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Binding of Basal Transcription Factor TFIIH to the Acidic Activation Domains of VP16 and p53

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Acidic transcriptional activation domains function well in both yeast and mammalian cells, and some have been shown to bind the general transcription factors TFIID and TFIIB. We now show that two acidic transactivators, herpes simplex virus VP16 and human p53, directly interact with the multisubunit human general transcription factor TFIIH and its *Saccharomyces cerevisiae* counterpart, factor b. The VP16- and p53-binding domains in these factors lie in the p62 subunit of TFIIH and in the homologous subunit, TFB1, of factor b. Point mutations in VP16 that reduce its transactivation activity in both yeast and mammalian cells weaken its binding to both yeast and human TFIIH. This suggests that binding of activation domains to TFIIH is an important aspect of transcriptional activation.

Accurate transcription in vitro by human RNA polymerase II involves the general transcription factors TFIIA, TFIIB, TFIID, TFIIE, TFIIF (RAP30 and RAP74), TFIIH (also known as BTF2), and TFIIJ (reviewed in references 13 and 121). Beginning with TFIID, which recognizes the TATA boxes present in many promoters for RNA polymerase II, these factors and RNA polymerase II can be assembled in a defined order onto a promoter (5). TFIIH and TFIIJ are the last of these factors to bind to an assembling initiation complex (12, 14, 26), and TFIIH, also known as BTF2, is the only factor known to possess associated enzymatic activities. These include an ATP-dependent DNA helicase activity (95, 96) and a protein kinase activity that can phosphorylate the carboxy-terminal heptapeptide repeat domain (CTD) of the largest subunit of RNA polymerase II (12, 21, 68). Evolutionarily related general initiation factors are utilized in the yeast *Saccharomyces cerevisiae*, and the yeast counterpart of human TFIIH was originally called factor b (21, 29).

Regulation of transcription by RNA polymerase II involves DNA-binding activator proteins, negative regulators, and coactivators (reviewed in references 91 and 121). Activator proteins often contain separate domains for site-specific DNA binding and transcriptional activation. Activation domains, which function even when attached to the DNA-binding domain of a heterologous protein, interact with the basal transcription machinery via DNA looping (reviewed in references 32, 80, and 81). Activation domains vary greatly in amino acid composition and sequence and include many that are highly acidic, others that are glutamine rich, and some that are proline rich (73). Acidic activation domains were originally identified in the yeast activators GCN4 (47) and GAL4 (69) and are also found in the C-terminal 78 amino acids of the herpes simplex virus transactivator VP16 (107) and in the N-terminal 73 amino acids of the mammalian tumor suppressor protein p53 (23). The p53 protein has a site-specific DNA-binding domain in its carboxy-terminal portion (101). VP16 does not, by itself, bind specifically to DNA, but instead its amino-terminal portion binds DNA in association with mammalian factors, including the POU homeodomain protein Oct-1 that recognizes octamer sequences in DNA (28, 60, 102). When fused to the DNA-binding domain of GAL4, the VP16 and p53 activation domains stimulate transcription in yeast and human cells of a gene bearing GAL4-binding sites (9, 16, 23, 74, 87, 92). These observations imply that common mechanisms for transcriptional activation by acidic activators may exist in fungi and mammals.

Many activation domains have been shown to interact with the TATA-box-binding protein (TBP) subunit of TFIID. These include the highly acidic activation domains in VP16 (50, 103), p53 (10, 67, 72, 84, 97, 108), c-Myc (39), v-Rel and c-Rel (55, 118), and E2F-1 (37), as well as other kinds of activation domains found in the adenovirus activator E1A (48, 62), the Epstein-Barr virus proteins Zta (64) and R (70), the human T-cell leukemia virus type 1 (HTLV-1) activator Tax1 (8), the transactivator Tat of human immunodeficiency virus type 1 (HIV-1) (53), and human c-Fos and c-Jun (85), PU-1 (38), and Sp1 (20). In certain cases, reduced binding of TBP by activation domains with point mutations that reduce transactivation in vivo has provided evidence for the biological relevance of these interactions (8, 50, 53, 62). The TBP-associated factors (TAFs) in the TFIID complex are required for transcriptional activation (82), and particular TAFs have also been shown to bind Sp1 (46) and VP16 (30). Although the ability to bind TAF40 has been attributed to a particular subdomain of VP16 (30), point mutations in the activation domain of VP16 that simultaneously affect both transcriptional activation and binding to TAFs have not yet been described.

