Factors (TAFs) required for activated transcription interact with TATA box-binding protein conserved core domain

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TFIID is a multisubunit protein containing the TATA box-binding polypeptide (TBP) and associated factors (TFIID-TAFs) required for activated transcription by RNA polymerase II. TBP from different eukaryotes contain a highly conserved carboxy-terminal domain and very divergent amino-terminal domains. Earlier studies proposed that the amino-terminal domains of metazoan TBPs are required for activated transcription. However, we report that a human TFIID complex containing an amino-terminal truncated TBP contains all the major TFIID-TAFs and supports in vitro transcriptional stimulation by different classes of activation domains and from a TATA-less promoter. Protein blotting experiments revealed direct interactions between the conserved domain of TBP and the two largest TAFs. The results suggest a model for the interaction of TFIID-TAFs with TBP.

[Key Words: TFIID; RNA polymerase II; transcription factor; transcriptional activation; TBP-associated factors]

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The TATA box-binding polypeptide (TBP) is required for transcription by all three nuclear RNA polymerases in eukaryotes (for review, see Sharp 1992; White and Jackson 1992). In higher eukaryotes the association of TBP with transcription by the different polymerases appears to be determined by its interaction with tightly associated polypeptides called TAFs (for TBP-associated factors; for review, see Pugh and Tjian 1992). Historically, the TBP complexes involved in RNA polymerase II transcription have been called TFIID (Matsui et al. 1980). Isolated TBP can function with the other polymerase II general transcription factors in basal level in vitro transcription from a TATA box promoter, but the TAFs associated with TBP in TFIID are required for transcriptional activation by activator proteins (Dynlacht et al. 1991; Tanese et al. 1991). Recently, TFIID activity from HeLa cells was shown to reside in what appears to be a single purified multisubunit complex of ~700 kD, termed holo-TFIID, composed of TBP and several TAFs ranging in size from ~250 to 28 kD (Zhou et al. 1992). Holo-TFIID was shown to support in vitro transcriptional activation by several classes of activation domains and from a polymerase II promoter lacking a TATA box.

Complementary DNAs encoding the TBP have been cloned from yeasts, insects, plants, and mammals

(Greenblatt 1991; Pugh and Tjian 1992). The carboxy-terminal ~180 residues of TBP are >80% identical in all organisms and form a protease-resistant domain that binds TATA boxes and is active for basal polymerase II transcription in vitro (Horikoshi et al. 1990; Lieberman et al. 1991). The amino-terminal domains of TBPs from these organisms differ significantly in length and sequence. A recent crystal structure of a TBP from *Arabidopsis thaliana* shows that the conserved carboxyl terminus folds into two symmetrical subdomains with the amino-terminal domain extending to one side (Nikolov et al. 1992).

Early in vitro experiments with cloned *Drosophila* and human TBP indicated that the "species-specific amino terminus" is required for the stimulation of transcription by the activator protein Sp1 that has glutamine-rich activation domains (Peterson et al. 1990; Pugh and Tjian 1990). However, later molecular genetic studies in yeast revealed that the species-specific amino-terminal domain of yeast TBP is not required for viability (Cormack et al. 1991; Gill and Tjian 1991; Reddy and Hahn 1991; Poon et al. 1991; Zhou et al. 1991). At first it seemed surprising that transcriptional activation might function through a different mechanism in yeast than in higher eukaryotes. However, Kelleher et al. (1992) recently reported that in both yeast and human in vitro transcription systems, either yeast or human TBPs, which have highly divergent amino-terminal domains, can support

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activation by Gal4-VP16 and GCN4. GAL4-VP16 and GCN4 have acidic activation domains that function *in vivo* in both yeast and higher eukaryotes, whereas the glutamine-rich activation domain of Sp1 does not function in yeast (cited in Pugh and Tjian 1992). This finding appeared to resolve the apparent conflict between the requirement of the human and *Drosophila* TBP amino-terminal domains for Sp1 activation and the lack of a requirement for the yeast amino-terminal TBP domain for yeast cell viability. It seemed that in both yeast and higher eukaryotic systems acidic activation domains required only the conserved domain of TBP to function, whereas activation through glutamine-rich activation domains required a mechanism developed in higher eukaryotes and absent in yeast, which depended on the much larger TBP amino-terminal domains present in these organisms.

