Multiple regions of TBP participate in the response to transcriptional activators in vivo

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We used mutant yeast and human TBP molecules with an altered DNA-binding specificity to examine the role of TBP in transcriptional activation in vivo. We show that yeast TBP is functionally equivalent to human TBP for response to numerous transcriptional activators in human cells, including those that do not function in yeast. Despite the extensive conservation of TBP, its ability to respond to transcriptional activators in vivo is curiously resistant to clustered sets of alanine substitution mutations in different regions of the protein, including those that disrupt DNA binding and basal transcription in vitro. Combined sets of these mutations, however, can attenuate the in vivo activity of TBP and can differentially affect response to different activation domains. Although the activity of TBP mutants in vivo did not correlate with DNA binding or basal transcription in vitro, it did correlate with binding in vitro to the largest subunit of TFIID, hTAF^n250. Together, these data suggest that TBP utilizes multiple interactions across its surface to respond to RNA polymerase II transcriptional activators in vivo; some of these interactions appear to involve recruitment of TBP into TFIID, whereas others are involved in response to specific types of transcriptional activators.

[Key Words: TBP, transcriptional activation, hTAF^n250, RNA polymerase II]

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RNA polymerase II transcription in higher eukaryotes is regulated by a diverse range of sequence-specific transcription factors, which act in combination to signal the transcriptional machinery to initiate transcription [for review, see McKnight and Yamamoto 1992]. Although much emphasis has been placed on how activators stimulate the basal machinery in vitro [for review, see Tjian and Maniatis 1994], little is known of the way in which the basal factors respond to activators in vivo.

Transcriptional activators appear to work in vitro by stimulating the ordered assembly of general transcription factors [GTFs] at a promoter, forming a preinitiation complex that can initiate transcription [for review, see Zawel and Reinberg 1993]. For TATA box-containing, RNA polymerase II-transcribed genes, formation of this complex begins with binding of the TATA box-binding protein [TBP] and its associated factors [TAFs] to the TATA box, followed by the sequential addition of other GTFs including RNA polymerase II [for review, see Buratowski 1994]. By targeting one or more of the general factors and facilitating their entry onto the promoter, activators can significantly enhance the rate of in vitro preinitiation complex assembly [Lieberman and Berk 1991, 1994; Lin and Green 1991; Choy and Green 1993].

The process of activation in vivo, however, may be different. For example, the response of the fractionated GTFs to activators during preinitiation complex assembly in vitro may not fully reflect the in vivo situation, because the GTFs may exist in large multicomponent complexes [Thompson et al. 1993; Kim et al. 1994; Koleske and Young 1994]; the mechanisms that activate such complexes may differ from those that affect the isolated GTFs in vitro. In addition, although most in vitro studies examine single rounds of transcription initiation, the processes of activated reinitiation are likely to be more important for activator function in vivo, as activators can sustain high levels of transcription from the one or two functional gene copies within a cell.

Of the general transcription factors, TBP is studied most extensively [for review, see Hernandez 1993]. It is a highly conserved protein, containing a 180-amino-acid carboxy-terminal domain of known structure [Niklov et al. 1992; J.L. Kim et al. 1993; Y. Kim et al. 1993] that shares >80% sequence identity between yeast and humans. TBP plays an important role in transcriptional activation in vitro, both as a direct target for activators [Ingles et al. 1991; Lieberman and Berk 1991; Truant et al. 1993; Emili et al. 1994] and by its association with other target proteins, including TFIIB [Lin and Green 1991] and the TAFs [for review, see Goodrich and Tjian 1994]. The association of TBP with the TAFs (forming the TFIID complex) is particularly important to the process...
of activation, as the TAFs are essential for activated transcription but dispensable for basal transcription in vitro (Dynlacht et al. 1991; Tanese et al. 1991; Zhou et al. 1993) and bridge activators to other components of the transcriptional machinery (for review, see Tjian and Maniatis 1994; Chen et al. 1994).

Previous studies of TBP in vivo have focused on the isolation of temperature-sensitive mutants in yeast. These studies have provided genetic evidence that TBP is involved in transcription by all three classes of RNA polymerase (Cormack and Struhl 1992; Schultz et al. 1992) and have identified a surface of TBP, spanning the two largest α-helices, that is required for RNA polymerase III transcription (Cormack and Struhl 1993). Such analyses are limited, however, to the isolation of mutants that generate a conditional growth phenotype, and to date no TBP mutants specifically defective for activated transcription by RNA polymerase II transcription have been identified by this approach. In addition, as some activation domains, such as those from the human transcription factor Sp1, do not display activity in yeast (Emili et al. 1994; Künzler et al. 1994), an analysis in human cells can better probe how a wide variety of nonconserved activators act on the relatively conserved basal transcriptional machinery.

In this paper we investigate the role played by TBP in activated RNA polymerase II transcription in human cells. We show that this activity of TBP in vivo involves numerous interactions across the surface of the molecule and that different regions of TBP participate in response to different transcriptional activators. Much of the behavior of TBP in vivo appears to reflect the nature of the association between TBP and the largest subunit of TFIIID, hTAF9250. These data underscore the importance of TBP–TAF interactions for activated transcription but also reveal that TBP functions in other ways to respond to transcriptional activators in vivo.

Results

Experimental design

The structure of the conserved carboxy-terminal domain of TBP is represented in Figure 1. The underside of the saddle-like structure (shown in Fig. 1B) contains eight antiparallel β-sheets (Niklov et al. 1992) that form the DNA-binding surface of TBP (J.L. Kim et al. 1993; Y. Kim et al. 1993). Accordingly, when TBP is bound to the DNA, much of the opposite surface of the molecule, including the four α-helices and many of the loops connecting the β-strands, should be available for interaction with other proteins.

To monitor the activity of TBP in human cells, we used the altered-specificity TBP assay first described by Strubin and Struhl (1992). This assay employs a TBP derivative with a triple amino acid substitution on its DNA-binding surface (indicated in orange in Fig. 1). This TBP derivative (TPBAS) has a relaxed DNA-binding specificity and recognizes both a canonical TATAAA box and an altered TGTAAA box (Strubin and Struhl 1992). The combination of TPBAS and the TGTAAA box circumvents the activity of endogenous wild-type TBP in yeast (Strubin and Struhl 1992), plant (Heard et al. 1993), and mammalian (Keaveney et al. 1993) cells and thus allows the effects of in vitro manipulations in TBP to be examined in vivo.

