TFIIB-facilitated recruitment of preinitiation complexes by a TAF-independent mechanism

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ABSTRACT

Gene activators contain activation domains that are thought to recruit limiting components of the transcriptional machinery to a core promoter. VP16, a viral gene activator, has served as a model for studying the mechanistic aspects of transcriptional activation from yeast to human. The VP16 activation domain can be divided into two modules—an N-terminal subdomain (VPN) and a C-terminal subdomain (VPC). This study demonstrates that VPC stimulates core promoters that are either independent or dependent on TAFs (TATA-box Binding Protein-Associated Factors). In contrast, VPN only activates the TAF-independent core promoter and this activity increases in a synergistic fashion when VPN is dimerized (VPN2). Compared to one copy of VPN (VPN1), VPN2 also displays a highly cooperative increase in binding hTFIIB. The increased TFIIB binding correlates with VPN2's increased ability to recruit a complex containing TFIID, TFIIA and TFIIB. However, VPN1 and VPN2 do not increase the assembly of a complex containing only TFIID and TFIIA. The VPN subdomain also facilitates assembly of a complex containing TBP: TFIIA:TFIIB, which lacks TAFs, and provides a mechanism that could function at TAF-independent promoters. Taken together, these results suggest the interaction between VPN and TFIIB potentially initiate a network of contacts allowing the activator to indirectly tether TFIID or TBP to DNA.

INTRODUCTION

Transcriptional activators increase gene expression by a variety of mechanisms including the stimulation of preinitiation complex (PIC) assembly [reviewed in (1,2)]. For protein-encoding genes in eukaryotes, the PIC contains RNA polymerase II, a Mediator complex, and a set of general transcription factors (GTFs) including TFIIA, TFIIB and TFIID [reviewed in (3–5)]. Human (h)TFIIA is composed of α, β and γ subunits. hTFIIB is a single polypeptide containing a zinc ribbon and a core domain that binds TBP. TFIIB, composed of the TATA-box binding protein (TBP) and a set of subunits called TBP-Associated Factors (TAFs), typically binds the core promoter and nucleates PIC formation. Initially, GTFs were identified as discrete proteins and activators were thought to facilitate step-wise assembly of the PIC. Subsequent evidence has accumulated for an RNA polymerase II holoenzyme, which contains many GTFs but typically not TFIID and TFIIA, that is recruited en masse by activators (6,7).

TFIID, TFIIA and TFIIB are putative activator targets (8–10). In vitro, activators facilitate the formation of complexes containing TFIID, TFIIA, TFIIB and/or RNA polymerase II bound to promoter DNA (11–20). Tethering TBP or TFIIB to DNA by fusing them to a DNA-binding domain can substitute in vivo for activator recruitment and stimulate transcription [(21) and references contained therein]. When both TBP and TFIIB are tethered to a promoter containing a weakened TATA box, synergistic gene activation was obtained (22). This result suggests that activators can facilitate PIC assembly by at least two different mechanisms.

Multiple mechanisms are reflected by the presence of TAF-dependent (TAF-dep) and TAF-independent (TAF-ind) promoters in yeast (23,24), although this classification does not currently extend to mammalian systems. Active TAF-dep promoters are bound by similar amounts of TBP and TAFs while TAF-ind promoters are bound by much higher levels of TBP than TAFs. The upstream activating sequences (UAS) and core promoters are typically TAF-dep or TAF-ind in parallel with the overall promoter (25). When the upstream activating sequences are exchanged, either class of UASs activated TAF-ind core promoters. In contrast, only the UAS from TAF-dependent genes generally activated TAF-dep core promoters. These results indicate that a UAS, and thus the activator(s) that binds it, can be classified by its ability to activate different core promoters.

VP16, a well-studied viral activator, contains a potent acidic activation domain in its C-terminus (26) that can be split into two functional subdomains or modules—VPN (comprising residues 411 to 456) and VPC (residues 452 to 490) (27–29). VPN and VPC use different mechanisms to activate transcription in mammalian cells (30). While each module activates...
transcription, each is weaker than intact VP16. The potency of VP16 may reside in having subdomains that function by different mechanisms.