Because TAFs are required for transcriptional activation and the amino-terminal domain of human TBP was thought to be required for Sp1 activation, we asked whether one or more TAFs associate specifically with the amino-terminal domain of human TBP to mediate activation by glutamine-rich activation domains. To address this question, we expressed an amino-terminal deleted form of TBP in HeLa cells and analyzed the TAFs associated with the deleted TBP polypeptide. We found that all the major TAFs associated with holo-TFIID also associated with the amino-terminal deleted form of TBP. Additional analyses of protein-protein interactions between TBP and immobilized TAFs confirmed that all detectable interactions were through the TBP conserved carboxy-terminal domain. Moreover, in contrast to the initial conclusion that the species-specific amino terminus of TBP is required for transcriptional activation by Sp1, we found that purified holo-TFIID complex assembled with the amino-terminal deleted form of TBP fully supported *in vitro* transcriptional activation by all activators tested including Sp1. These observations together with the discovery of specific interactions between the TBP core domain and specific TFIID-TAFs allow us to refine a molecular model of the TFIID complex.

Results

Expression of amino-terminal-deleted TBP in HeLa cells

A gene encoding an epitope-tagged (Field et al. 1988) TBP mutant (e Δ TBP) was stably introduced into HeLa cells using a retrovirus vector (Muller et al. 1991). The e Δ TBP mutant has a deletion of residues 1–154, removing nearly the entire amino-terminal domain. (The first residue in the conserved core of human TBP is residue 159.) A 9-residue epitope recognized by monoclonal antibody 12CA5 was appended to the amino terminus to facilitate purification (Field et al. 1988). Clones of transformed HeLa cells expressed varying levels of e Δ TBP (Fig. 1, lanes 2–9). Further experiments were performed with clone 20 (lane 9) in which the level of e Δ TBP is ~10–20% the concentration of the epitope-tagged form of full-length TBP (eTBP) in clone LTR α 3 (lane 1; Zhou

Figure 1. Clones of transformed cells expressing e Δ TBP. Extracts of LTR α 3 cells expressing full-length eTBP (Zhou et al. 1992; lane 1), extracts of e Δ TBP-expressing cell lines (lanes 2–9), and phosphocellulose fractions A, B, C, and D (Dignam et al. 1983; 30 μ g each, lanes 10–13) prepared from cell line 20 nuclear extract were subjected to SDS-gel electrophoresis on a 11% polyacrylamide gel and subjected to Western blotting with monoclonal antibody 12CA5. Lane 2 contains extract from a pool of G418-resistant cell lines generated with the e Δ TBP retrovirus vector. The positions of eTBP and e Δ TBP are shown.

et al. 1992). This represents ~7–15% of total TBP in clone 20 cells.

The nuclear extract was prepared from clone 20 cells, fractionated by chromatography on a phosphocellulose column (Dignam et al. 1983), and the resulting fractions analyzed by Western blotting with monoclonal antibody 12CA5 (Fig. 1, lanes 10–13). The highest concentration of e Δ TBP was found in the D fraction (0.5–1.0 M KCl fraction), similar to the full-length TBP protein present in the parental HeLa cells (data not shown). Isolated human TBP and its carboxy-terminal domain elute from phosphocellulose at a lower salt concentration (Peterson et al. 1990). Consequently, the elution profile of e Δ TBP expressed at low level in clone 20 cells suggests that it had become associated with the TAF(s) responsible for the relatively salt-stable interaction of the TFIID complex with phosphocellulose.

e Δ TBP binds to the same TFIID-TAFs as full-length TBP

A multisubunit protein complex containing e Δ TBP was affinity purified from the phosphocellulose D fraction of clone 20 cells using a column of 12CA5 monoclonal antibody followed by elution with synthetic epitope peptide (Field et al. 1988), as described for full-length epitope-tagged TBP (Zhou et al. 1992). This protein complex (referred to as e Δ NTFIID) and the eTFIID complex containing epitope-tagged full-length TBP were analyzed by SDS-polyacrylamide gel electrophoresis and silver staining (Fig. 2). Significantly, the e Δ NTFIID fraction contained each of the TAFs found associated with full-length eTBP. The low molecular weight e Δ TBP