We introduced various mutations into TPBAS and analyzed the in vivo effects of these mutations on response to a battery of different transcriptional activation domains, each fused to the heterologous yeast GAL4 DNA-binding domain (residues 1–94; Carey et al. 1989). The structure of these GAL4–fusion activators is represented in Figure 2A. Sp1GQ contains two copies of the glutamine-rich Sp1 B domain (Courey and Tjian 1988) and was chosen because it activates transcription in human but not yeast cells (Künzler et al. 1994). Oct-2OQ and Oct-2p are both derived from the human transcription factor Oct-2 but have distinctly different amino acid compositions: Oct-2OQ consists of four copies of an 18-amino-acid glutamine-rich segment from the amino-termina activa tion domain of Oct-2 (Tanaka and Herr 1994), whereas Oct-2pQ carries two copies of a 42-amino-acid proline-rich activation domain from the carboxy1 terminus of Oct-2 (Tanaka et al. 1994). CTFp and VP16 both carry single-copy activation domains from CTF (Mermod et al. 1989) and the herpes simplex virus trans-activator VP16 (Triezenberg et al. 1988), respectively.

Figure 2A also lists the relative activity of the various GAL4–fusion activators on the wild-type counterpart of the TGTAAA-containing c-fos reporter used in these studies (see below). Because the VP16 activation domain was much more active than the other activation domains examined, the amino- (VP16a) and carboxy- (VP16c) terminal halves of this domain (Regier et al. 1993) were also assayed individually. These truncated VP16 activation domains displayed activities similar to those of the other activators (see Fig. 2A).

Figure 2B shows the structure of the wild-type and TGTAAA-containing c-fos reporters. The wild-type TATAAA reporter construct contains the c-fos promoter to position −56 (Berkowitz et al. 1989) linked to four upstream synthetic GAL4 DNA-binding sites. The TGTAAA reporter is identical, except for the single A → G transition in the TATAAA box (TATAAA to TG TAAA). As shown below, this mutation in the c-fos promoter disrupts activation in the presence of wild-type TBP but not TPBAS. The disruption of wild-type TBP activity by the TGTAAA mutation was promoter specific; a corresponding mutation in the β-globin promoter did not attenuate in vivo transcriptional activity (data not shown).

TPB and GAL4–fusion activator molecules were transiently coexpressed with the c-fos reporter constructs in human HeLa cells. Correctly initiated transcripts from the c-fos reporters and from an α-globin internal control plasmid were quantitated by RNase protection analysis. The α-globin signals were used to normalize for differences in transfection efficiency and were not detectably affected by transient expression of exogenous TBP at the
Figure 1. Ribbon diagram of TBP structure, showing the positions of the systematic sets of mutations generated for in vivo analysis. (A) Top view; (B) front view. Depicted is the solved crystal structure for Arabidopsis thaliana TBP-2 [Niklov et al. 1992]; the yeast TBP structure is similar [Y. Kim et al. 1993] and is likely to be the conformation adopted by human TBP in this region. The α-carbon backbone is shown in white, and the position of the three altered-specificity (AS) substitutions [Strubin and Struhl 1992] are shown in orange. (Red) Position of the alanine substitution mutations H1 and H1'; (green) position of the H2 and H2' mutations; (purple) position of the mutations on the two loops connecting strands S3'/S4'. These mutations are described in detail in Table 1.

levels used in these experiments [data not shown]. Expression of the mutant TBPAS molecules was monitored by quantitative Western blot analysis of protein extracts from transfected cells [data not shown]. In all experiments the amount of TBPAS expression construct used was adjusted to give equivalent levels of expression for each TBPAS.

Figure 3 shows the response of wild-type and mutant TBPAS molecules to each of the GAL4-fusion activators. Because the different activators possess very different relative activities [see Fig. 2A], different length exposures are shown for each activator. The wild-type TATAAA c-fos promoter responded well to the different activation domains examined [Fig 3, cf. lanes 1 and 2, panels A–G, see also Fig. 2A]. Introduction of the TG-TAAA mutation in the c-fos promoter significantly attenuated the transcriptional response [cf. lanes 2 and 4], from between 12-fold for VP16 [E] to 17-fold for Oct-2Q [B]. Transient coexpression of full-length human TBPAS [lane 6], but not an equivalent amount of wild-type human TBP [lane 5], restored transcriptional activation [cf. lanes 2 and 6, A–G]. Activation by TBPAS [lane 6] was still dependent on expression of the GAL4 activator [data not shown]. The difference in c-fos promoter activity observed in the absence [lane 4] and the presence [lane 6] of TBPAS provides the assay for wild-type and mutant TBPAS function in response to each of the activators.

The activity of TBPAS was not dependent on the nonconserved amino-terminal region of human TBP [residues 2–159], as its deletion [ΔN, cf. lanes 6 and 7, A–G] did not diminish response to any of the GAL4–fusion activators examined—indeed, the Oct-2Q and VP16E activators were three- to fivefold more active with the amino-terminally truncated TBP [cf. lanes 6 and 7, C and F]. These data show that the nonconserved region of TBP is dispensable for RNA polymerase II transcriptional activation in human cells as it is in vitro [Kelleher et al. 1992; Zhou et al. 1993] and in yeast cells [Cormack et al. 1991] and enabled us to confine our further mutational analysis to the conserved carboxy-terminal region of the protein.

Human and yeast TBP support equivalent levels of transcriptional activation in human cells

The carboxy-terminal 180 amino acids of human and yeast [Saccharomyces cerevisiae] TBP are 81% identical and are interchangeable for basal transcription and response to acidic activators in vitro [Kelleher et al. 1992] and in vivo [Strubin and Struhl 1992; Keaveney et al. 1993]. Despite this high degree of sequence conservation, yeast TBP cannot replace human TBP for response to an “E1A-like” activity in mouse embryonic carcinoma (EC) cells [Keaveney et al. 1993] and does not respond to Sp1 activation domains in yeast [Emili et al. 1994; Künzler et al. 1994]. To investigate functional differences between human and yeast TBP, we therefore assayed the ability of yeast TBP to respond to our panel of activation domains in human cells.

As shown in Figure 3, yeast and human TBPAS responded similarly to all of the GAL4–fusion activators examined [cf. lanes 6 and 14, A–G]. Thus, yeast TBP can respond to a wide array of mammalian activation domains in human cells. In particular, there was no significant difference in human and yeast TBPAS response to Sp1Q [A], demonstrating that although the activation domains of Sp1 do not activate transcription in yeast, yeast TBP can support activation by Sp1 in human cells. This result suggests that the role TBP plays in activated transcription has been conserved between yeast and humans but that the accessory factors required for Sp1 activation are not conserved between these species.
TBP response to activators in vivo

A

<table>
<thead>
<tr>
<th>GAL4-Activator</th>
<th>Activation Domain</th>
<th>Activation Domain Character</th>
<th>Relative Activity</th>
</tr>
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<tr>
<td>Sp1°</td>
<td>Sp1(2x8)</td>
<td>Q-rich</td>
<td>1.0</td>
</tr>
<tr>
<td>Oct-2°</td>
<td>Oct-2(4xQ18)</td>
<td>Q-rich</td>
<td>3.3</td>
</tr>
<tr>
<td>Oct-2p</td>
<td>Oct-2(2xP42)</td>
<td>P-rich</td>
<td>4.7</td>
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<tr>
<td>CTFP</td>
<td>CTF</td>
<td>P-rich</td>
<td>5.1</td>
</tr>
<tr>
<td>VP16</td>
<td>VP16(413-490)</td>
<td>Acidic</td>
<td>97</td>
</tr>
<tr>
<td>VP16N</td>
<td>VP16(413-456)</td>
<td>Acidic</td>
<td>7.8</td>
</tr>
<tr>
<td>VP16C</td>
<td>VP16(452-490)</td>
<td>Acidic</td>
<td>16</td>
</tr>
<tr>
<td>Δ</td>
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<td>none</td>
<td>0.04</td>
</tr>
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</table>