Several transcriptional activation domains have also been shown to bind TFIIB directly. These include activation domains in VP16 (30, 66), Epstein-Barr virus R (70), and human c-Rel (118) and CTF (57), as well as COUP-TF and other

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members of the nuclear receptor superfamily (1, 49). Consistent with these observations, activator proteins characteristically affect early steps in the assembly of the preinitiation complex. Some experiments suggest that acidic activators accelerate a slow step involving TFIID and TFIIA (112, 114). Other experiments indicate that a direct interaction of the activator with TBP and/or TFIIB leads to recruitment of TFIIB into the preinitiation complex (11, 57, 65, 66, 105). When the DNA template contains multiple activator-binding sites, acidic activators also stimulate a subsequent step in the assembly of the preinitiation complex involving recruitment of the other general initiation factors and RNA polymerase II (11). However, none of these studies on the mechanism of activation exclude the possibility that transactivation can also affect a later step in transcription by RNA polymerase II, such as open-complex formation or chain elongation. Indeed, several types of mutations in VP16 that reduce transcriptional activation also reduce open-complex formation (51). Moreover, the *Drosophila* heat shock factor prevents pausing by RNA polymerase II about 20 to 40 bp downstream of the hsp70 initiation site (86) and both VP16 and the Tat protein of HIV-1 increase the processivity of chain elongation by RNA polymerase II (52, 61, 71, 119). Here we show that two acidic activators, VP16 and p53, interact directly and analogously with human TFIIH and its yeast counterpart, factor b. This interaction could be important for a late step in the pathway leading to production of a transcript by RNA polymerase II.

MATERIALS AND METHODS

Protein purification. Bacterially expressed human TBP was purified as previously described (45) with modifications. Strain BL21 containing plasmid pET27 (79) was grown in 2× YT medium at 37°C to an optical density at 600 nm of 0.5. The expression of human TBP was induced with 0.4 mM isopropyl thiogalactoside at 30°C for 30 min. Then 15 g of cells was harvested by centrifugation for 20 min at 4,000 rpm and stored frozen at −70°C. The frozen cell pellets were thawed and ground with 22.5 g of alumina. The mixture was resuspended in 22.5 ml of HEMGN buffer containing 0.3 M KCl. The alumina and cell debris were removed by centrifugation at 40,000 rpm in a Beckman TI70 rotor for 2 h. HEMGN buffer was added to the supernatant to adjust its KCl concentration to 100 mM. Then 75 ml of diluted supernatant was loaded on a 125-ml DEAE-Sepharose column (Pharmacia) which was preequilibrated with HEMGN buffer containing 100 mM KCl. Flowthrough and wash fractions were collected as 50-ml fractions. Fractions (200 ml) containing the human TBP were loaded onto a 6-ml heparin–Sepharose CL-6B (Pharmacia) column preequilibrated with HEMGN buffer containing 100 mM KCl. The column was washed with 120 ml of HEMGN buffer containing 100 mM KCl and then eluted with a 120-ml gradient from 0.2 to 1 M KCl in HEMGN buffer. Fractions of 3 ml each were collected. The TBP was monitored by Western immunoblotting with a cross-reacting antiserum raised against yeast TBP. The peak fractions were pooled and dialyzed against 0.1 M KCl in nuclear dialysis buffer (buffer D [98]). The aliquots were stored at −70°C.