Figure 2. Comparison of purified e Δ NTFIID complex and eTFIID complex. A silver-stained 11% SDS-polyacrylamide gel is shown. Lane 1 contains molecular weight markers. Lane 2 shows eTFIID eluted with HA1 peptide from a 12CA5 monoclonal antibody affinity column incubated with the phosphocellulose D fraction of LTR α 3 cells (Zhou et al. 1992). Lane 3 shows e Δ NTFIID complex eluted from the affinity column incubated with the phosphocellulose D fraction of e Δ NTBP-expressing cell line clone 20. The 30- and 28-kD TAFs reported earlier (Zhou et al. 1992) are difficult to see in this reproduction.

subunit was not visible on this silver-stained gel, but was detected readily by Western blotting using the monoclonal antibody 12CA5 (see Fig. 4C). It is difficult to determine the stoichiometry of the different TAFs in eTFIID and e Δ NTFIID from silver-stained gels (e.g., Figs. 2 and 4) because different polypeptides stain to different extents and the relative stain intensities vary somewhat from gel to gel. On the basis of the appearance of several silver-stained gels of these purified proteins, we do not believe the stoichiometry of the TAFs to TBP is different for full length and Δ NTBP, but we cannot rule out this possibility.

This and other preparations of e Δ NTFIID clearly revealed a 43-kD TAF that was not visualized earlier (Zhou et al. 1992), probably because it migrates close to eTBP on SDS-polyacrylamide gels. We questioned whether this 43-kD polypeptide might be the endogenous TBP in clone 20 cells. This would be possible if the TFIID complex contained two copies or more of TBP per complex. This is an important point because if the 43-kD polypeptide were TBP, some of the TAFs might be associated

with the purified e Δ NTFIID through the amino-terminal domain of the full-length TBP. However, a rabbit polyclonal serum that recognizes human TBP (Lee et al. 1991) failed to detect the 43-kD polypeptide in Western blots (data not shown). Because the e Δ NTFIID complex preparation did not contain the amino-terminal domain of TBP but did contain each of the major TFIID TAFs, we conclude that the species-specific amino terminus of human TBP is not required for the interaction between TBP and the major TAFs in TFIID.

Interaction of TBP with immobilized TAFs

To confirm and extend this result, interactions between TBP and TAFs were analyzed using a Far-Western protein blotting assay (Lee et al. 1991; Fig. 3). Purified eTFIID complex was subjected to SDS-gel electrophoresis, blotted onto nitrocellulose, subjected to a protein renaturation protocol, and probed with 35 S-labeled full-length TBP or TBP deletion mutants TBP Δ C273, TBP Δ C167, or TBP Δ N157, as diagrammed in Figure 3A. Figure 3C shows that the two largest TAFs (TAF₂₅₀ and TAF₁₂₅) were specifically bound by full-length TBP. These two TAFs were also bound by the amino-terminal truncated TBP derivative TBP Δ N157, but not by a TBP deletion mutant lacking almost the entire carboxy-terminal conserved domain (TBP Δ C167). TBP Δ C167 failed to interact with any TAF on the blot. Moreover, when part of the second imperfect direct repeat of TBP (for review, see Greenblatt 1991) was removed from the conserved domain, the resulting mutant TBP Δ C273 recognized TAF₁₂₅ but not TAF₂₅₀.

In control experiments, none of the TAFs were bound by in vitro translated brome mosaic virus (BMV) coat protein, which has a high isoelectric point similar to that of human TBP (data not shown). This indicates that the interactions between TBP and TAFs detected in this protein-blotting experiment were not due to simple nonspecific electrostatic interactions. The specificity of the interactions is also demonstrated by the observation that TBP Δ 273, which contains the TBP basic repeat region, is bound to TAF₁₂₅ but not to TAF₂₅₀. These Far-Western results are consistent with the analysis above of TAFs associated with e Δ NTBP in vivo. Interactions were detected between TAFs and the conserved carboxy-terminal domain of TBP, but not with the TBP amino-terminal domain.

