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Characterization of the interaction between the acidic activation domain of VP16 and the RNA polymerase II initiation factor TFIIB

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ABSTRACT

Contact between a transcriptional activator and one or more components of the RNA polymerase II transcription initiation machinery is generally believed important for activators to function. Several different molecular targets have been suggested for direct contact by herpes simplex virus virion protein VP16, including the general initiation factor TFIIB. In this report we have used several strategies to critically assess this interaction between VP16 and TFIIB. Affinity columns of VP16 bound TFIIB activity from HeLa cell extracts and the binding was reduced by mutations in the activation domain of VP16. In assays of direct binding, VP16 bound recombinant human TFIIB but not *Drosophila* or yeast TFIIB. Unlike binding from an extract, however, we found that the interaction between VP16 and recombinant human TFIIB was not affected by mutations in VP16 that reduce transactivation. Point mutations within human TFIIB that reduce transactivation by VP16 have been shown to reduce VP16 binding, but we show here that these same mutations critically affect both the important TBP–TFIIB interaction and the ability of TFIIB to support activator-independent basal transcription *in vitro*. Taken together our results suggest more evidence is needed to support the notion that TFIIB is a functionally important target for the activator VP16.

INTRODUCTION

Transcriptional activator proteins regulate the expression of genes in eukaryotic cells that are transcribed by RNA polymerase II. These activator proteins often contain separable domains, one for site-specific binding of DNA and others for transcriptional activation (1,2). Activation domains function even when attached to the DNA binding domain of a heterologous protein. They are believed to make contact with the RNA polymerase II transcription machinery and effect the assembly and/or the activity of the transcription initiation complex (3,4).

Within the RNA polymerase II transcription initiation complex, a plethora of putative targets has been suggested for direct contact by the activation domains of *cis*-binding positive acting transcription factors. The earliest studies had suggested that the TFIID fraction was a target of several activators (5,6) and both the TATA binding protein (TBP) (7,8) and certain of its associated TAF polypeptides (9,10) have since been shown to be capable of direct binding to an increasingly large number of proteins with diverse activation domains (11–22). *In vitro* interactions of activators with other polymerase II general initiation factors have also been reported. A number of activators bind the initiation factor TFIIB (23–27) and the multi-component factor TFIIH has also been implicated as a target for several activators (28). Furthermore, the potential targets for activators are not limited to just the minimal components required for basal level transcription. In yeast cells the ADA2 protein, identified genetically as a co-activator (29), has been shown to bind to the acidic activation domain of VP16 (30). In mammalian cells another factor, PC4, a component of the USA fraction required for efficient activated transcription *in vitro*, also appears capable of direct activator contact (31). While this multiplicity of potential targets for activators may be indicative of a complex and dynamic exchange of interactions resulting in more transcription initiation by RNA polymerase II (4), evidence supporting a role within cells for some of these interactions detected *in vitro* is either entirely incomplete or is lacking. Furthermore, the concept of an ordered multi-step pathway for the initiation of transcription by RNA polymerase II with the potential for having several rate limiting steps in the formation of the initiation complex accelerated by activators (32,33) has been challenged by evidence that a large multi-component RNA polymerase II holoenzyme complex may pre-exist within cells (34–37).

Work with the acidic activator VP16 from our laboratories provided the first evidence of direct activator–TBP interactions (7,8). Our initial reports, however, seemed to be at variance with very similar experiments suggesting that TFIIB rather than TBP was an important target for VP16 (23,38). Because apparently different results were obtained with ostensibly similar experiments, we have now re-examined the interactions between VP16 and the TFIIB polypeptide from human, *Drosophila* and yeast

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cells. Our results suggest that if TFIIB is indeed an important target of this particular activator, it is so only in mammalian cells. We also show that mutations within the activation domain of VP16 or within the putative target human TFIIB that result in reduced levels of activated transcription either do not affect the VP16–TFIIB interaction or they alter additional important protein–protein interactions within the transcription initiation complex. Taken together, our results suggest that more compelling evidence is still required to support the notion of TFIIB as a target for the activator VP16.