Figure 2. Activators and reporter constructs. (A) GAL4–fusion activators. Activation domains are classified as either glutamine-rich (Q-rich), proline-rich (P-rich), or acidic and were expressed as fusions with the yeast GAL4 DNA-binding domain (GAL4 DBD, residues 1–94). Sp1° was constructed by duplicating the B domain of Sp1 (Courey and Tjian 1988). The other activation domains have been described [4xQmi8 (Tanaka and Herr 1994); 2xP41 (Tanaka et al. 1994); CTF (Mermod et al. 1989); VP16N (413–456); VP16C(452–490); and VP16(413–490) (Triezenberg et al. 1988; Regier et al. 1993)]. (A) GAL4 DNA-binding domain alone. Relative activity refers to the maximal level of activation of the wild-type (TATAAA) c-fos reporter by each of the GAL4–fusion activators, setting the level of activation by the weakest activator, Sp1°, to 1.0.

B

Promoter

c-fos TATAAAA

GAL4 Sites

TATAAA

Figure 2. Activators and reporter constructs. (A) GAL4–fusion activators. Activation domains are classified as either glutamine-rich (Q-rich), proline-rich (P-rich), or acidic and were expressed as fusions with the yeast GAL4 DNA-binding domain (GAL4 DBD, residues 1–94). Sp1° was constructed by duplicating the B domain of Sp1 (Courey and Tjian 1988). The other activation domains have been described [4xQmi8 (Tanaka and Herr 1994); 2xP41 (Tanaka et al. 1994); CTF (Mermod et al. 1989); VP16N (413–456); VP16C(452–490); and VP16(413–490) (Triezenberg et al. 1988; Regier et al. 1993)]. (A) GAL4 DNA-binding domain alone. Relative activity refers to the maximal level of activation of the wild-type (TATAAA) c-fos reporter by each of the GAL4–fusion activators, setting the level of activation by the weakest activator, Sp1°, to 1.0. (B) The wild-type (TATAAA) and altered (TGTTAA) c-fos reporter constructs. Both reporters contained the c-fos (−56) promoter [Berkowitz et al. 1989] downstream of four synthetic GAL4 DNA-binding sites (Tanaka et al. 1994).

TBP activity in vivo is not greatly affected by clustered point mutations

To examine the regions of TBP that are involved in response to different activation domains in vivo, we constructed 10 amino acid substitution mutants as described in Table 1. Two sets of mutant TBPs were generated. The first set, IIA and pol III, was based on temperature-sensitive mutations that had been described for yeast TBP (Buratowski and Zhou 1992; Schultz et al. 1992) and were introduced at corresponding positions in both yeast and human TBPAs. The IIA mutation is a double lysine to leucine substitution on the surface of helix 2 that disrupts interaction of yeast TBP with human TFIIA in vitro (Buratowski and Zhou 1992); it is not known whether this double leucine substitution in human TBP affects association with human TFIIA in vitro. The pol III mutation changes a conserved proline near the amino terminus of the TBP core to serine and disrupts transcription by RNA polymerase III but not RNA polymerase II (Schultz et al. 1992; S. Hahn, pers. comm.).

The second set of mutations consisted of a series of systematic alanine substitutions on the solvent-exposed surface of DNA-bound human TBP, targeting the four α-helices {H1, H1', H2, and H2'} and two prominent loops connecting strands S3 and S4 {S3'/S4'} and S3' and S4' {S3'/S4'}. Figure 1 shows the position of these mutations on the TBP structure. In an attempt to disrupt human TBP activity, for each mutation we made double and, in one case {H2}, triple alanine substitutions and focused on residues whose charge is conserved in TBPs from many species. Mutations at corresponding positions were made in each symmetrical half of the molecule except in helices H2 and H2', where the position of charged residues is not strictly conserved between each repeat. Curiously, two of these TBPAs mutants, H1 and H2' as well as the yeast pol III mutant could not be expressed in HeLa cells (Table 1). The reason for this lack of expression is not known, but a similar phenomenon has been described for other TBP mutants in yeast (Reddy and Hahn 1991) and may reflect incorrect protein folding (Parsell and Sauer 1989).

The ability of the mutant TBPs to support activation by the GAL4–fusion activators is shown in Figure 3 (lanes 8–13,15). The most notable feature of this analysis is that none of the mutations had a greater than threefold effect on the activity of TBP in vivo. In the context of yeast TBPAs, the IIA mutation reduced response to all activators approximately twofold (cf. lanes 14 and 15, A–G). When the corresponding mutation was placed in the context of human TBPAs, it modestly impaired acti-
Figure 3. Effects of single regional mutations on TBP activity in human cells. RNase protection analysis of RNA isolated from HeLa cells transiently transfected with the c-fos TATAAA (lanes 1, 2) or TGTAAA (lanes 3–15) reporters, an appropriate TBP-expression construct (lanes 5–15), and an expression construct for each GAL4-fusion activator, as indicated. [h] Wild-type human TBP. All other TBPs carried the altered-specificity substitutions and are described in Table 1. Only the correctly initiated c-fos transcripts, normalized to the α-globin signal, are shown. Transient expression of TBPAS in the absence of a GAL4-fusion activator did not detectably increase transcription from the c-fos TGTAAA reporter or affect the α-globin signal (data not shown). As these GAL4-fusion proteins activate transcription to different overall levels [Fig. 2A], exposure time for each autoradiograph is different.

Curiously, the transcriptional effects of the systematic set of multiple alanine substitution mutations were also small and mainly confined to activation by the VP16-derived activation domains [Fig. 3, cf. lane 6 with lanes 10–13]. The only reproducible effect was observed with the H2 mutant TBPAS, which was reduced two- to threefold in response to VP16. Comparison of full-length VP16 [lane 11, E] with its two less active subdomains [Fig. 3, F, G; see Fig. 2A] shows that the small selective effects of these mutations on VP16 activation do not simply result from its significantly greater transcriptional activity, because VP16AS, which displays an activity similar to that of CTFP, mirrored the response of full-length VP16 and not CTFP to each of the TBP mutations [cf. lanes 10–13, D–F].

The resistance of TBP activity in vivo to the effects of the multiple alanine substitutions was unexpected, given the high degree of sequence conservation in TBP across many species. It suggests that either these mutations are not in regions of the protein that are involved in supporting activated transcription in vivo or that important regions of TBP were being targeted, but the mutations are not themselves sufficient to debilitate TBP activity.