Activators stimulate transcription synergistically—a greater than additive increase with regard to parameters such as activator concentration, number of activator-binding sites or number of activation subdomains (31,32). For example, in mammalian cells as well as extracts from both yeast and mammalian cells, activators containing one, two or four copies of VPN synergistically increase gene expression with respect to both the number of activation subdomains and the number of activator binding sites in the promoter (31,33). Similar results have been observed with the glutamine-rich and proline-rich activation subdomains of Oct-2, and an acidic 11-amino acid region of RelA (34–36).

In this study, we initially examined the ability of VPN and VPC to activate TAF-dependent or TAF-independent core promoters. VPC stimulates both classes while VPN only activates the TAF-independent core promoter. When dimerized, VPN increases transcription in a synergistic manner, exhibits a synergistic increase in binding TFIIIB, but not TBP, TAF9 or TFIIA, and has an increased ability to recruit a complex containing TFIIID, TFIIA and TFIIB, but not TFIIID and TFIIA. Interaction with TFIIIB could allow an activator to function in a TAF-independent manner and VPN also exhibits the ability to recruit a complex containing TBP, TFIIA and TFIIIB. These results suggest that an activator:TFIIB interaction could indirectly tether TFIIID or TBP to DNA and stabilize the preinitiation complex, possibly without the involvement of TAFs.

**MATERIALS AND METHODS**

**Plasmid constructs**

pSH18-34A Spe, the parental β-galactosidase (β-gal) reporter construct, was constructed by digesting pSH18-34 (Invitrogen) with SpeI to remove the 2μ origin and ligating the remaining plasmid DNA. DNA fragments containing one or two upstream copies of a LexA DNA-binding site and either the ADH1 or RPS5 core promoter were generated by PCR, digested with Xmal and BamHI and ligated into pSH18-34A Spe.

The yeast vectors that express the LexA derivatives were generated by digesting pBXG-VPN1 and pBXG-VPN2 (31) with EcoRI and Drai, and ligating the fragment into EcoRI and SmaI sites of pBTM116 (a generous gift from Stan Hollenberg, Oregon Health Sciences). Glutathione S-transferase (GST)–hTFIIB was constructed by using PCR to generate a BamHI–BglIII fragment encoding hTFIIB that was inserted at the BamHI site of pGEX2TK (Amersham Pharmacia). GST-VPN1 and GST-VPN2 were constructed by excising a BamHI fragment from the LexA-VPN1 and Lexa-VPN2 yeast expression vectors and ligating it into the BamHI site of pGEX-KG (a generous gift from Yi Zheng, Children’s Hospital Research Foundation, Cincinnati, OH). pGEX-dmTAF9, pGEX-TBP and pGEX-TFIIBαβ were generous gifts of Jim Goodrich and Robert Tjian (UC Berkeley), Arnie Berk (UCLA), and Dongmin Ma and Danny Reinberg (UMD—New Jersey), respectively.

**Yeast culture and transformations**

Yeast strains were grown as described (37). The β-gal reporters were integrated into the isogenic wild-type strain of taf6-19 (a generous gift of Sukesh Bhaumik and Michael Green, University of Massachusetts) at the URA3 locus (37). Competent yeast were prepared and transformed using the EZ Yeast Transformation II kit (ZymoResearch).

**β-Galactosidase assay**

Yeast strains were grown to log phase in the appropriate minimal media, then harvested by centrifugation of 1 ml at 2000 g for 5 min. The cell pellet was resuspended in 200 μl of SCE, then lysed by adding 5 units of Zymolyase (ZymoResearch) and incubation at 37°C for 60 min. The samples were subjected to two cycles of freeze/thaw using a dry ice/ethanol bath and the lysate was isolated after centrifugation at 15 000 g for 3 min. β-Galactosidase activity was assayed using the Lumin sensor Chemiluminescent Beta-galactosidase Kit II (Clontech). The protein concentration was determined using the QuantPro BCA Assay Kit (Sigma). The values for specific β-gal activity were determined by assaying and averaging at least three independent transformants grown in parallel. The expression of the LexA derivatives was assayed by western blots using an anti-LexA antibody (Invitrogen). LexA-VPN2, LexA-VPC1 and Lexa-VPC2 were expressed at similar levels, which is less than 3-fold lower than expression of LexA or Lexa-VPN1 (S. Xu, X. Hu and R. Hori, unpublished data).