MATERIALS AND METHODS

Protein purification

The bacterially expressed RNA polymerase II initiation factors yeast TBP (39), human TBP (28) and *Drosophila* TBP (40), human TFIIB (41), yeast TFIIB (42) and *Drosophila* TFIIB (43), human TFIIF (44) and the GAL4–VP16 derivatives (45) were prepared as previously described, as were protein A (pA)–VP16 (7) and GST–TFIIB fusion proteins (46). The human TFIIB mutants R185E/R193E and K198E/K200E (46) (kindly provided by D. Reinberg), along with wild-type human TFIIB, were expressed in BL21 (DE3) cells with a 10 histidine N-terminal tag after subcloning TFIIB cDNAs into pET19b (Novagen) and purified on Ni^{2+}–NTA columns (15). Human TFIIB proteins were further purified on a 0.5 ml heparin column (16). All purified proteins were dialyzed against affinity chromatography buffer (7) containing 0.1 M NaCl and stored at –70°C. Highly purified calf thymus RNA polymerase II (47) was kindly provided by M. Sopta. HeLa nuclear extract fractions (48) were kindly provided by D. Fitzpatrick and heat-inactivated nuclear extract was prepared as previously reported (49).

Affinity chromatography

Affinity chromatography columns of pA and pA–VP16 derivatives were prepared and used as previously described (7).

In vitro transcription assay

In vitro transcription assays using as template DNA a G-less reporter cassette driven by the adenovirus 2 major late (Ad2ML) promoter were performed essentially as described previously (7,28).

Electrophoretic mobility shift assay

TFIIB–TBP complex formation between yeast TBP and human TFIIB proteins was analyzed by an electrophoretic mobility shift assay (50). A ^{32}P-labeled probe containing the TATA element of the Ad2ML promoter from –53 to +33 (a gift from B. Coulombe) was used. DNA binding reactions were performed for 30 min at 30°C and resolved on a 4% polyacrylamide gel in Tris–glycine buffer (50) lacking EDTA.

In vitro translation of [^{35}S]methionine-labeled proteins

^{35}S-Labeled GAL–VP16 proteins were synthesized by a coupled transcription–translation procedure or by transcribing RNA from a T7 promoter and then using this RNA for *in vitro* translation reactions in a rabbit reticulocyte lysate system (Promega).

In vitro protein binding assay

Human TFIIB fused to GST was bound to glutathione–Sepharose beads at 0.05, 0.5 and 2 mg/ml in buffer A [20 mM Tris–HCl, 0.2 mM EDTA, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride, 0.5 mM benzamidine hydrochloride and 28 µM tosyl-phenylalanine chloromethyl ketone] containing 1 M NaCl. GST–TFIIB-coupled beads were first equilibrated three times in 10 vol buffer B (40 mM HEPES, pH 7.9, 5 mM $MgCl_2$, 1 mM DTT, 0.5% NP-40 and 100 or 150 mM KCl) for 1 h. Aliquots of 20 µl of these beads were incubated with the ^{35}S-labeled GAL4–VP16 proteins in 80 µl buffer B containing 100 or 150 mM KCl for 1 h and finally washed three times with 200 µl buffer B. The beads were subsequently boiled in gel sample buffer and the eluates were resolved by SDS–PAGE and monitored by a phosphoimager (Bio-Rad) or by autoradiography.

Transcription initiation assay

Initiation of transcription was performed on a synthetic template containing Ad2ML promoter sequence with a single-stranded bubble from nucleotides –9 to +3 (51). The template was incubated with wild-type or mutant human TFIIB, yeast TBP, human TFIIF, calf thymus RNA polymerase II, [^{32}P]CTP and the primer dinucleotide CpA for 25 min at 23°C. RNA trimers were analyzed on an 18% polyacrylamide denaturing gel containing 7 M urea as previously described (51).

RESULTS

VP16 affinity columns retain both human TFIID and TFIIB

The first experiments identifying targets amongst the RNA polymerase II initiation factors contacted by the potent acidic activator VP16 implicated both TFIID and TFIIB as potential targets. In one set of experiments, TFIID activity was depleted by passage of HeLa cell nuclear extracts competent for *in vitro* transcription over columns of immobilized VP16 (7); in another TFIIB activity was depleted (23). The ability of these affinity columns of VP16 to quantitatively remove any particular initiation factor may depend upon salt and ligand concentrations, upon the bed volume of the columns and also upon the relative levels of particular initiation factor activities, which undoubtedly vary amongst different extract preparations. Rather than assess whether initiator factors are depleted by chromatography of an extract over VP16, we have now monitored the ability of wild-type and mutant VP16 columns to retain a portion of either TFIIB or TFIID activity. TFIIB activity in column eluates was assessed in a reconstituted *in vitro* transcription system using partially fractionated HeLa cell components. TFIID activity was assessed using heat-treated (TFIID-deficient) HeLa nuclear extracts (49). As shown in Figure 1 (lanes 1–3), these assay systems show a near complete dependence upon the addition of either TFIIB or TFIID (TBP). A comparison of lanes 4 and 7 indicated that the eluate from the wild-type pA–VP16 column, but not the control pA column, contained both TFIIB and TFIID activities. Consistent with results reported earlier by Lin and Green for TFIIB (23) and Ingles *et al.* (8) for the TBP component of yeast TFIID, the ability of VP16 to bind TFIIB or TBP was reduced by mutations in the activation domain of VP16. Truncation of the activation domain to amino acid 456 reduces transactivation by VP16 to ~50% when assessed in murine cells with the herpes virus ICP4 promoter (52) and to an undetectable