Δ NTFIID supports activated transcription and transcription from a promoter lacking a TATA box

These results indicated that the amino-terminal domain of human TBP is not required for TBP binding to the major TFIID-TAFs. However, it seemed possible that minor TAFs not visualized on our gels might be required for activation by some activator proteins. Consequently, it was important to test the transcriptional activity of the e Δ NTFIID complex fraction. The activity of e Δ NTFIID was analyzed in in vitro transcription reactions reconstituted with partially purified general tran-

Figure 3. Far-Western protein-blotting analysis of the interaction between TBP and TBP-associated factors (TAFs). (A) Diagrams of the primary structures of the full-length TBP and three TBP deletion mutants. The rectangle represents the conserved carboxy-terminal domain. Arrows represent the two imperfect direct repeats. Positive signs indicate the basic repeat region, HLH, potential helix-loop-helix motifs. (B) SDS-PAGE analysis of the full-length TBP and three TBP deletion mutants. Proteins were expressed in a rabbit reticulocyte lysate in vitro translation system in the presence of Trans³⁵S-Label (ICN). (C) Equal amounts of the eTFIID complex was subjected to SDS-gel electrophoresis, blotted onto nitrocellulose, subjected to a renaturation protocol and incubated with equal cpm of ³⁵S-labeled full-length TBP, TBPΔC273, TBPΔC167, or TBPΔN157 preparations shown in B. An autoradiogram of the washed blots is shown. The positions of TAF₂₅₀ and TAF₁₂₅ are indicated by arrows. (D) Western analysis of the same nitrocellulose blots shown in C using monoclonal antibody 12CA5 specific for eTBP, demonstrating that the same amount of the eTFIID complex was on each of the blot strips.

scription factors. The first activator tested was GAL4-AH, which contains an acidic activation domain (Giniger and Ptashne 1987). The template, pG5E4T (Carey et al. 1990), contained five Gal4-binding sites upstream of the adenovirus E4 TATA box and the initiation region. Activation by Gal4-AH requires one or more TAFs, as activation is observed in reactions with eTFIID complex, but not with recombinant TBP (Zhou et al. 1992).

In the absence of any source of TFIID activity, no specific transcription could be detected (Fig. 4A, lanes 1–2). Significantly, addition of eΔNTFIID supported GAL4-AH activated transcription (lane 6) to the same extent as the eTFIID fraction (lane 4). The amounts of eΔNTFIID and eTFIID used in these reactions were assayed by gel electrophoresis and silver staining (Fig. 4B), and by Western blotting with monoclonal antibody 12CA5 (Fig. 4C).

On the basis of these silver-staining and Western blotting results, we estimate that the amount of eΔNTFIID protein used in the transcription reaction was within a factor of two of the amount of eTFIID used. (From the Western blotting result the amount of eΔNTBP appeared to be ~50% the amount of eTBP (Fig. 4C), whereas the silver-stained gel (Fig. 4B) indicated slightly more eΔNTFIID protein compared with eTFIID protein. We think these slight variations are due, on the one hand, to differences in transfer of polypeptides of different size in Western blotting, and on the other hand, to variations in recovery and staining of polypeptides in the SDS gel.) Given the limitations of these techniques to measure the

low protein concentrations involved we conclude that the eΔNTFIID fraction had similar (within a factor of two) transcriptional-specific activity as the eTFIID fraction for GAL4-AH activated transcription.

Next, we tested whether purified eΔNTFIID could support transcriptional activation by Sp1. As a stringent test of Sp1 activation we assayed transcription from a promoter lacking a TATA box. It was shown previously (Smale et al. 1990; Pugh and Tjian 1991; Zhou et al. 1992) that one or more TAFs are required for transcription from a template lacking a TATA box but containing the initiator element of the terminal transferase gene (Smale and Baltimore 1989) and upstream Sp1-binding sites. The eΔNTFIID fraction supported Sp1 activated transcription from this TATA-less promoter with similar specific activity to that observed for the eTFIID complex (Fig. 5). The eΔNTFIID fraction also supported Sp1-activated transcription from a promoter containing a TATA box and transcriptional activation by the Zta transcription factor encoded by Epstein-Barr virus, assayed as described earlier (Zhou et al. 1992; data not shown).