Recombinant human RAP74 was expressed in strain BL21 and purified as previously described (24). Purification of RAP74 was monitored by Western blotting with an antiserum against RAP74. The peak fractions were dialyzed against 0.1 M KCl in buffer D and stored at −70°C. Recombinant TFIIEα and TFIIEβ were purified as previously described (78). Recombinant RAP30 was purified as previously described (24). Highly purified calf thymus RNA polymerase II (99) was provided by S. McCracken. Recombinant p62 (plasmid pET11a-BTF2; generously provided by R. Roy and J.-M. Egly) was produced in a strain carrying plasmid pET11a-BTF2. The procedure used for purification of p62 was similar to that used for purification of the human TBP described above, except that the cells were grown in 2× YT medium to an optical density at 600 nm of 0.5 at 30°C and induced with 0.4 mM isopropylthiogalactopyranoside for 3 h. The DEAE-Sepharose flowthrough fraction was dialyzed against ACB buffer containing 0.1 M NaCl.

The TFIIA/J (104) and the TFIIH–mono-S fraction (26) were purified as previously described. To purify TFIIH by VP16 chromatography, 80 ml of HeLa whole-cell extract was chromatographed as previously described (103) on a 4-ml affinity column containing the acidic activation domain of VP16 (amino acids 412 to 490) fused to glutathione *S*-transferase (GST-VP16) (4 mg/ml). Bound proteins were eluted with 16 ml of 1 M NaCl in ACB buffer and dialyzed against 2 liters of buffer C (89). To further purify this TFIIH, 15 ml of bound proteins in 0.1 M KCl buffer C was loaded onto a 1-ml phosphocellulose column (89). The column was washed with 0.1 M KCl in buffer C and step eluted with 5 ml each of 0.3, 0.5, and 0.85 M KCl in buffer C. Eluted fractions (1 ml each) were dialyzed against 0.1 M KCl in buffer D (98). The 0.85 M KCl eluate was found to have most of the TFIIH activity, and no other general transcription factors were detected by activity assay and in the cases of TBP and TFIIB, also by Western blotting (data not shown). This chromatographic behavior on phosphocellulose of VP16-bound TFIIH was identical to that previously described for TFIIH (26). The peak fractions eluted with 0.85 M KCl were dialyzed against 0.1 M KCl in buffer D, and 20 μl was loaded on 20-μl GST or GST-VP16 affinity columns (4 mg/ml) under identical conditions. The columns were washed with 20 μl of 0.1 M KCl in buffer D, and 40 μl was collected as the flowthrough fraction. The columns were washed again with 180 μl of 0.1 M KCl in buffer D and eluted with 80 μl of 1 M KCl in buffer D. The eluted fractions were dialyzed against 0.1 M KCl in buffer D before being assayed for TFIIH activity.

Affinity chromatography. The GST, GST-VP16, GST-VP16^{FP442}, GST-VP16$^{\Delta 456}$, GST-VP16$^{\Delta 456FP442}$ and GST-p53 (4-mg/ml each) affinity columns were prepared by immobilizing GST or GST fusion proteins on glutathione-Sepharose beads as previously described (65). The affinity columns coupled with PA-VP16, PA-VP16^{FA442}, PA-VP16^{FP442}, PA-VP16^{FA442FP473}, PA-VP16$^{FA473/475}$, PA-VP16$^{FA442/475}$, or PA-VP16$^{FA442/473/475}$ (3 mg/ml each) were prepared as previously described (103). For Fig. 5a, the GST-p53 column and control GST column were prepared as previously described (108). Affinity chromatography was performed as previously described (103). However, bound proteins were usually eluted with ACB buffer containing 1 M NaCl except when indicated otherwise. Eluates used in transcription reactions were dialyzed against 0.1 M KCl in buffer D.

Antibodies and Western blotting. Monoclonal antibody M.Ab3c9 (25) against p62 of BTF2 was provided by J.-M. Egly. Anti-yeast TFB1 serum (22) was provided by J. Feaver and R. Kornberg. Western blotting was performed with an alkaline phosphatase-coupled goat anti-rabbit or anti-mouse immunoglobulin G as described by the manufacturer (Bio-Rad). For Fig. 4c and 5d, the ECL Western blotting detection system (Amersham) was used.