level when measuring GAL4–VP16 activation with a reporter having only a single GAL4 binding site (53). Within the context of a full-length activation domain, substitution of Phe with Pro at position 442 moderately reduces transactivation by VP16 (54). When combined with the truncation to position 456, this FP442 mutation completely inactivates transactivation by VP16 (52,53). As lanes 5, 6 and 8 of Figure 1 indicate, the ability of these mutant forms of pA–VP16 to retain either TFIIB or TFIID activity was very similar. Truncation to position 456 markedly affected binding of both factors, as did the Phe→Pro mutation at 442 in full-length VP16. In the context of truncated VP16, the FP442 mutation completely prevented retention of either factor. This correlation between transactivation activity *in vivo* and initiation factor binding *in vitro* argues that these interactions could be important during the activation of transcription. These experiments do not, however, address whether these activator–initiation factor interactions are direct or are mediated by one or more of the components present in the HeLa cell extracts loaded on these columns.

Human TFIIB, but not *Drosophila* or yeast TFIIB, can bind directly to VP16

To examine whether the retention of human TFIIB by immobilized VP16 was the result of a direct interaction between VP16 and TFIIB, human TFIIB was produced in and purified from *Escherichia coli* cells (41). For these affinity chromatography experiments we used truncated derivatives of the activation domain of VP16; the effect of mutations at position 442 on transcription being more marked in this context. When the concentration of ligand VP16 on the columns was >2.0 mg/ml a significant portion of the recombinant human TFIIB was retained by the columns. The 0.1 M NaCl wash fractions eluted some of the TFIIB, but the majority of applied TFIIB was only eluted by the 0.6 M NaCl step (E in Fig. 2A). While the mutations in VP16 that reduced transactivation did markedly affect the recovery of TFIIB when unfractionated HeLa cell extracts were applied to the columns (Fig. 1), these mutations in VP16 had no effect on the binding of purified recombinant human TFIIB. The data shown in Figure 2A indicates that the VP16 derivative Δ456-FP442, which is completely defective in transactivation and failed to interact with TBP (8), bound human TFIIB just as well as did the matrix made with the corresponding wild-type but truncated derivative, a result similar to that reported by Goodrich *et al.* (10). The effect of other missense mutations at the 442 position is similar. Changes from Phe to Ala or Ser, which markedly compromised TBP binding (8) and the transactivation potential of VP16 (52), had no effect on the binding of human TFIIB (data not shown, but see Fig. 3). In the context of a full-length activation domain, the mutations at position 442 were also without effect on the binding of recombinant human TFIIB (data not shown).

Since the activation domain of VP16 functions as a potent activator of transcription in a variety of eukaryotic species, we also asked if VP16 would bind purified recombinant *Drosophila* or *Saccharomyces cerevisiae* TFIIB. As indicated in Figure 2A, however, only a very minor portion of the applied *Drosophila* or yeast TFIIB was retained by VP16. The amount of TFIIB bound was equivalent to that bound to the control pA matrices (data not shown) and this residual binding was unaffected by the FP442 mutation in VP16. When these binding experiments were conducted at either ligand concentrations greater than that required to bind human TFIIB or lower ionic strength, somewhat more of the *Drosophila* and yeast TFIIB bound to VP16, but this binding was still unaffected by the FP442 mutation (data not shown).