Discussion

In contrast to what has been widely believed on the basis of early studies with cloned human and *Drosophila* TBP, the experiments reported here demonstrate that the species-specific amino terminus of human TBP is not re-

Figure 4. eΔNTFIID complex supports GAL4-AH stimulated transcription. (A) Primer extension assays of reconstituted in vitro transcription. Reactions in the absence (–) or presence (+) of GAL4-AH are shown with the indicated sources of TFIID activity. The template contained five GAL4-binding sites upstream of the adenovirus E4 TATA box and initiation region. The arrowhead marks the position of the major specific transcripts. (B) The amounts of eTFIID and eΔNTFIID used in the transcription reactions shown in A were analyzed by SDS-polyacrylamide gel electrophoresis and silver staining. (C) The amounts of eTFIID and eΔNTFIID used in the transcription reactions shown in A were designated as one unit. The indicated units of either the eTFIID or eΔNTFIID fractions were subjected to Western blotting using monoclonal antibody 12CA5.

quired for transcriptional *trans*-activation by three different activator proteins tested. These activators have different classes of activation domains: Sp1 contains glutamine-rich activation domains (Courey and Tjian 1988); Gal4-AH has an acidic activation domain (Giniger and Ptashne 1987); Zta contains an activation domain that is not particularly rich in any one amino acid residue (for discussion, see Lieberman and Berk 1990). The amino-terminal domain of TBP was also found to be dispensable for transcription from a promoter lacking a TATA box.

Analysis of the TAFs associated with the conserved core domain of TBP provides a molecular explanation for why the species-specific amino-terminus is dispensable for activated transcription. TAFs are required for transcriptional activation by all activator proteins tested (Dynlacht et al. 1991; Tanese et al. 1991; Zhou et al. 1991), and the TBP conserved core domain associates with all the TAFs complexed to full-length TBP (Fig. 2). These results emphasize the importance of the TAFs in the mechanism of activation.

Our results also resolve what initially appeared to be a contradiction between studies of TBP in yeast and higher eukaryotes. The conclusion that the species-specific amino termini of human and *Drosophila* TBPs were re-

quired for transcriptional activation (Pugh and Tjian 1990; Peterson et al. 1990) seemed at odds with the observation that the amino-terminal domain of yeast TBP is not required for viability (Cormack et al. 1991; Gill and Tjian 1991; Reddy and Hahn 1991; Poon et al. 1991; Zhou et al. 1991). Initially it seemed that despite the remarkable conservation of many of the features of RNA polymerase II transcription among all eukaryotes, the mechanism of transcriptional activation might be fundamentally different in yeast and metazoans. The finding of Kelleher et al. (1992) that both yeast and human TBP supported in vitro activation by GAL4-VP16 and GCN4 went a considerable way in dispelling the apparent discrepancy, as it argued that the TBP core domain is sufficient for activation by acidic activation domains. The new result that the amino-terminal domain of TBP can be entirely deleted without affecting activation by Sp1, the activator used in the initial studies suggesting the importance of the TBP amino-terminal domain, argues that the mechanisms of activation in yeast and higher eukaryotic systems are in fact likely to be highly homologous. Moreover, our finding that the conserved carboxy-terminal domain of human TBP associates with the full set of TFIID-TAFs makes it seem very likely

Figure 5. eNTFIID complex supports Sp1-activated transcription from a promoter lacking a TATA box. The template used in these reconstituted *in vitro* transcription reactions (plasmid VI; Smale et al. 1990) contains the Sp1 binding SV40 21 base-pair repeat region 42 bp upstream of the terminal transferase gene initiator element (Smale and Baltimore 1989). The sources of TFIID activity are indicated. The units of eTFIID and eNTFIID were the same as for Fig. 4C. Sp1 purified by three cycles of affinity chromatography (Kadonaga and Tjian 1986) was added to the transcription reactions as indicated (+).

that the highly homologous yeast TBP core domain interacts with a similar set of yeast TAFs in the process of polymerase II transcriptional activation.