In vitro transcription. Transcription in vitro on linear DNA templates and RNA analyses were performed essentially as previously described (15), except that 0.5 U of RNase T_1 per ml and 0.5 mM 3′-*O*-methyl-GTP (Pharmacia) were added to

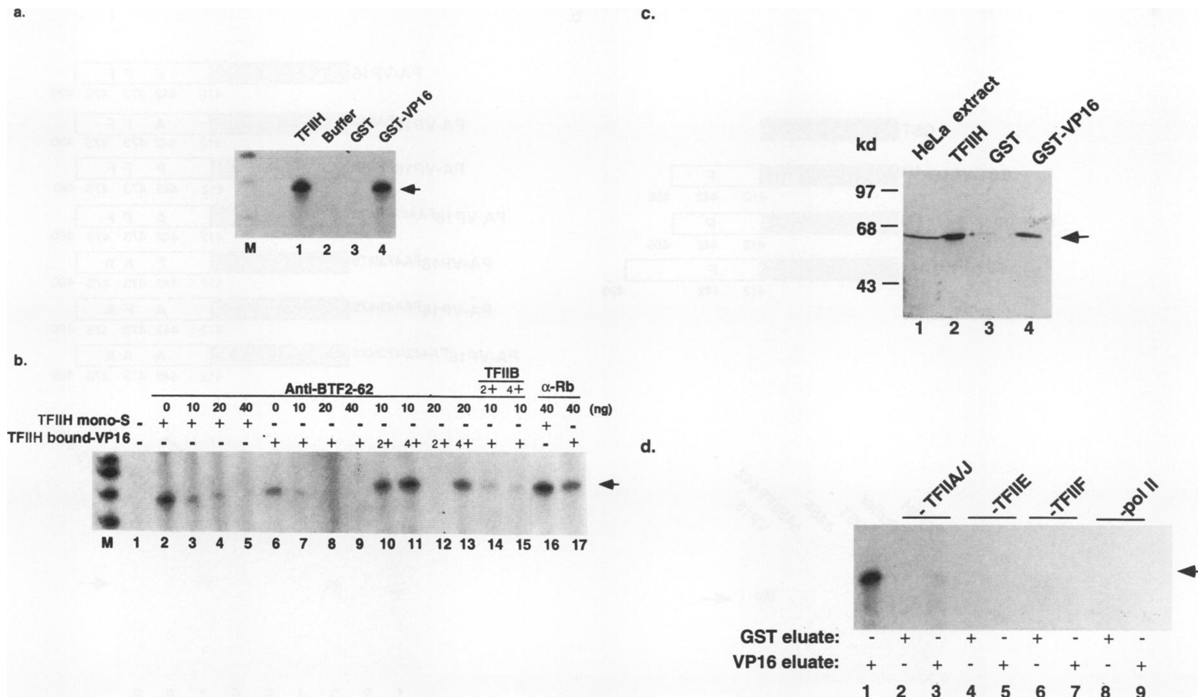

FIG. 1. The acidic activation domain of VP16 interacts with the basal transcription factor TFIIH. (a) Transcripts (arrow) were initiated at the adenovirus type 2 major late promoter on plasmid pML(C2AT)-50 (93) in reactions involving bacterially expressed recombinant TFIIB, TBP, TFIIEα, TFIIEβ, RAP30, and RAP74, a TFIIA/J fraction, and calf thymus RNA polymerase II. The reaction mixtures were supplemented with either 1 μl of HeLa cell TFIIH mono-S fraction (26) (lane 1) or buffer D or with 4 μl of the bound proteins from a GST column (lane 3) or a GST-VP16 column (lane 4). (b) Inhibition of transcription by M.Ab3c9 against BTF2. Transcription reactions were as in panel a. TFIIH mono-S fraction (lanes 2 to 5 and 16) and TFIIH activity that had bound to a GST-VP16 column and was further purified by phosphocellulose chromatography as described in the legend to Fig. 2a (lanes 6 to 15 and 17) were incubated with M.Ab3c9 or the monoclonal antibody Ab-1 directed against Rb (Oncogene Science) as indicated. Some reactions, as indicated, were supplemented with additional VP16-bound TFIIH (lanes 10 to 13) or TFIIB (lanes 14 and 15). (c) Protein immunoblotting with M.Ab3c9 against p62 of BTF2. Lanes: 1, 20 μl of HeLa whole-cell extract; 2, 10 μl of TFIIH mono-S fraction; 3, 40 μl of GST column eluate; 4, 40 μl of GST-VP16 column eluate. The arrow indicates the p62 polypeptides. (d) The binding of TFIIH to VP16 is selective. Transcription reaction mixtures missing TFIIH and the other indicated general transcription factor were supplemented with 4 μl of the bound proteins from either a GST-VP16 or a control GST column.