**Protein purification**

The GST derivatives, GAL4 derivatives, TBP, TFIIA and TFIIIB were purified as described previously (33,38–40). TFIIID was purified using a cell line expressing a hemagglutinin-tagged TBP as described previously (41). Protein concentrations were quantitated according to the method of Bradford using the Bio-Rad Protein assay (Bio-Rad) and by comparison on denaturing protein gels.

**Protein–protein interaction assays**

Ten micrograms of GST, GST-TFIIB, GST-TAF9, GST-TBP and GST-TFIIBαβ coupled to glutathione–agarose were pre-equilibrated twice for 5–10 min at 22°C with 100 μl Buffer A (12 mM HEPES, pH 7.9, 12% glycerol, 0.12 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF and 0.025% NP-40) containing 60 mM KCI and 200 μg/ml BSA, centrifuged for 2 min at 3000 g and the supernatant removed. In the binding reaction, the pre-equilibrated resin was incubated in a 1 ml mixture containing 2.5 μg of each GAL4 derivative in Buffer A plus 60 mM KCl for 60 min at 22°C with gentle rocking, then centrifuged at 3000 g for 2 min. The resin was washed typically eight times with 200 μl of Buffer A containing the specified KCI concentration, then 30 μl of 2X SDS–PAGE loading dye was added. The samples were heated at 95°C for 2 min, centrifuged at 10 000 g for 2 min, loaded onto 12% SDS–polyacrylamide gels and separated by electrophoresis at 100 V for 1 h. The samples were transferred to Hybond-C (Amersham Pharmacia) and blocked using 1X PBS containing 5% non-fat dried milk and 0.1% Tween-20. The blots were probed with a polyclonal antibody serum directed against GAL4 1-147 (a generous gift of Michael Carey, UCLA), and detected using a secondary goat anti-rabbit serum (Bio-Rad) and the ECL detection system (Amersham Pharmacia) according to the manufacturer’s specifications.

The interaction assays with GST-VPN1 and -VPN2 contained 7.5 μg of GST, GST-VPN1 and GST-TBP or 4 μg of
Figure 1. VPN and VPC function differently. (A) VPN activates the TAF-ind ADH1 core promoter synergistically. The various activators (denoted on the left) were expressed in yeast strains containing either the LexA1-ADH1-lacZ or LexA2-ADH1-lacZ reporter gene. The bars represent the specific β-gal activity and the error bars denote standard deviations. The value of the β-gal activity is placed on the right side of the respective bar. The asterisk denotes P < 0.05 when this value is compared to the β-gal activity generated by LexA alone. (B) VPC, but not VPN, activates the TAF-dep RPS5 core promoter. The various activators (denoted on the left) were expressed in strains containing either the LexA1-RPS5-lacZ or LexA2-RPS5-lacZ reporter gene. The bars represent the specific β-gal activity and the error bars denote standard deviations. The value of the β-gal activity is placed on the right side of the respective bar.

Electrophoretic mobility shift assays

Electrophoretic mobility shift assays (EMSAs) were performed using 1.4% agarose gels containing Mg²⁺ as described previously (15,43). These magnesium-containing agarose gels are used because TFIIID’s size and an induced DNA bend of 90° upon TBP binding make it difficult to monitor complexes containing TFIIID using native acrylamide gels. The bands observed are sometimes diffuse in this system and can cause variation in the detectable level of labeled DNA although equivalent amounts are added to each reaction.