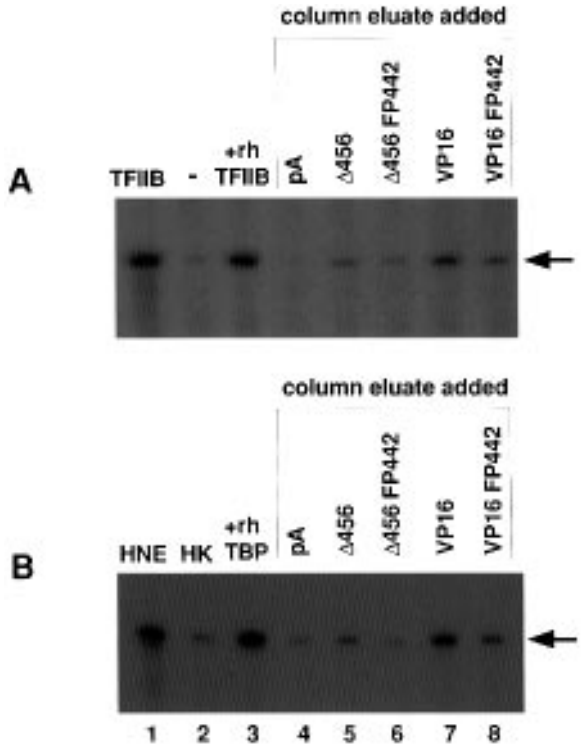

Figure 1. Both TFIIB and TFIID activities in HeLa cell extracts interact with the activation domain of VP16. The ability of wild-type and mutant forms of the activation domain of VP16, expressed as pA fusions and covalently coupled to Affi-Gel 10 matrices at 1.2 mg/ml, to retain either TFIIB or TFIID activities present in a HeLa cell extract was assessed by monitoring transcription initiated at the Ad2ML promoter on plasmid pML(C2AT)-50 in *in vitro* transcription systems that showed a dependence on addition of either TFIIB (**A**) or TFIID (**B**). HeLa whole cell extract (2.7 ml) (7) was chromatographed on 0.07 ml affinity columns containing pA, pA–VP16-Δ456, pA–VP16-Δ456-FP442, pA–VP16 (wild type) or pA–VP16-FP442 (lanes 4–8 as indicated). TFIIB activity (A) was assessed by adding either 2 µl of a human TFIIB fraction (lane 1), 10 ng recombinant human TFIIB (lane 3) or 4 µl aliquots of the 0.57 M KCl eluates of the different columns to a mixture containing the partially purified HeLa cell fractions, TFIIA, TFIID and TFIIE/F/H and calf thymus RNA polymerase II. TFIID activity (B) was assessed by supplementing a heat-treated TFIID-deficient HeLa nuclear extract (49) with either 25 ng recombinant human TBP (lane 3) or 8 µl aliquots of the different column eluates. The dependence of these two transcription systems on added TFIIB or TFIID activity is shown (lane 2) in (A) and (B) respectively. Arrows indicate the position of the specifically initiated transcript.

These preparations of purified recombinant TFIIB from different species appear equally active in other assays. First, we assessed the ability of these recombinant TFIIB polypeptides to form a complex in a TBP-dependent fashion on an oligonucleotide containing a TATA box. An electrophoretic mobility shift assay (EMSA) with ^{32}P-labeled Ad2ML promoter DNA was used (50). Consistent with results reported by others, we observed that TFIIB from each of these species can form a 'DB' complex with the yeast TBP polypeptide (Fig. 2B). Titrations with increasing quantities of each TFIIB preparation indicated that there were no significant differences in the relative activities of