**Mutations in TBP that do not affect activity in vivo have large effects in vitro**

In a parallel series of experiments, we examined the potential of the systematic set of multiple alanine substitution mutants to bind DNA and to support basal transcription in vitro. We analyzed the H1', H2, S3/S4, and S3'/S4' mutants because they are expressed in HeLa cells, allowing a comparison of in vitro and in vivo activities.

Full-length H1', H2', S3/S4, and S3'/S4' TBPAS molecules were expressed in *Escherichia coli* and purified as fusions with glutathione S-transferase (GST) sequences. The DNA-binding capability of these GST–TBPAS mutants was then examined by DNase I footprinting analysis, as shown in Figure 4. Titration of wild-type TBPAS on the TGTAAA c-fos promoter produced two clear regions of protection (cf. lane 2 with lanes 3–5); a region encompassing 6 bases 5' and 4 bases 3' of the TGTAAA sequence and a region containing an A/T-rich vector sequence upstream of the GAL4 DNA-binding sites. In
Table 1. Altered-specificity TBP molecules used in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Species</th>
<th>Mutation*</th>
<th>Color</th>
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<td>yTBPAS</td>
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<td>yeast</td>
<td>none</td>
<td>+</td>
</tr>
<tr>
<td>.IIA</td>
<td></td>
<td>yeast</td>
<td>K(133)L</td>
<td>+</td>
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<tr>
<td>.pol III</td>
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</table>

*All TBPs carry the triple amino acid altered-specificity substitutions, indicated in orange in Fig. 1. The coordinates for these substitutions are hTBPAS—I(292)F, V(301)T, L(303)V; yTBPAS—I(194)F, V(203)T, L(205)V. Coordinates are given for full-length human and yeast TBP, where appropriate.

In contrast, titration of identical amounts of the mutant TBPs produced patterns of DNase I protection that differed from wild-type TBPAS. Binding of the H1' and S3'/S4' mutants, although detectable, was reduced ~10-fold (cf. lanes 3–5 with lanes 6–8 and 12–13), whereas the S3'/S4' mutant TBPAS did not bind DNA to any significant degree (cf. lanes 3–5 with lanes 15–17). By comparison, the H2 mutant bound DNA at levels similar to wild-type TBPAS (cf. lanes 3–5 with lanes 9–11); the binding of this mutant TBPAS, however, produced a unique DNase I hypersensitive site 3 bases 5’ to the protected region (see arrow), possibly reflecting a different conformation of the TBP–DNA complex. As the H1', H2, S3/S4, and S3'/S4' TBPAS molecules can support activated transcription in vivo, these in vitro-binding data indicate that the ability of TBP to bind DNA in vitro does not necessarily correlate with its ability to function for activated transcription in vivo. Similar discrepancies between the DNA-binding potential of TBP and its ability to support transcription in vitro and in yeast cells have been reported (Cormack and Struhl 1992; Lee et al. 1992; Schultz et al. 1992; Yamamoto et al. 1992).

Figure 5 shows the effects of the four sets of systematic alanine substitutions on the capacity of TBPAS to support basal transcription from the TGTAAA c-fos reporter in vitro. The results of RNase protection analysis performed on RNA transcribed in crude HeLa cell nuclear extract reveal that in the absence of added wild-type GST–TBPAS, no basal transcription was observed from this promoter (lane 1). Addition of wild-type GST–TBPAS resulted in a high level of correctly initiated transcripts (lane 2), whereas the two mutant TBPAS molecules H1' and S3/S4, which bound DNA poorly, supported nearly wild-type levels of basal transcription (cf. lane 2 with lanes 3 and 5). In contrast, mutants H2 (lane 4) and S3'/S4' (lane 6) showed no detectable basal activity in this assay, even though the H2 mutant binds well to DNA (Fig. 4). Taken together with the in vivo functional data (Fig. 3), this result demonstrates that the ability of

![Figure 4](https://genesdev.cshlp.org)

Figure 4. Alanine substitution mutations on the surface of human TBP affect DNA binding in vitro. For DNase I footprinting analysis, increasing amounts of recombinant GST–hTBPAS fusion proteins were incubated with an end-labeled DNA fragment spanning the altered c-fos TGTAAA box, the four synthetic GAL4 DNA-binding sites, and an A/T-rich vector sequence, as indicated. Each titration represents a 10-fold range in the amount of full-length GST–hTBPAS added, from 15–150 ng. The region of protection spanning the TGTAAA box is bracketed, and the position of the hypersensitive site induced by the H2 mutant TBP is indicated by an arrow. (G+A) Chemical sequencing reactions on the same labeled probe.
TBP to support basal transcription in vitro is not a prerequisite for its ability to support activated transcription in vivo. Moreover, this result, together with the DNA-binding studies described above, suggests that interaction of TBP with cellular components in vivo can counteract the effects of mutations in TBP that disrupt DNA binding ([H1', S3/S4, and S3'/S4']) or basal transcription (H2 and S3'/S4') in vitro.

**Combined sets of mutations in different regions of TBP disrupt transcriptional activation in vivo**

As the activity of TBP in vivo was relatively unaffected by mutations in single regions of the protein, we reasoned that the functioning of TBP may depend on multiple, redundant interactions at different points across its surface. One prediction of this hypothesis is that combined mutations in different regions of TBP may have a significant effect on its activity in vivo. To explore this possibility, we made all combinations of the [H1', H2, S3/S4, and S3'/S4'] mutations in TBP to respond to the different activation domains in vivo.

Figure 6 shows the response of Oct-2Q, CTF*, full-length VP16, and VP16N to each of the combined sets of mutations in TBPAS. Also examined were the Oct-2K and Spl* activators, which yielded results similar to those of Oct-2Q [data not shown]. The combination of all four [H1', H2, S3/S4, and S3'/S4'] mutations in TBPAS (lane 1, A–D) disrupted the ability of TBP to respond to any of the activation domains examined. Analysis of the double (lanes 3–8) and triple (lanes 9–12) combined sets of mutants, however, revealed that the activity of TBP in vivo is sensitive only to specific combinations of these mutations. Any double combination of the [H1', S3/S4, and S3'/S4'] mutations [lanes 4,5,8] had little if any effect on the response to each activator, except for VP16N, which was reduced threefold by the S3'/S4 + S3'/S4' combination (cf. lanes 2 and 8, D). In contrast, combining the H2 mutation with any of the other mutations [lanes 3,6,7] disrupted response to all of the activators, especially VP16 [lanes 3,6,7, C]. For the other activators, the H2 + S3'/S4' combination was the most severe of any of the double combinations [lane 7, A,B,D]. The sensitivity of TBP to combined sets of mutations across different regions of the protein, but not to individual mutations, suggests that multiple surfaces of TBP participate in response to RNA polymerase II transcriptional activity. However, because TBP can apparently tolerate loss of function in isolated regions of the protein, the individual participation of each surface of TBP is not obligatory for transcriptional activity.

