acidic activators can stimulate the formation of a TFIID-TFIIA-TATA complex in vitro (6, 33, 51), a step that may be analogous to TBP recruitment to the promoter in vivo. On the other hand, acidic activators can increase recruitment of TFIIB or subsequent factors to complexes containing TBP (or TFIID) bound to the TATA element (7, 34). The relationship between these postrecruitment steps in vitro to the postrecruitment step we have defined here in vivo is unknown, particularly in regard to the role of TBP.

Acidic activators can stimulate transcription in reactions reconstituted in two distinct ways. In one approach, acidic activators are added to reactions containing TFIID, highly purified or recombinant general factors (TFIIA, -B, -E, -F, -H, and -J), and purified Pol II that presumably lacks Srb (and other) proteins (39, 41, 48). A critical aspect of this approach is that the TAFs are required for activation; i.e., TBP cannot be substituted for the TFIID complex. The second type of reaction contains recombinant TBP and the Pol II holoenzyme, a multiprotein complex that contains the Pol II subunits, most of the general transcription factors, and the Srb (and other) proteins (25, 28). In this situation, TAFs do not appear to be required for activation. In vivo, direct recruitment of Pol II holoenzyme can bypass the requirement for an activation domain, but not the need for TBP (2). For these reasons, it is tempting to speculate that the two steps of activation in vivo defined here by the TBP mutants are related to the two mechanisms of activation in vitro. One possibility is that the TBP mutations defective in recruitment to the promoter might correspond to TAF-dependent activation in vitro, whereas the TBP mutations defective after recruitment might correspond to holoenzyme-dependent activation. In this view, both the TAF- and the holoenzyme-dependent activation mechanisms observed in vitro would be required for the full response to acidic activators observed under physiological conditions.

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