The earlier experiments that suggested that the amino-terminal domain of human TBP is required for activated transcription used unfractionated nuclear extracts in which TFIID activity was heat inactivated (Peterson et al. 1990). Addition of full-length TBP partially restored activated transcription, whereas the conserved core domain did not. In hindsight, it seems likely that the full-length TBP must have reassociated with one or more TAFs required for activated transcription, whereas the conserved core domain did so less efficiently. Consequently, the amino-terminal domain may increase the association of TAFs with TBP *in vitro* under the conditions of these earlier experiments. However, our findings indicate that the amino terminus is not required for the assembly of functional TFIID complex *in vivo*. Recent transfection studies have also found that the amino-terminal domain of human TBP is not required for TBP to synergize with the β -retinoic acid receptor *in vivo* (Berkenstam et al. 1992).

Although the region amino-terminal to the conserved core domain varies greatly between TBPs isolated from different phyla; the amino-terminal domain of murine TBP is >90% identical to human TBP (Tamura et al. 1991). This high conservation among mammals suggests that the amino-terminal region does perform a significant function. Although we found that the amino termi-

nus of human TBP is not required for activated transcription by RNA polymerase II *in vitro*, we noted in earlier *in vivo* studies that deletion of the amino-terminal region of yeast TBP decreased induction from some inducible promoters (Zhou et al. 1991). Also, TBP participates in specific transcription initiation by each of the three eukaryotic RNA polymerases (Sharp 1992; White and Jackson 1992). Perhaps the amino-terminal domain of TBP has an important function in transcription by RNA polymerase I or III.

The Far-Western protein-blotting analysis (Fig. 3) demonstrated that the TBP conserved core domain interacts directly with the two largest TFIID-TAFs, TAF₂₅₀ and TAF₁₂₅. The carboxy-terminal 66 residues are required for TBP binding to TAF₂₅₀, but not for binding to TAF₁₂₅. These results suggest a model in which the remaining lower molecular weight TAFs interact with TBP indirectly, through TAF₂₅₀ and TAF₁₂₅. However, we emphasize that a negative result in the Far-Western analysis cannot be interpreted because it could be due to a failure to renature a domain of a smaller TAF required for TBP binding. Also, functionally significant interactions between TBP and smaller TAFs might occur, but not be sufficiently stable to be demonstrated by this technique.

On the basis of a comparison of the DNase I footprint produced by holo-TFIID on the adenovirus major late promoter to the footprint produced by isolated TBP, we proposed a model in which TBP is not at the center of the holo-TFIID complex, but rather at one side (Zhou et al. 1992). With the new information on the direct interactions between TBP and the 250- and 125-kD TAFs, we can modify this model, placing the two largest TAFs at the center of the multisubunit complex, with the smaller TAFs and TBP bound to their surfaces (Fig. 6). This diagram also emphasizes the finding that the amino-terminal domain of TBP does not make any significant contacts with TFIID TAFs or with promoter DNA. Further analyses of holo-TFIID should indicate

Figure 6. Model of holo-TFIID. The diagram is drawn to emphasize the interaction of the TBP carboxy-terminal core domain with TAF₂₅₀ and TAF₁₂₅, and the lack of interaction of the TBP amino-terminal domain with TFIID-TAFs or DNA. The complex is shown extending downstream from the TATA box on promoter DNA, in accordance with earlier footprinting results (Zhou et al. 1992). The arrangement of TAFs in the complex and the association of TAF 125 with DNA is purely hypothetical.

which TAFs interact with each other and with DNA downstream from the TATA box.

Materials and methods

Construction of HeLa cells expressing eΔNTBP

A gene encoding the 12CA5 epitope-containing sequence MGY-PYDVPDYAV (Field et al. 1988) fused to residues 155–339 of human TBP was synthesized by PCR amplification of a TBP cDNA clone (Kao et al. 1990) using synthetic primers for introduction of the amino-terminal epitope and flanking *EcoRI* sites. The amplified DNA fragment was inserted into the *EcoRI* site of pSRαMSVtkneo (Muller et al. 1991) to create pLTReΔNTBP. A helper-free retroviral stock was prepared by cotransfection of COS cells with pLTReΔNTBP and pSV-Ψ⁻-A-MLV that contains Ψ⁻ MLV proviral sequences expressing an amphotropic coat cloned into an SV40-based expression vector. Calcium phosphate transfection precipitates containing 15 μg of each plasmid were transfected into 2 × 10⁶ COS cells and media containing recombinant retrovirus was harvested at 48 hr post-transfection. Freshly seeded HeLa S3 cells were infected and selection of G418-resistant clones was performed as described (Zhou et al. 1992). Clones were expanded in medium containing 200 μg/ml G418 and screened by Western blotting of total cell protein using monoclonal antibody 12CA5, as shown in Figure 1. Clone 20 was selected, grown, and maintained in suspension in S-MEM plus 5% calf serum with 200 μg/ml G418 in a volume of 500 ml. A 20-liter culture was grown in media without antibiotic; cells were harvested and used for preparation of eΔNTBP.