In contrast to these universal effects on TBPAS response to activators, certain combined mutations predominantly affected activation by the VP16 activation domain. In particular, the H1' + S3/S4 + S3'/S4' triple combination supported wild-type levels of activation by CTF, but was <10% active for response to VP16 [Fig. 6, lane 11, cf. B and C]. Curiously, unlike the response to single sets of mutations [see Fig. 3], the response of each...
of the combined sets of mutations to VP16, more closely mirrored the response to Oct-2 and CTF than to full-length VP16. This comparison raises the possibility that the VP16-specific defect of the H1’ + S3/S4 + S3’/S4’ mutant TBPAS is a result of the high activity of the full-length VP16 activation domain. The mutation domain-specific defect of this mutant TBP makes two important points: (1) It shows that, although the H1’ + S3/S4 + S3’/S4’ mutant TBP is only weakly active for VP16, this loss of activity is not the result of a generalized disruption of the integrity or accessibility of the TBP, because it functions at wild-type levels for CTF, and (2) it demonstrates that activators can differ in how they directly or indirectly utilize different residues in TBP to activate transcription.

Interaction of TBP with hTAFn250 is resistant to mutations in single regions of TBP but is disrupted by specific combinations of mutations

The ability of different mutant TBPs to support activated transcription in vivo did not correlate with their ability to interact with DNA or to support basal transcription in vitro. To probe the mechanisms that dictate TBP activity in vivo, we investigated the effect of our alanine substitution mutations on the interaction of TBP with two other components of the transcriptional apparatus. Because TBP only supports activated transcription in vitro when assembled into a partial or complete TFIIID complex (Dynlacht et al. 1991; Tanese et al. 1991; Zhou et al. 1993; Chen et al. 1994), we examined mutant TBP association with hTAFn250 in vitro. Human TAFn250 binds directly to TBP and appears to form a scaffold for the assembly of TFIIID (Goodrich et al. 1993; Ruppert et al. 1993; Weinzierl et al. 1993; Verrijzer et al. 1994; Chen et al. 1994), thus, the ability of TBP to interact with hTAFn250 is probably a good measure of its ability to be recruited into TFIIID. Additionally, hTAFn250 has been shown to be crucial for transcriptional activation in vivo (Wang and Tjian 1994). However, although the carboxy-terminal core of TBP is sufficient for the assembly of an active TFIIID complex (Zhou et al. 1993), little is known about the residues in the core of TBP that are required for association of TBP with hTAFn250. In addition, we also examined interaction of these mutant TBP molecules with TFIIB in vitro, as TFIIB is known to interact with both TBP and activator proteins in vitro (Lin et al. 1991; Ha et al. 1993) and has been implicated as an important functional target for transcriptional activators (Lin and Green 1991).

In vitro-translated radiolabeled wild-type and mutant TBPASs were mixed with hemagglutinin influenza virus (HA) epitope-tagged hTAFn250 or GST-TFIIB and either coimmunoprecipitated with antibodies against the HA-epitope tag [hTAFn250] or cobound to glutathione beads [TFIIB]. After extensive washing, the labeled TBP that remained bound to the precipitated hTAFn250 or TFIIB was analyzed by SDS-PAGE followed by autoradiography. The presence of the altered-specificity substitutions did not affect the ability of wild-type TBP to interact with either hTAFn250 or human TFIIIB in vitro (data not shown). Figure 7 shows the results of these experiments. Figure 7A shows 10% of the input TBP for each assay. As a negative control for the TBP–hTAFn250 interaction, we also examined the interaction of labeled mutant TBPASs with beads carrying just the anti-HA antibody. Binding of labeled TBPASs to these control beads was ~1% that of binding to beads containing hTAFn250. In a similar control experiment for the interaction of TBP with GST–TFIIB, we found that ~10-fold more TBPAS bound to GST–TFIIB beads than to GST-alone beads (data not shown).

None of the mutations, including the quadruple combination mutation H1’ + H2 + S3/S4 + S3’/S4’ (lane 16), had a large effect on the interaction of TBP with human TFIIIB (cf. Fig. 7, A and C). This result suggests that these mutations do not target the residues of TBP that interact with TFIIIB under these conditions and is consistent with the finding that large regions of TBP can be deleted without disrupting the TBP–TFIIIB interaction in this type of assay (Ha et al. 1993).

In contrast, the ability of these TBP molecules to interact with hTAFn250 was strongly affected by some of the mutations (cf. Fig. 7, A and B). As with in vivo transcriptional activity, the interaction of hTAFn250 with TBP was largely resistant to the effects of mutations in single regions of TBP. The only single regional mutation that had a detectable effect was the H2 mutation, which reduced twofold the ability of TBPAS to interact with hTAFn250 (Fig. 7B, cf. lanes 1 and 3). When different mutations on the surface of TBP were combined, interaction between TBP and hTAFn250 could be strongly affected. In particular, any combinations of the H2 and S3'/S4' mutations (lanes 10, 13, 15, 16) reduced the ability of TBP to interact with hTAFn250 to 10% or less of wild-type levels. These data suggest that TBP interacts with hTAFn250 by making multiple contacts across its surface and that any one point of interaction between TBP and hTAFn250 can be disrupted without disrupting the overall integrity of the TBP–hTAFn250 association.

In Figure 7E, the interaction of the various mutant TBP molecules with hTAFn250 is summarized and compared with the results of CTF and VP16 activation in vivo. Generally, the ability of wild-type and mutant TBP molecules to respond to transcriptional activators in vivo closely paralleled their ability to interact with hTAFn250 in vitro. In particular, all mutations in TBP that reduced interaction with hTAFn250 in vitro by 10-fold (columns 10, 13, 15, 16, E) those that carry the H2 + S3/S4' combination were defective for transcriptional activation in vivo. Similarly, the mutant TBP molecules carrying double or triple combinations of the H1', S3/S4, and S3'/S4' mutations (columns 7, 8, 11, 14) retain considerable in vivo activity for response to CTF and associate better with hTAFn250 than the less active mutants. The VP16-derived activator, however, did not activate transcription at full potential even when TBP could associate effectively with hTAFn250, albeit at a lower level, as in the case of the H1' + S3/S4 + S3'/S4' triple mutant (column 14). This result suggests that to
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**Figure 7.** Combined mutations in different regions of TBP disrupt interaction with hTAF₁₅₂₅₀ in vitro. (A–D) Coimmunoprecipitation and GST-pulldown assays. ³⁵S-Labeled, in vitro-translated human TBP₆₅ molecules, as indicated, were incubated with either HA-tagged, baculovirus-expressed hTAF₁₅₂₅₀ (B), or E. coli-expressed GST-linked human TFIIB (C). The interaction of these mutant TBPs with hTAF₁₅₂₅₀ was measured by their ability to be coimmunoprecipitated with antibodies against the HA epitope tag; interaction with TFIIB was measured by their ability to be precipitated by coupling of GST–TFIIB to glutathione-agarose. Also shown are 10% of the labeled input TBP (A) and background binding to the αHA beads (D). (E) Interaction of mutant TBPs with hTAF₁₅₂₅₀ compared with in vivo response to VP₁₆ and CTFP. hTAF₁₅₂₅₀ interaction with wild-type TBP₆₅ was set to 1.0 and was calculated as follows: ³⁵S-Labeled TBP bands from the gels shown in B were quantitated by PhosphorImaging, and each value was corrected for background binding to the αHA beads alone and for minor differences in input protein. In vivo activity was calculated by quantitation of the gels shown in Figs. 3 and 6 by PhosphorImaging, subtraction of the activity observed in the presence of activator but in the absence of TBP₆₅, and setting the rescued level of transcription by wild-type TBP₆₅ for each activator to 1.0.