RESULTS

The VPN and VPC activation domain modules of VP16 function differently

VPN and VPC both activate the TAF-independent ADH1 promoter in yeast, but differ in their ability to function synergistically when dimerized. VP16 was not assayed because it is toxic at high levels in yeast (44). β-Galactosidase expression from a promoter containing one LexA binding site upstream of the ADH1 core promoter (LexA1-ADH1-lacZ) was assayed. LexA-VPN1 and LexA-VPN2 stimulated expression by 3- and 150-fold, respectively (Figure 1A). When dimerized, VPN activated transcription synergistically. LexA-VPC1 and LexA-VPC2 stimulated LexA1-ADH1-lacZ by 89- and 113-fold, respectively, relative to LexA (Figure 1A). Similar results were obtained on the LexA2-ADH1 promoter. When dimerized, VPC did not activate the ADH1 core promoter synergistically.

VPN and VPC differ in their ability to activate the TAF-dependent RPS5 core promoter. LexA-VPC1 and/or LexA-VPC2 activated LexA1- or LexA2-RPS5-lacZ (one or two LexA sites upstream of the RPS5 core promoter), but was not consistently synergistic upon dimerizing VPC (Figure 1B). In contrast, neither LexA-VPN1 nor LexA-VPN2 stimulated these promoters above background levels (Figure 1B). When compared to VPN, VPC activated the ADH1 and RPS5 promoters, as well as the TAF-independent GAL1 promoter (45), to higher levels.

The ability of VPN2 to function synergistically correlates with increased TFIIIB binding

Next, the association of VPN1 and VPN2 with putative targets was compared. Upon dimerizing VPN, there may be a specific interaction that increases synergistically and parallels the increased activation observed in Figure 1A. VPC was not characterized further because it did not display synergistic gene activation in these experiments and a recent study has measured the affinity between VPC and the four putative targets (that are characterized next) and identified TBP as having the highest affinity for VPC (46). We tested hTBP, (Drosophila melanogaster) dmTAF9 [formerly called dTAF9 (47)], hTFIIA and hTFIIB because they have been implicated as putative targets of acidic activators within a complex containing TFIIID, TFIIIA and TFIIIB (DAB) (8–10). Since there is a ‘one-to-one correspondence’ between the subunits composing all eukaryotic general transcription factors (3), it is expected that the human, Drosophila and yeast proteins will have similar activities. Affinity resins that display hTBP, dmTAF9 or hTFIIB, as GST fusion proteins, were incubated with either VPN1, VPN2, or VP16 fused to the DNA-binding domain (DBD) of GAL4. The amount of bound activator was
measured using an anti-GAL4 DBD antibody. Because the GAL4 DBD is common to each activator, the amounts of each activator can be directly compared.

The binding of VPN2 to TFIIB increases synergistically relative to VPN1. TFIIB bound approximately 60-fold more VPN2 than VPN1 (Figure 2A, lanes 4 and 9), when the reaction was performed at 50 mM KCl (similar to an in vitro transcription reaction). Interactions formed under these conditions were challenged with higher ionic strength buffers (containing 500 mM KCl). Under these conditions, VPN1 and VPN2 did not interact with TFIIB (Figure 2A, lanes 5 and 10), but there was still significant association between the more potent VP16 activation domain and TFIIB (Figure 2A, lane 15). As a control for specificity, VPN1, VPN2 and VP16 displayed no detectable binding of GST (Figure 2A, lanes 2, 3, 7, 8, 12 and 13).

The synergistic increase in the association between TFIIB and dimerized VPN was also observed when the GST pull-down assay was 'reversed'. GST-VPN1 or GST-VPN2 affinity resins were incubated with increasing amounts of TFIIB. At the two higher concentrations of TFIIB, the amount of TFIIB bound to GST-VPN2, relative to VPN1, increases synergistically (Figure 3A, compare lane 10 and 11 with lanes 7 and 8, respectively). There was no detectable interaction between