Affinity purification of eΔNTFIID complex

Nuclear extract was prepared from clone 20 cells and fractionated by phosphocellulose chromatography as described (Dignam et al. 1983). One milliliter of the undialyzed D (1.0 M KCl) fraction, ~1 mg protein/ml, was incubated with 0.1 ml packed beads of protein A–Sepharose covalently coupled to monoclonal antibody 12CA5 with rotation at 4°C for 8 hr. Further steps in purification were as described for eTFIID complex in Zhou et al. (1992). Briefly, the protein A–Sepharose beads with bound complex were washed extensively with buffer containing 0.5 M KCl, then with buffer containing 0.1 M KCl, and then eluted in 0.1 ml buffer containing 0.1 M KCl and 1 mg/ml peptide YPYDVPDYA at room temperature for 30 min.

In vitro transcription

One hundred nanograms of template plasmid [pG5E4T (Carey et al. 1990), pZ₇E4T (M. Carey, unpubl.), or plasmid VI (Smale et al. 1990) containing the Sp1-binding sites of SV40 and the initiator promoter element of the terminal transferase gene, but lacking a TATA box] was incubated with 30 μg fraction TFIIA, 60 ng recombinant TFIIB, 12 μg fraction polymerase II/E/F, and 10 μl of eΔNTFIID complex or eTFIID complex as indicated, with or without activator protein in 50 μl 60 mM KCl, 7.5 mM MgCl₂, 12 mM HEPES (pH 7.9), 10 mM β-mercaptoethanol, 12% glycerol, 4% polyethylene glycol, and 0.6 mM of ATP, GTP, CTP, and UTP for 60 min at 30°C. Fractions TFIIA and polymerase II/E/F were purified from HeLa cell nuclear extract by chromatography on phosphocellulose and DEAE–Sepharose as described by Lin and Green (1991). Recombinant TFIIB was purified from engineered *Escherichia coli* as described by Ha et al. (1991). Activator proteins were 100 ng Gal4-AH purified from engineered *E. coli* as described by Lin et al. (1988); 200 ng Zta

purified from engineered *E. coli* as described by Lieberman and Berk (1990); or Sp1 purified from HeLa nuclear extract by chromatography on Sephacryl S-300 followed by three passes over an oligonucleotide-affinity column as described by Kadonaga and Tjian (1986). Transcripts were quantitated by primer extension as described (Schmidt et al. 1989) using primers described in Zhou et al. (1992).

Far-Western protein blotting

Interactions between TBP and TFIID–TAFs were analyzed by a protein-blotting protocol described in detail in Lee et al. (1991). Briefly, eTFIID complex isolated, as described in Zhou et al. (1992), was subjected to SDS–gel electrophoresis, electrophoretically transferred to nitrocellulose, and subjected to a renaturation protocol by incubating the nitrocellulose filter in buffers containing decreasing concentrations of guanidine HCl. The blot was then incubated at room temperature for 14 hr in 10 ml buffer CZ 0.05 [10 mM HEPES (pH 7.9), 50 mM KCl, 2.5 mM MgCl₂, 50 μM ZnCl₂, 50 μM EDTA, 1 mM DTT, 8.5% glycerol] plus 100 μl reticulocyte lysate containing ³⁵S-labeled TBP or TBP deletion mutants prepared by in vitro transcription–translation. The blot was then washed with 20 ml buffer CZ 0.05 for 20 min two times and autoradiographed at –70°C with an intensifying screen for 1–2 days.

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