reach its full activation potential, VP₁₆ requires more than simply the ability of TBP to associate with hTAF₁₅₂₅₀.

Given the marked differences in the assays used to measure TBP activity in vivo and its interaction with hTAF₁₅₂₅₀ in vitro, the qualitative similarities in the activation and hTAF₁₅₂₅₀-association profiles are striking. These data suggest that the resilient nature of the interaction of TBP with hTAF₁₅₂₅₀ is the reason why TBP activity in vivo is resistant to single sets of amino acid substitutions. They also suggest that interaction of TBP with hTAF₁₅₂₅₀ plays a central role in the response of TBP to a diverse range of transcriptional activation domains.
Discussion

TBP performs a range of diverse activities within the cell. It is involved in transcription by all three nuclear RNA polymerases, from promoters with or without a TATA box. TBP achieves much of this diversity by interacting with different sets of TAFs, depending on the polymerase and promoter context (see Hernandez 1993; Goodrich and Tjian 1994). These many different physical associations probably place large constraints on the polymerase and promoter context (see Hernandez 1993; TBP performs a range of diverse activities within the Discussion

We have studied the surfaces of TBP that are required in vivo for just one of its many functions: activated RNA polymerase II transcription from a TATA box-containing promoter. The highly conserved TBP core is sufficient to respond to a battery of different activation domains in human cells, but, to our surprise, the high degree of sequence conservation in this region of the molecule is not required (Fig. 3). Indeed, TBP can withstand multiple amino acid substitutions within individual regions of the conserved domain without losing the ability to respond to the activators tested in our transient expression assay. This result suggests that it is likely to be other aspects of TBP function that constrain its evolutionary sequence divergence.

One possible constraint is the role played by TBP in RNA polymerase III transcription. When single amino acid substitutions within the top surface of TBP [as represented in Fig. 1A] that affect yeast cell viability have been identified, they predominantly affect RNA polymerase III transcription [Cormack and Struhl 1993; Cormack et al. 1994]. This result could simply mean that the top surface of TBP has little involvement in transcription by RNA polymerase II. Alternatively, the activity of yeast TBP in RNA polymerase II transcription may also be resistant to single amino acid substitutions; perhaps combinations of amino acid substitutions in yeast TBP, as in human TBP, would affect its activity in RNA polymerase II transcription.

Transcription by RNA polymerase II differs significantly from that by the other RNA polymerases, because RNA polymerase II must respond to many different sequence-specific transcription factors. The coactivator hypothesis (Pugh and Tjian 1992) posits that complexes with TBP are proteins—coactivators—that serve as intermediaries between activators and RNA polymerase II. Indeed, TAFs display coactivator activity [Dynlacht et al. 1991; Tanese et al. 1991; Chen et al. 1994]. Coactivators thus permit the activity of diverse sequence-specific activators to be directed toward activation of a single RNA polymerase. By virtue of its ability to bind to both the TATA box and TAFs, TBP targets coactivators to TATA box-containing promoters. The correlation described here between TBP–TAF interaction in vitro and the response of TBP to many different activation domains in vivo [Fig. 7E] is consistent with this hypothesis.

The ability of yeast TBP to respond to a variety of transcriptional activation domains in human cells [Fig. 3] suggests that, during evolution, the interface between TBP and other components of the transcriptional machinery has been conserved. This conserved interface could well be that between TBP and the TAFs. Like human cells, yeast contain TAFs [Poon and Weil 1993], including a direct structural homolog of the human TBP-binding TAF1250 [A. Weil, pers. comm.]. Furthermore, yeast TBP can bind hTAF1250 in vitro [S. Ruppert and R. Tjian, unpubl.]. Thus, this TBP–TAF interaction, which is involved in recruiting TBP into the TFIIID complex, has been evolutionarily conserved, perhaps reflecting its universal importance to activated RNA polymerase II transcription in vivo.

Although the activation domains of Spl [Künzler et al. 1994] and Oct-2Q [C. Hinkley and W. Herr, unpubl.] do not function in yeast, they can activate transcription with yeast TBP in human cells [Fig. 3]. This finding suggests that one or more elements other than the interface between TBP and the transcriptional machinery have diverged between yeast and humans. One possibility is that the full complement of TFIIID TAFs has not been universally conserved. For example, Drosophila TAF110 responds to a glutamine-rich activation domain from Spl [Hoey et al. 1993; Chen et al. 1994]; perhaps in yeast either this TAF is absent or a homolog has lost the ability to respond to the Spl activation domain. Such changes in TAFs could result in changes in transcriptional response to entire classes of activation domains during speciation or even during cell differentiation if TAF expression is developmentally controlled.

To contrast the activity of TBP point mutants in vivo with their in vitro activities, we assayed the effects of clustered alanine substitution mutations in four in vitro assays: [1] basal transcription, [2] binding to DNA, [3] association with TFIIA, and [4] association with hTAF1250. Only the association with hTAF1250 correlated with in vivo activity [Fig. 7]. TFIIA association was unaffected by any of the mutations [Fig. 7], whereas both DNA binding and basal transcription in vitro [Figs. 4 and 5] were disrupted by individual sets of point mutations that had little if any effect in vivo. It is known that defects in DNA binding by yeast TBP can be overcome in vivo and during in vitro basal transcription [Cormack and Struhl 1992; Lee et al. 1992; Schultz et al. 1992; Yamamoto et al. 1992].

We were surprised, however, that human TBP mutants that supported activated transcription in vivo could be defective for basal transcription in vitro. We imagined that the ability to support basal transcription would be a likely prerequisite for TBP to respond to activators. As suggested above, perhaps the interactions of TBP in vivo with cellular components can counteract the effects of these TBP mutations. A good candidate for such an interaction is that between TBP and TAFs in TFIIID and, in particular, between TBP and hTAF1250, which recruits TBP into TFIIID [Ruppert et al. 1993; Weinzierl et al. 1993; Chen et al. 1994].