the reaction mixtures. Transcripts were initiated at the adenovirus type 2 major late promoter on the plasmid pML(C2AT)-50 (93) in reaction mixtures containing bacterially expressed recombinant TFIIB (100 ng/ml), TBP (38 ng/ml), TFIIEα (100 ng/ml), TFIIEβ (100 ng/ml), RAP30 (50 ng/ml) and RAP74 (50 ng/ml), a TFIIA/J fraction (1 μl), a TFIIH fraction (2 μl), and calf thymus RNA polymerase II (2.5 μg/ml).

In vitro protein-binding assays. ^{35}S-labeled proteins were synthesized by using the plasmids encoding p62 (25), TFB1 (29), SSL1, SSL2, and RAD3 (22) and the TNT transcription-translation system (Promega), following the protocol provided by Promega. The input fractions used for binding assays contained these ^{35}S-labeled proteins diluted 10-fold with ACB buffer containing 0.1 M NaCl, 0.5% Nonidet P-40, and 1 mg of bovine serum albumin (BSA) per ml. Glutathione-Sepharose beads (20 μl) containing immobilized proteins were preincubated for 2 h with ACB buffer containing 0.1 M NaCl, 0.5% Nonidet P-40, and 1 mg of BSA per ml, incubated with input fractions (200 μl) at 4°C for 2 h, and washed four times with 1 ml of ACB buffer containing 0.1 M NaCl and 0.5% Nonidet P-40. The bound proteins were eluted with ACB buffer containing 1 M NaCl (80 μl), and the beads were then boiled in 80 μl of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. For Fig. 6b, the ^{35}S-labeled proteins were diluted 10-fold with ACB buffer containing 0.1 M NaCl and 1 mg of BSA per ml and then used as inputs for affinity chromatography. Bound proteins were eluted with ACB buffer containing 1 M NaCl.

RESULTS

Interaction of the VP16 activation domain with TFIIH. To determine in a systematic way which general initiation factors bind to VP16, GST-VP16 fusion proteins were produced in *Escherichia coli* and used as ligands for affinity chromatography (66). The affinity columns were loaded with HeLa whole-cell extracts, and bound proteins were eluted with salt and assayed for the general factors required for initiation by RNA polymerase II in vitro at the adenovirus major late promoter. Our reconstituted in vitro transcription system consisted of the recombinant human general factors TBP (79), TFIIB (36), TFIIE (α and β) (78), and TFIIF (RAP30 and RAP74) (24, 99) all produced in *E. coli*; highly purified calf thymus RNA polymerase II (100); and the general initiation factor fractions TFIIA/TFIIJ (104) and TFIIH (26) partially purified from HeLa nuclear extracts. The TFIIA/TFIIJ fraction used in our experiments contained both TFIIA and TFIIJ, but only the TFIIJ activity is necessary for production of runoff transcripts from the adenovirus major late promoter in reactions involving recombinant TBP (14). As is shown in Fig. 1a, no transcript