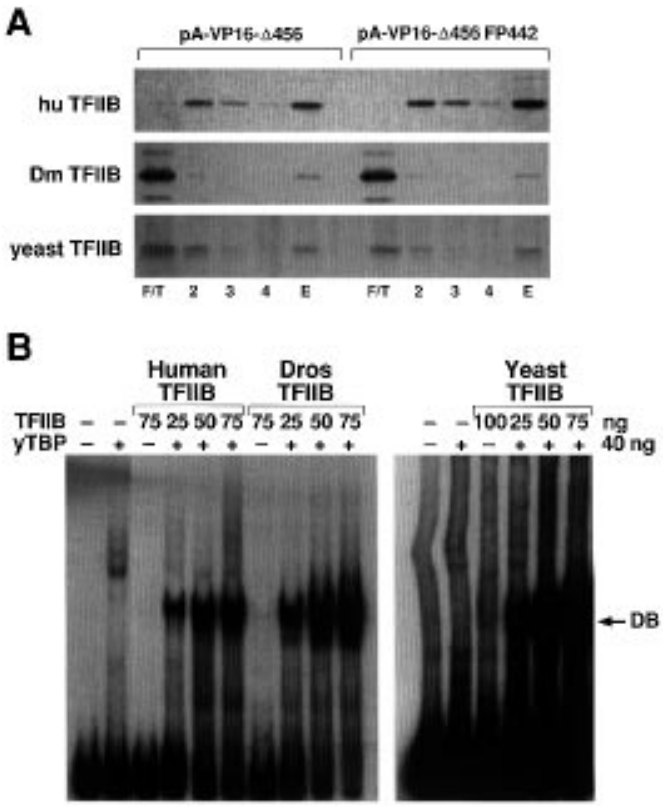

Figure 2. (**A**) Both wild-type and mutant forms of the activation domain of VP16 bind directly to human TFIIB but not to *Drosophila* or yeast TFIIB. Recombinant forms of human, *Drosophila* and yeast TFIIB (2 µg) were chromatographed on 10 µl affinity columns of wild-type but truncated (pA–VP16-Δ456) and mutant (pA–VP16-Δ456-F442P) matrices coupled to a ligand concentration of 3 mg/ml. Equivalent aliquots of the flow-through (F/T), 0.1 M NaCl wash fractions (2–4) and the 0.6 M NaCl eluate fractions (E) were analyzed by SDS–PAGE and silver-staining. (**B**) Recombinant forms of human, *Drosophila* and yeast TFIIB are equally active in forming a TFIIB–TBP (DB) complex. An EMSA was used to assess the ability of the indicated amounts of the different TFIIB preparations to bind with yeast TBP to a ^{32}P-labeled oligonucleotide containing the Ad2ML promoter TATA box.

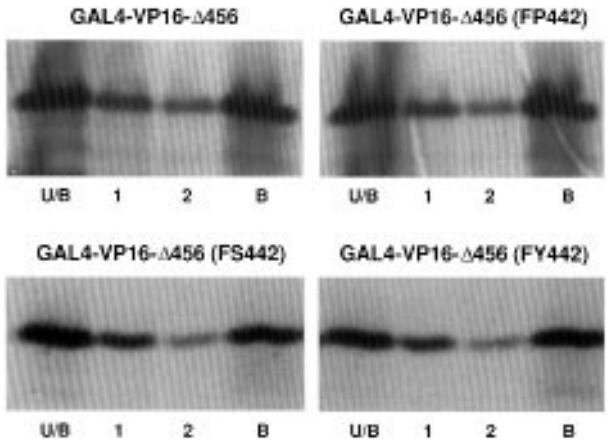

Figure 3. Immobilized GST–human TFIIB binds equally well to wild-type and mutant forms of VP16 expressed as GAL4–VP16 fusion proteins. GST–TFIIB was bound to glutathione–Sepharose beads at 0.5 mg/ml and 20 µl aliquots of these beads were then incubated with the indicated *in vitro* translated [^{35}S]methionine-labeled GAL4$^{1–96}$–VP16 derivatives. Fifty percent of the unbound protein (U/B), 25% of each of two washes with buffer containing 100 mM NaCl and all of the bound protein (B) were resolved by SDS–PAGE and analyzed by autoradiography.

each of these polypeptides in this assay system. Secondly, the recombinant human and *Drosophila* TFIIB preparations were also found to be active in reconstituted *in vitro* transcription assays, with 10 ng of each protein being sufficient to support comparable levels of activator-independent transcription in a HeLa cell-derived system (data not shown).

Binding of GAL4–VP16 to immobilized TFIIB

As the mutations in VP16 at position 442 had no effect on the ability of immobilized pA–VP16 to directly bind human TFIIB, we decided to examine these interactions another way. Since a direct interaction between human TFIIB and the activation domain of VP16 has also been detected when the TFIIB protein rather than VP16 is immobilized (46), we purified a similar GST–human TFIIB chimeric polypeptide and bound it to glutathione–Sepharose beads at several ligand concentrations. The GST–TFIIB-containing beads were then incubated with ^{35}S-labeled GAL4–VP16 derivatives, washed and finally TFIIB-bound GAL4–VP16 was eluted by boiling in gel sample buffer and analyzed by SDS–PAGE. A ligand concentration of 0.5 mg/ml GST–TFIIB was required to obtain significant GAL4–VP16 binding. With this ligand concentration ~30% of the input GAL4–VP16 Δ456 was bound by GST–TFIIB. We then tested the effects of various mutations in VP16 at amino acid 442. As shown in Figure 3, the Phe to Pro or Ser changes, which completely inactivate the transactivation potential of VP16 (52), had no effect whatsoever on the binding of GAL4–VP16 to immobilized human TFIIB. The conservative FY442 change, which permits a moderate level of transactivation (52), also had no effect on binding of GAL4–VP16. Increasing the salt concentration to 0.15 M reduced the binding of these GAL4–VP16 derivatives to GST–TFIIB, but still no effect of the mutations in VP16 was evident (data not shown). The lack of effects of these mutations in VP16 on binding to immobilized human TFIIB is consistent with the data presented in Figure 2A using immobilized VP16.