The association of hTAF1250 with TBP in vitro appears to involve interactions with multiple surfaces of TBP and is resistant to changes in isolated regions of TBP...
These effects correlate with the in vivo activity of TBP and suggest an extensive and stable interaction between TBP and hTAFn250. Although we do not discount the possibility that the effects of these mutations on hTAFn250 interaction could be indirect, such an extensive interaction is consistent with the stable association of TBP with TAFs in the TFIID complex (Dynlacht et al. 1991; Tanese et al. 1991; Zhou et al. 1993). It is also consistent with the observation that multiple regions of hTAFn250 can independently interact with TBP in vitro (S. Ruppert and R. Tjian, unpubl.). But why does TBP rely on an extensive interaction with hTAFn250 across its surface in this way?

We propose that by utilizing multiple points of contact across its surface, TBP is able to interact with hTAFn250 and, in turn, a network of other TAFs in a way that is both strong and flexible. This may have several consequences for the functioning of TBP in vivo. First, the resilience of the interaction of TBP with hTAFn250 raises the possibility that once TBP enters TFIID, it may not be available for interaction with the other TBP-containing complexes (such as SL1 and TFIIIB, for review, see Goodrich and Tjian 1994). As TBP is apparently limiting for RNA polymerase II and III transcription (Cormack and Struhl 1993), the distribution of TBP into the various TAF-containing complexes may have important consequences for coordinating transcription within the cell. Second, as there are multiple points of contact between TBP and hTAFn250, it is possible that one or more of these individual interactions could be disrupted without destroying the overall integrity of the TBP–hTAFn250 association. In this way, the precise nature of the TBP–hTAFn250 association could change, perhaps allowing TBP to also make direct contact with activators or with other GTFs.

In addition to the importance of the TBP–hTAFn250 interaction for activated transcription we observe with all activators tested, there are elements of TBP function that selectively affect the activity of one but not other activation domains. This property is exemplified by the sensitivity of the VP16 activation domain to mutations that do not affect the activity of other activation domains, in particular, the multiple regional point mutant H1' + S3/S4 + S3'/S4', which severely disrupts activation by VP16 but not CTFP [Fig. 6]. The behavior of this mutant TBP demonstrates that TBP itself, a single component of the basal transcriptional machinery, can be used in different ways to achieve transcriptional activation.

The differential effects of the H1' + S3/S4 + S3'/S4' mutant may reflect the unusual potency of VP16. Although this combined mutation in TBP reduced VP16 activation by >10-fold, the absolute level of activation achieved by VP16 in this instance is very similar to that seen with CTFP (Fig. 2A). One possible mechanism for such an unusual potency is that VP16 has a greater range of targets in the transcriptional apparatus. This mechanism is implied by the biochemical characterization of multiple potential targets for VP16, which thus far include TFIIA [Lin and Green 1991, Lin et al. 1991], TBP [Ingles et al. 1991], and TAFp40 [Goodrich et al. 1993]. It is also consistent with the observation that VP16, when affected less severely by the H1' + S3/S4 + S3'/S4' mutation than the more active full-length activation domain [see Fig. 6]. VP16 may thus achieve greater levels of activation by stimulating additional or different targets than CTFP. The H1' + S3/S4 + S3'/S4' mutation may disrupt the interaction of TBP with these VP16-specific targets, either directly or by inducing changes in the hTAFn250–TBP complex that in some way weaken recognition by critical VP16 coactivators, such as a human homolog of the Drosophila TAFp40 [Goodrich et al. 1993].

In conclusion, our in vivo and in vitro studies of TBP function suggest that TBP responds to activators by more than one pathway: one broadly used pathway involving association with TFIID through an extensive interaction with TAFp250 and a second pathway that may only be used by a selected set of activation domains, including the very potent VP16 activation domain.

Materials and methods

Construction of mutant TBP molecules and GAL4–activators

TBP-encoding sequences were subcloned into the mammalian expression vector pCGN [Tanaka and Herr 1990], which adds a 15-amino-acid HA epitope tag to the amino terminus of the expressed protein. To minimize translation initiation after the epitope tag, the full-length wild-type and altered-specificity human TBP pCGN clones [gifts from M. Tanaka and R. Mittal, respectively [Cold Spring Harbor Laboratory]] were modified by site-directed in vitro mutagenesis [Kunkel et al. 1987] to remove sequences encoding the initiator methionine of human TBP. Yeast TBP sequences (encoding amino acids 2–240 of S. cerevisiae TBP) were amplified from genomic DNA [gift of K. Gavin, Cold Spring Harbor Laboratory] by the polymerase chain reaction [PCR] with 5’ Nhel- and 3’ BamHI-adapter primers. After restriction enzyme digestion, the resulting fragment was subcloned into the unique Xbal and BamHI sites of pCGN and site-directed mutagenesis was used to introduce (1) the triple amino acid altered-specificity substitution [Strubin and Struhl 1992] and (2) two silent mutations at codons 61 and 62, which removed a cryptic splice-site donor that prevented expression of full-length yeast TBP in HECA cells [W.P. Tansey and W. Herr, unpubl.]. Single regional point mutations in human and yeast TBP [H1, H1’, H2, H2’, S3/S4, S3'/S4’, I1A, and pol III] were generated by site-directed mutagenesis with single synthetic oligonucleotides carrying the desired mutations; multiple regional point mutants were generated with a pool of the H1’, H2, S3/S4, and S3'/S4’ mutagenic oligonucleotides.

To prepare the pT8–TBPm constructs for in vitro translation, inserts of the pCGNTPBM constructs were amplified by PCR with 5’ Ndel- and 3’ BamHI-adapter primers. The products were digested with Ndel and BamHI and subcloned into pT8STOP [gift of H. Jantzen, Jantzen et al. 1992] to yield the corresponding pT8–TBPm constructs used for in vitro transcription/translation. The sequence integrity of all constructs was confirmed by dideoxy sequence analysis of the entire conserved region of TBP.

GAL4–fusion activators were constructed by subcloning various activator-derived sequences into the vector pCG–GAL1–94 [Tanaka et al. 1994; modified by C. Hinkley—to be described elsewhere]. The GAL4–CTFP fusion was produced by PCR amplification of CTF sequences [encoding amino acids
Altered-specificity TBP assay

HeLa cells were transiently transfected by the calcium phosphate coprecipitation method as described (Tanaka et al. 1992). Each transfection included [1] 200 ng of c-fos (−56)[4xGAL] reporter, either wild-type (gift of M. Tanaka, Tanaka et al. 1994) or carrying the TATAAA to TGTAAA mutation introduced by site-directed mutagenesis; [2] between 160 ng and 1.76 μg of pCGN-TBP expression plasmid (adapted to give equivalent levels of expression for each HA-tagged TBP); [3] between 350 and 720 ng of pCG–GAL4-activator expression plasmid (the amount for each determined empirically to give maximal activation of the wild-type c-fos reporter; [4] 80 ng of α-globin internal reference plasmid p4x(A+C) (Tanaka et al. 1988), and [5] pUC119, taking the total amount of transfected DNA to 20 μg. At 36 hr post-transfection, cells were harvested and cytoplasmic RNA prepared as described (Tanaka et al. 1992). RNase protection analysis (Tanaka et al. 1992) was used to quantitate correctly initiated transcripts from the c-fos reporter and from the α4x(A+C) internal control plasmid. TBP expression levels were determined by transfecting threefold dilutions of each expression construct, followed by Western blot analysis of protein extracts from transfected cells with an antibody probe (12CA5) against the HA epitope tag.