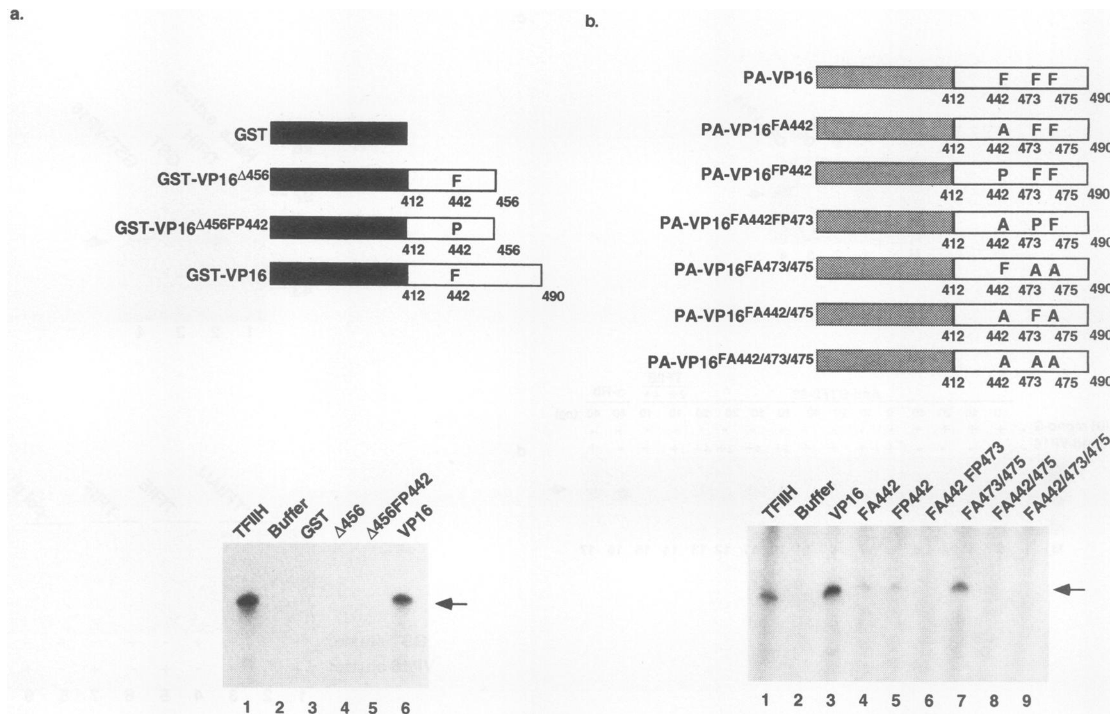

FIG. 2. Effects of VP16 mutations on binding of TFIIH. (a) The C-terminal portion of the VP16 activation domain is important for binding TFIIH. The top panel shows diagrams of the proteins used as affinity column ligands. The numbers indicate the positions of residues in VP16. HeLa cell extract was chromatographed on four 0.5-ml affinity columns containing 4 mg of GST, GST-VP16$^{\Delta456}$, GST-VP16$^{\Delta456FP442}$, or GST-VP16 wild-type proteins per ml. Transcription reaction mixtures containing all the general transcription factors, except TFIIH, were complemented with a TFIIH mono-S fraction (lane 1), buffer D (lane 2), or bound HeLa cell proteins eluted from affinity columns containing immobilized GST, GST-VP16$^{\Delta456}$, GST-VP16$^{\Delta456FP442}$, or GST-VP16 wild type (lanes 3 to 6). (b) Point mutations in VP16 affect its ability to bind TFIIH. The top panel shows diagrams of the proteins used as affinity column ligands. The numbers indicate the residues of VP16. Transcription reaction mixtures containing the other general factors (see the legend to Fig. 1) were supplemented with TFIIH mono-S fraction (lane 1), buffer D (lane 2), or bound HeLa cell proteins from 20-μl affinity columns containing 3 mg of immobilized PA-VP16, PA-VP16^{FA442}, PA-VP16^{FP442}, PA-VP16^{FA442FP473}, PA-VP16$^{FA473/475}$, PA-VP16$^{FA442/475}$, or PA-VP16$^{FA442/473/475}$ per ml (lanes 3 to 9, respectively). Arrows indicate the position of the specific transcript.