Pleiotropic effects of mutations in the VP16 binding domain of TFIIB

A VP16-interacting region of human TFIIB has been shown by analysis of deletion clones of human TFIIB to map between amino acids 178 and 201 (46). This same region of TFIIB has, however, also been shown in several studies to be important for interaction with TBP (55–57). Missense mutations in this region of TFIIB have been shown to affect binding to VP16 (46). We have now carefully analyzed the effects of two different double point mutations in human TFIIB used in these earlier studies. As reported by Roberts *et al.* (46), we too found that recombinant human TFIIB proteins containing either the R185E/R193E or K189E/K200E mutation were defective in binding to VP16 (data not shown). However, since other charge-change mutations in this region of the *Drosophila* TFIIB protein compromised the interaction between TBP and TFIIB (56) and crystallographic studies of a TFIIB–TBP–DNA complex indicate that this region

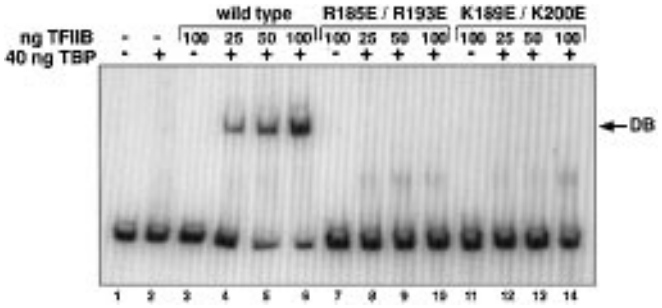

Figure 4. Mutations in the human TFIIB protein that compromise the response to the activation domain of VP16 are also defective in forming a TFIIB–TBP complex on promoter DNA. EMSAs with the indicated proteins were used to assess the formation of a complex of wild-type (lanes 3–6) and mutant forms of human TFIIB (lanes 7–10 and 11–14) with the yeast TBP polypeptide on a ^{32}P-labeled oligonucleotide containing the Ad2ML promoter TATA box sequence.

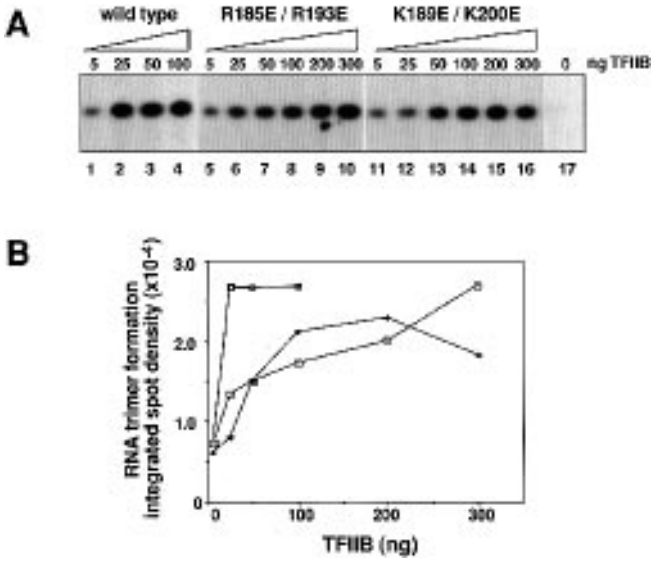

Figure 5. The R185E/R193E and K189E/K200E mutations in human TFIIB affect activator-independent initiation of transcription. Increasing quantities of wild-type (lanes 1–4) and mutant TFIIB protein (lanes 5–16) were used with calf thymus RNA polymerase II, yeast TBP and human TFIIF in assays of abortive initiation (51) on a template containing the Ad2ML promoter sequence with a mismatched bubble from nucleotides –9 to +3 (51). The ^{32}P-labeled RNA trimers were resolved by 18% urea–PAGE, analyzed by autoradiography (**A**) and then quantitated (**B**) by phosphoimaging. Wild-type TFIIB, ; R185E/R193E TFIIB, □; K189E/K200E TFIIB, ♦.

of TFIIB contacts TBP (57), we also assessed the ability of these particular mutant forms of human TFIIB to interact with TBP. As assessed by formation of a TFIIB–TBP complex on Ad2ML promoter DNA (Fig. 4), addition of as much as 100 ng of each of these mutant forms of TFIIB failed to make a stable complex with TBP. In contrast, formation of a readily detected TFIIB–TBP complex on DNA was possible with as little as 25 ng of wild-type TFIIB.