Production of recombinant proteins

Altered-specificity TBP sequences were subcloned into the unique XbaI and BamHI sites of the pET11α-GST (Lai et al. 1992) and expressed in E. coli as GST fusion proteins by use of the T7 expression system developed by Studier et al. (1990). E. coli BL21(DE3) cultures of 500 ml carrying the appropriate pET11α-GST–TBPAs construct were grown at 30°C to an OD595 of 0.6. At this point, IPTG was added (0.5 mM final concentration) and the cultures induced for 4 hr at 30°C, growth and induction at this temperature resulted in the highest yield of soluble induced protein. After induction, cells were harvested by centrifugation, resuspended in 30 ml of lysis buffer [25 mM HEPES-KOH (pH 7.9 at 4°C), 20% glycerol, 12.5 mM MgCl2, 2 mM EDTA, 2 mM DTT, 0.2 mM PMSF, 5 μg/ml of aprotinin, 5 μg/ml of leupeptin, and lysed by treatment with lysosyme [final concentration of 100 μg/ml] and the addition of NP-40 to 0.1% [final concentration]. The lysate was then sonicated to shear bacterial DNA, and insoluble proteins were removed by centrifugation. GST–fusion proteins were purified from the soluble fraction by binding to 200 μl of glutathione–agarose [Sigma] for 1 hr at 4°C, washing extensively with HEMGN buffer (Lieberman and Berk 1991) containing 0.15 M KCl, and eluting with two lots of 100 μl of 5 mM reduced glutathione [in HEMGN with 0.15 M KCl]. Samples of the eluates were analyzed by SDS-PAGE combined with Coomassie staining and Western blot analysis. Typically, a 500-ml culture yielded between 5 and 20 μg of full-length GST–TBPAs, in a form that was essentially free of non-TBP-related proteins; these preparations did, however, contain a significant proportion of inactive carboxy-terminal TBP breakdown products.

DNA-binding and basal transcription analyses

DNase I footprinting analysis was performed with purified recombinant GST–TBPAs fusion proteins. The c-fos TGTAAA probe was prepared by PCR from the c-fos (−56)[4xGAL] TGTAAA reporter with an unlabelled reverse sequencing primer and an end-labeled primer that hybridized to c-fos promoter sequences spanning +6 to +23. Each binding reaction included 10,000 cpm of probe, 15-150 ng of full-length GST–TBPAs, 50 ng of poly[d(G-C)], 2% polyvinyl alcohol, 2 μg of BSA, 12.5 mM HEPES–KOH (pH 7.9), 75 mM KCl, 0.1 mM EDTA, 10% glycerol, 5 mM MgCl2, 1 mM DTT, 0.1 mM PMSF, and 0.05% NP-40, in a total volume of 25 μl and was allowed to proceed for 40 min at 30°C. DNase I digestion was then carried out as described (Schmidt et al. 1989), and reaction products were analyzed by denaturing polyacrylamide gel electrophoresis. G+A chemical sequencing reactions of the c-fos TGTAAA probe were run alongside the footprinting reactions.

In vitro transcription from the c-fos (−56)[4xGAL] TGTAAA template was performed in crude HeLa cell nuclear extract (Dignam et al. 1983) supplemented with purified recombinant GST–TBPAs proteins. Reactions contained 4 μl of nuclear extract (32 μg of total protein), 160 ng of c-fos (−56)[4xGAL] TGTAAA reporter plasmid, 15 ng of the appropriate full-length GST–TBPAs fusion protein, 20 mM HEPES–KOH (pH 7.9), 62.5 mM KCl, 5 mM ammonium sulfate, 3% polyethylene glycol 8000, 8 mM MgCl2, 10% glyc erol, 1 mM DTT, and 0.7 mM each of ATP, CTP, UTP, and GTP. After a 60-min incubation at 30°C, the reaction mixture was treated with proteinase K and SDS and extracted with phenol/chloroform, and nucleic acids were precipitated with ethanol. RNA transcribed in vitro was then analyzed by RNase protection analysis (Tanaka et al. 1994).

Coimmunoprecipitations and GST pulldowns

HA epitope-tagged hTAFii250 was expressed in baculovirus-infected SF9 cells and whole-cell extracts were prepared as described (Ruppert et al. 1993). After diluting the extract to HEMGN containing 0.3 μM KCl (0.3 HEMGN) and clearing it by centrifugation, the supernatant was incubated for 4 hr on ice with anti-HA [12CA5] antibodies [BarbCO]. The extract was cleared again by centrifugation, and the 12CA5 antibody and immunopurified hTAFii250 was precipitated from the supernatant by binding to protein A–Sepharose CL-4B beads [Pharma cia]. The beads were washed extensively with 0.1 HEMGN and analyzed by SDS-PAGE and Coomassie staining. β3-S-Labeled TBPAs proteins were generated from the pTP–TBPAs constructs by in vitro transcription/translation using the TNT coupled reticulocyte lysate system [Promega]. The in vitro-translated proteins were diluted with four volumes of 0.1 HEMGN and cleared by centrifugation. Fifty microliters of the supernatant was incubated with 25 μl of packed protein A beads containing either 12CA5 and −200–300 ng of full-length hTAFii250 or 12CA5 alone. After mutating 3–4 hr at 4°C, bound TBPAs proteins were recovered by low speed centrifugation and the beads were washed extensively with 0.1 HEMGN. The bound proteins, 10% of the input TBPAs, and an aliquot of the final wash were analyzed by SDS-PAGE, followed by silver-staining and
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autoradiography. Bound TBP<sub>As</sub> proteins were quantitated by Phosphorimaging and corrected for background binding to αHA [12CA5] beads and for minor variations in protein input. GST–TFIIB and control GST proteins were expressed from the T7 expression vectors (gift of D. Reinberg; Ha et al. 1993) in E. coli DH5α, purified by binding to glutathione–Sepharose 4B beads [Pharmacia], and analyzed by SDS-PAGE and Coomassie staining. Beads containing 200–300 ng of GST–TFIIB fusion protein or 500 ng of GST alone were incubated with <sup>35</sup>S-labeled TBP<sub>As</sub> and analyzed as described above. Approximately 10-fold more TBP<sub>As</sub> proteins were bound to the GST–TFIIB beads than to the control GST beads.

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Multiple regions of TBP participate in the response to transcriptional activators in vivo.

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