Figure 2. The interaction between GAL4-VPN2 and hTFIIB but not dTAF9, or hTBP increases synergistically relative to GAL4-VPN1. (A) GST or GST-hTFIIB (10 μg) was incubated with 2.5 μg of either GAL4-VPN1 (lanes 2 to 5), GAL4-VPN2 (lanes 7 to 10) or GAL4-VP16 (lanes 12 to 15), washed extensively with buffer containing either 50 or 500 mM KCl and 50% of the bound fraction was analyzed by electrophoresis and an immunoblot using an anti-GAL4 antibody. Lanes 1, 6 and 11 contain 1% of the input protein. (B) GST or GST-dTAF9 (10 μg) was incubated with 2.5 μg of either GAL4-VPN1 (lanes 2 to 5), GAL4-VPN2 (lanes 7 to 10) or GAL4-VP16 (lanes 12 to 15), washed extensively with buffer containing either 50 or 500 mM KCl and 25% of the bound fraction was analyzed by electrophoresis and an immunoblot using an anti-GAL4 antibody. Lanes 1, 6 and 11 contain 1% of the input protein. (C) GST or GST-hTBP (10 μg) was incubated with 2.5 μg of either GAL4-VPN1 (lanes 2 and 3) or GAL4-VPN2 (lanes 5 and 6), washed extensively with buffer containing 50 mM KCl and 25% of the bound fraction was analyzed by electrophoresis and an immunoblot using an anti-GAL4 antibody. Lanes 1 and 4 contain 1% of the input protein.

Figure 3. The association between GAL4-VPN2 and hTFIIB, but not hTFIIA, increases synergistically relative to GAL4-VPN1. (A) GST (lanes 3 to 5) and GST-VPN1 (lanes 6 to 8) of 7.5 μg or 4 μg of GST-VPN2 (lanes 9 to 11) were incubated with 3-fold increasing amounts (1.6, 4.8 and 14.4 μg) of TFIIB, washed extensively and 18% of the bound fraction was analyzed by electrophoresis and an immunoblot using an anti-TFIIB antibody. Lane 2 contains 0.8% of the input from the highest concentration of TFIIB and lane 1 contains molecular mass markers that have been labeled on the left side. (Note: Equimolar amounts of VPN were available because approximately half as much GST-VPN2 affinity resin, by mass, was used.) (B) GST (lanes 2 and 3), GST-VPN1 (lanes 4 and 5) and GST-TBP (lanes 8 and 9) of 7.5 μg or 4 μg of GST-VPN2 (lanes 6 and 7) were incubated with 4-fold increasing amounts of holo-TFIIA, washed extensively and 29% of the bound fraction was analyzed by electrophoresis and an immunoblot using an anti-His6 antibody. Lane 1 contains 20% of the input from the highest concentration of TFIIA. The positions of molecular mass markers have been labeled on the left side.
TFIIB and GST alone (Figure 3A, lanes 3 to 5). The synergistic increase of VPN2 binding TFIIB in both assays is similar to the synergistic increase in the ability to activate transcription [this study; (31,33)].

The dramatic increase in binding dimerized VPN was specific to TFIIB and did not occur with dmTAF9, TBP and TFIIA. These proteins exhibited only modest or no increase when their association with VPN1 and VPN2 was compared. dmTAF9 bound approximately 3-fold more VPN2 than VPN1 (Figure 2B, lanes 4, 5, 9 and 10). VPNC interacts more strongly with dmTAF9 than VPN, and VP16 was used as a control protein known to bind dmTAF9 strongly (39). As expected, more VP16 bound dmTAF9 than either VPN1 or VPN2 (Figure 2B, compare lanes 14 and 15 to lanes 4, 5, 9 and 10). Similar to dmTAF9, TBP bound approximately 3-fold more VPN2 than VPN1 (Figure 2C, lanes 3 and 6).

There was no interaction observed between VPN and holo-TFIIB (TFIIAβ + TFIIAγ). The GST-VPN1 and GST-VPN2 affinity resins were incubated with increasing amounts of holo-TFIIB and no significant binding was detected (Figure 3B, lanes 4 to 7). GST-TBP was used as a control protein that binds holo-TFIIB (4) and they associated as expected (Figure 3B, lanes 8 and 9). From the combination of the above experiments, we conclude that the association between VPN and TFIIB is the only one (among these putative targets in the DAB complex) that increases in a synergistic manner.