These double mutations, R185E/R193E and K189E/K200E, in human TFIIB were reported to be selectively defective in activated but not basal transcription (46). In view of our observation that the TBP–TFIIB interaction was compromised by these particular TFIIB mutations and the general belief that a TBP–TFIIB interaction is important for initiating transcription by RNA polymerase II (41,50), we next assessed the ability of these mutant forms of human TFIIB to function in transcription initiation. For these experiments we used a very sensitive assay of transcription initiation, quantitating the formation of RNA trinucleotides on an Ad2ML promoter template containing a DNA mismatch bubble from nucleotide positions –9 to +3 (51). Synthesis of RNA trimers in this system is dependent only upon addition of RNA polymerase II, TBP and TFIIB and is stimulated by TFIIF. As shown in Figure 5, addition of 25 ng of the wild-type TFIIB polypeptide permitted a maximal level of transcription. With the R185E/R193E and K189E/K200E mutant forms of human TFIIB, however, addition of between 100 and 300 ng of TFIIB was required to approach similar levels of transcript initiation. These data, taken together with those reported by Roberts *et al.* (46), indicate that the effects of these particular mutations on TFIIB function are multiple. An interaction with VP16 and the response to this activator may well be compromised, but so too is the important interaction between TFIIB and TBP. As a result, both the basal level, activator-independent transcription and activator-dependent transcription appear to be adversely affected by mutation of this TBP- and VP16-interacting region of TFIIB.

DISCUSSION

The experiments described in this report were undertaken because we felt it was important to more carefully assess the different conclusions reached with the rather similar experimental protocols used by Stringer *et al.* (7) and Lin and Green (23) in the identification of targets of the acidic activation domain of the herpes simplex transactivator VP16. We now have shown, in agreement with the initial report of Lin and Green (23), that TFIID and TFIIB activities can both be retained by affinity columns of immobilized VP16. The retention of both these initiation factors was markedly reduced by several mutations in the activation domain of VP16 that reduce its transactivation potential. Whether TFIID or TFIIB is quantitatively depleted by passage over VP16 may simply be a reflection of the particular chromatographic conditions (e.g. ligand and salt concentrations and column volumes) and the relative concentration of each factor in different extract preparations. The binding of these initiation factors to immobilized VP16 need not be direct, however. Documented factor–factor interactions (58) and the recent reports of the existence of polymerase II holoenzyme complexes containing many of the polymerase II initiation factors (34–37) raise the possibility that certain activator–initation factor interactions could indeed be indirect.

To examine this issue, different polymerase II initiation factors have been either highly purified or expressed as recombinant proteins. Our experiments with recombinant human TFIIB largely confirm the observations of Lin *et al.* (38). Immobilized VP16 bound TFIIB and, as reported by Roberts *et al.* (46), immobilized TFIIB can bind the chimeric activator GAL4–VP16, albeit only at ligand concentrations considerably higher than the 0.05 mg/ml reportedly used by these authors. In one crucial aspect, however, our results differ in a substantive way. While Lin *et al.*, using glutathione–Sepharose-bound GST–VP16 matrices, did see reduced binding of recombinant

human TFIIB to a single VP16 mutant (38), we found, under a variety of salt and ligand concentrations with both pA derivatives (Fig. 2A) and GST derivatives (data not shown), that not only this mutation but others in the activation domain of VP16 at the critical Phe442 residue did not alter the strength of the direct interaction of VP16 with recombinant TFIIB. Our results are similar to those reported by Goodrich *et al.* (10) for the FP442 mutation of VP16 when they examined the interaction between recombinant human TFIIB and glutathione–Sepharose-bound GST–VP16 derivatives. In this respect we also note that mutations at several other positions within the C-terminal region of the activation domain of VP16 which contribute to inactivation of the transactivation potential of VP16 were reported by Walker *et al.* (53) to be without effect on the binding of human TFIIB present in a nuclear extract to covalently coupled GST–VP16–Sepharose matrices. The lack of effect of these VP16 mutations on the direct interaction with TFIIB contrasts with the marked effects of these same mutations on the binding of VP16 to recombinant TBP (8) and TFIIH (28). A correlation between the effects of mutations within a second acidic activation domain, that of the yeast activator GAL4 and binding to yeast TBP has also been reported (59). GAL4, like VP16 (see Fig. 2A), was reported not to bind yeast TFIIB (59).