The ability of VPN2 to recruit TFIIB-containing complexes correlates with the increase in its ability to bind TFIIB

We tested the hypothesis that the increased association between VPN2 and TFIIB could result in increased recruitment of preinitiation complexes containing TFIIB. Electrophoretic mobility shift assays, using magnesium-containing agarose gels initially developed to examine TFIIID-containing complexes (15) and optimized with human proteins, were performed. These EMSAs employed promoter DNA containing five GAL4-binding sites upstream of the adenovirus E4 core promoter (G2/E4T) because VP16 has been demonstrated to facilitate formation of complexes containing TFIIID:TFIIA (DA), DAB and hTBP:hTFIIIB complexes on the E4 core promoter (11,16,48). Since the ability of activators to recruit complexes increases with respect to the number of activator binding sites (11), a promoter bearing five activator-binding sites was employed to enhance the ability to assay activator function.

While both VPN1 and VPN2 facilitated assembly of the DAB complex, VPN2 had a larger effect. Assembly of the DAB complex was measured at three concentrations of human TFIID in the presence of hTFIIA and hTFIIIB. In the absence of activators, 9% of the promoter DNA was assembled into the DAB complex at the lowest concentration of TFIID (Figure 4A, lane 2). At the highest concentration of TFIID, 39% of the promoter DNA was assembled into the DAB complex (Figure 4A, lane 4). The addition of either GAL4-VPN1 or GAL4-VPN2 alone resulted in a slower mobility species (Figure 4A, compare lanes 1, 5 and 9). VPN1 increased DAB formation by 1.3-fold at the lowest concentration of TFIID (Figure 4A, compare lanes 6 and 2), but had no significant effect at the higher TFIID concentrations (Figure 4A, compare lanes 7 and 8 with lanes 3 and 4). In the presence of VPN2, the promoter fragment was completely assembled into the DAB complex at all TFIID concentrations (Figure 4A, lanes 9 to 12). At the lowest concentration of TFIID, VPN2 produces an approximately 10-fold increase...
in recruitment of the DAB complex (Figure 4A, compare lanes 10 and 2). The enhanced DAB recruitment by VPN2 correlates with its increased TFIIIB binding.

TFIIB was required for the VPN subdomain to recruit transcription complexes. Formation of a complex containing TFIIID and TFIIA in the absence of activator was measured (Figure 4B, lane 2). Under conditions where GAL4-VP16 resulted in 10-fold increased DA formation (Figure 4B, compare lane 10 with 2), the addition of the activators GAL4-AH (amphipathic helix) (49), GAL4-VPN1 or GAL4-VPN2 results in a less than 2-fold variation in DA assembly (Figure 4B, compare lanes 7 to 9 with lane 2). Consistent with this result, a previous study demonstrated that VP16 and VPC facilitate DA assembly, but VPN does not (16).

VPN recruits a complex containing TBP, TFIIA and TFIIIB (TAB). Since VPN only activated the TAF-independent core promoter (Figure 1), the ability of the VPN module to recruit the TAB complex, which lacks TAFs, was determined. Complex assembly was examined at four concentrations of hTBP. In the absence of activator, the amount of promoter DNA assembled into the TAB complex ranged from approximately 20% at the lowest concentration of TBP to 80% at the highest concentration of TBP (Figure 4C, lanes 2 to 5). When VPN1 and VPN2 were added to the same mixtures, both activators facilitated assembly of the TAB complex (Figure 4C, compare lanes 7 to 10 and 12 to 15 with lanes 2 to 5). Compared with VPN1, VPN2 generated a larger increase in TAB assembly at the three lowest concentrations of TBP (Figure 4C, lanes 12 to 14 compared with lanes 7 to 9). The increased ability of VPN2 (relative to VPN1) to recruit the TAB complex is consistent with the increased association between VPN2 and TFIIIB. This experiment suggests VPN has the potential to stabilize pre-initiation complex formation in the absence of TAFs.