Correlations between transactivation potential of wild-type and mutant transactivation domains and the strength of interaction between an activator and its targets can help establish the biological relevance of activator–initiation factor contact. In addition, mutations within the putative target(s) within the polymerase II initiation complex may, if their effects are limited to just the activator–factor interaction, be used to establish the importance of contacts of activators with their putative targets. Such mutations within both TBP (60) and TFIIB (46) have been described. These mutations reportedly diminish interaction with the activator VP16 and compromise VP16-activated, but not activator-independent, basal transcription *in vitro*. Close examination of the published data detailing the selective effect of several missense mutations in human TFIIB on activated transcription reveals, however, that the mutant forms of human TFIIB may not have been equivalent to wild-type TFIIB in supporting basal transcription (46). In particular, it seems that with 5 ng of wild-type TFIIB transcription was maximized and that with comparable amounts of the mutant K189E/K200E form of TFIIB equivalent levels of transcription were not attained. The data shown in Figure 5 in this report explores this finding in greater detail. While additions of as little as 25 ng of wild-type TFIIB supported a maximal rate of transcript initiation in our reconstituted system, additions in the range 100–300 ng of the same two mutant forms of TFIIB used by Roberts *et al.* (46) were needed to approach wild-type levels of transcript initiation in our system. As studies from several laboratories had already indicated, the region of TFIIB believed to interact with VP16 is also important for interaction of TFIIB with TBP (55–57). We have now shown that these same double point mutations in human TFIIB are defective in binding to a TBP–DNA complex, a result that could account for the effects of these mutations on basal transcription and which may also explain the crippled response to the activator VP16.

How then is transcription activated and just which are the important interactions between transcriptional activators and the polymerase II machinery? Several lines of evidence (reviewed in 61) suggest that an important aspect of activator function is to promote assembly of the polymerase II transcription initiation complex at a promoter. In particular, some experiments have shown that the VP16 activation domain can facilitate the recruitment of TFIIB into the assembling pre-initiation complex (23,32,60). It is unclear whether this recruitment of TFIIB is a consequence of the interaction of VP16 with TFIIB or with TBP (32,60). Although a multi-step assembly pathway, with the opportunity of having one or more rate limiting steps in assembly be accelerated by activators, was for many years an attractive model (32,33), it now appears that much if not all of the polymerase II initiation machinery could exist as a pre-assembled holoenzyme (34–37). Contact with one or more components of such a polymerase II holoenzyme may help bring polymerase II to the promoter in a single step. Recent experiments with yeast cells bearing a mutation within one component of the holoenzyme, GAL11, argue persuasively for this view (62). Other studies have suggested that, for certain promoters, activators can function by promoting recruitment of TBP to the promoter (63–65). In this respect it is interesting to note that TBP was initially reported to co-purify with the polymerase II holoenzyme (34). Although subsequent preparations of the yeast polymerase II holoenzyme did not contain TBP, it is not yet clear whether the absence of either TBP, or certain other of the polymerase II initiation factors, reflects the *in vivo* situation or is a consequence of the purification procedure. Since both TFIIB and TFIIH are present within at least one preparation of yeast holoenzyme (35), the contacts between the activator studied in this report, VP16, and either of these initiation factors could lead to holoenzyme recruitment at promoters. Our data, however, suggests that if VP16 does indeed contact the factor TFIIB within cells, then this contact differs from the contact between VP16 and either TBP (8) or TFIIH (28) in being insensitive to mutations in VP16 that compromise transactivation. Since mutations within TFIIB that reduce the transactivation response to VP16 also critically affect other important functions of this initiation factor, it may be premature at this time to conclude that direct contact of activators with TFIIB is an important aspect of the transactivation process. It should be noted, however, that the *in vivo* relevance of direct activator–TBP contact has also been questioned recently. The ability of TBP to interact with activation domains *in vitro* was not required for TBP to support activated transcription *in vivo* (66). If contact between an activator and virtually any subunit of holoenzyme can lead to recruitment of RNA polymerase II to the promoter and transcriptional activation (62), there may indeed be multiple contacts between a strong activator and the transcription apparatus and no one particular contact may be essential for activation. It seems that if the molecular details of the mechanism of transactivation are going to be securely established, then perhaps the application of new and quite different experimental approaches may be required.

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