**DISCUSSION**

In this study, it was demonstrated that the VPN and VPC activation domain modules function differently on the TAF-independent ADH1 and TAF-dependent RPS5 core promoters in yeast. VPC activated both TAF-ind and TAF-dep core promoters, which suggests that VPC functions by a TAF-dependent mechanism. In contrast, VPN stimulated transcription from the TAF-independent ADH1 core promoter, but not the TAF-dep one. This suggests that VPN functions by a TAF-independent mechanism. When VPN is dimerized (VPN2), it synergistically activated the ADH1 core promoter relative to VPN1. VPN2 also exhibited a strong synergistic increase in its ability to bind TFIIIB, but not TBP, TAF9 and TFIIA. Taken together, these results are consistent with TFIIIB as a potential target of activators that function by a TAF-independent mechanism.

VPN2 was more proficient than VPN1 at facilitating the formation of a complex containing TFIIID, TFIIA and TFIIB. Neither VPN1 nor VPN2 increased assembly of a complex containing only TFIIID and TFIIA. In contrast, VP16 and VPC are able to recruit the DA complex (11, 16). These results support the idea of at least two different mechanisms by which activators facilitate the formation of TFIIID-containing complexes. Furthermore, the VPN subdomain exhibited the ability to recruit a complex containing TBP:TFIIB, which lacks TAFs. These results indicate that activator contact with TFIIIB can potentially generate a network of interactions that leads to stabilization of the binding of TFIIID or TBP to DNA, and can potentially occur in the absence of TAFs.

**Mechanism of increased TFIIIB binding**

One issue raised by our studies is how two copies of an activation subdomain would increase binding to TFIIIB. TFIIIB contains two direct repeats and the C-terminal ends of each repeat have been identified as VP16 binding sites (50, 51). An activator that contains two regions capable of binding TFIIIB might simultaneously occupy both sites more easily and thus, generate increased stability. In this scenario, additional activation domain modules beyond two would produce only minimal increases in binding. Consistent with this idea, the largest increase in activation potential of RelA was upon dimerization (36) and the binding of GAL4-VPN4 for TFIIIB only increases slightly relative to VPN2 (S. Pyo, R. Hori and M. Carey, unpublished results).

Alternatively, two copies of VPN could facilitate the allosteric change in TFIIIB caused by activators; this allosteric change disrupts an interaction between the N-terminal and the C-terminal domains of TFIIIB (52, 53). The ability to form two (simultaneous) binding interactions might increase an activator’s ability to disrupt this intramolecular association.

**Mechanism of transcriptional activation**

Our study provides evidence that the interaction between VPN and TFIIIB facilitates complex assembly, potentially by tethering TFIIID or TBP indirectly. A TBP mutant that is deficient in its ability to interact with TFIIIB and support transcriptional activation has been identified (54). This defect is dependent on the UAS within the promoter and this suggests that a key step for certain gene activators is the entry of TFIIIB into the pre-initiation complex. However, the topic of activator targets and activator function is controversial. For example, some studies have found a poor correlation between activators binding to TFIIIB and gene activation (29, 55, 56). In contrast, other studies have strongly supported a correlation between the affinity of VP16 for TFIIIB and activation (50, 57, 58). These differences may reflect the properties of TAF-dependent and TAF-independent promoters (25). For example, inactivation of TFIIIB decreases the binding of TBP to TAF-independent promoters, but has little effect on TAF-dependent promoters (23). A TFIIIB mutant that specifically decreases stimulation by certain gene activators, but not others, has also been identified (54, 59).

This study indicates that recruitment of TFIIIB-containing complexes by an activator could lead to more transcription. Mutations in TFIIIB that specifically decrease both its ability to be recruited by activators and transcription in vitro have been identified (50, 53). The ability of p53 to recruit TFIIIB and TBP, but not TAF9, to a promoter correlated with activation in vivo (60). In an in vitro study, TAF-dependent activation required only PC4 and TFIIID to be pre-incubated with activator, while TAF-independent activation required the pre-incubation of PC4, TBP, TFIIIB and RNA polymerase II with activator, (20). This result suggests a role for TFIIIB in a TAF-independent activation pathway.
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