was produced in the absence of TFIIH (lane 2) and the eluate from a GST-VP16 column (lane 4) contained an activity that could substitute for the TFIIH fraction (lane 1) to support transcription. In contrast, the eluate from a control GST column lacked TFIIH activity (lane 3). To confirm that the TFIIH activity binding to the VP16 column corresponded to the same factor, known as TFIIH or BTF2, that was described in previous experiments (26, 27), we used a monoclonal antibody (M.Ab3c9) (25) against the 62-kDa subunit (p62) of BTF2. This monoclonal antibody inhibited the transcription activities of both TFIIH purified by a conventional procedure (26) (Fig. 1b, lanes 3 to 5) and the TFIIH activity that was eluted from a VP16 column (lanes 7 to 9). In contrast, an irrelevant monoclonal antibody against the retinoblastoma protein had no effect (lanes 16 and 17). Moreover, the inhibition by M.Ab3c9 was specific because it could be reversed by the addition to the reaction of excess TFIIH that had been highly purified by VP16 affinity chromatography and subsequent phosphocellulose chromatography (lanes 10 to 13) but not by the addition of excess TFIIB (lanes 14 and 15). Furthermore, when the affinity column eluates were subjected to Western blotting with M.Ab3c9, only the GST-VP16 column eluate, and not the control GST column eluate, contained the immunoreactive p62 polypeptide (Fig. 1c). Therefore, VP16 binds authentic TFIIH. This binding of TFIIH to VP16 was selective, since the binding of TFIIE and TFIIF polypeptides could not be detected by Western blotting with appropriate antisera (data not shown) and since no measurable amounts of TFIIE, TFIIF, or RNA polymerase II activity and only a trace amount of TFIIA/J activity bound specifically to the GST-VP16 column (Fig. 1d, lanes 3, 5, 7, and 9). Previous studies have shown that the VP16 activation domain can bind TFIIB (65) and two subunits of TFIID, TBP (103) and TAF40 (30). Consistent with these studies, we found that GST-VP16 columns abound TFIID and TFIIB activities in addition to TFIIH activity (data not shown).

The interaction between VP16 and TFIIH is related to transcriptional activation. To examine the specificity and biological relevance of the interaction between VP16 and TFIIH, we tested whether mutations in the VP16 activation domain (17, 88, 107) that affect its transcriptional activity also affect the binding of TFIIH. The structures of the fusion proteins used in these experiments are shown in Fig. 2. Truncation of the activation domain of VP16 to position 456 reduced the transactivation by VP16 to approximately 50% of maximal activity when a reporter gene containing the herpes simplex virus ICP4 promoter was used (17) and to an undetectable level when a GAL4-VP16 fusion protein was used for activation and the target gene had only one GAL4-binding site (111). This carboxy-terminal deletion (Δ456) had a large effect on the binding of TFIIH activity (Fig. 2a, lanes 4 and 6).

In the context of a full-length VP16 activation domain, a

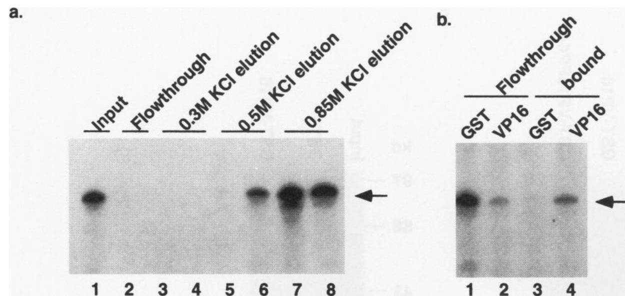

FIG. 3. Highly purified TFIIH binds to the acidic activation domain of VP16. (a) Further purification by phosphocellulose chromatography of TFIIH that had been purified by GST-VP16 chromatography. Transcription reactions were used to assay 4-μl aliquots of phosphocellulose column fractions for TFIIH activity as described in the legend to Fig. 1a. Salt concentrations used for eluting the column are indicated. For each salt concentration, the two fractions with the highest protein concentrations were assayed. (b) Transcription reaction mixtures lacking TFIIH (see the legend to Fig. 1a) were supplemented with phosphocellulose-purified TFIIH activity (0.85 M KCl eluate) that flowed through a GST column or a GST-VP16 column (lanes 1 and 2) or with TFIIH activity bound to the GST column or GST-VP16 column (lanes 3 and 4) (4 μl of each). Arrows indicate the position of the specific transcript. Note that equal volumes of the flowthrough and eluted fractions were assayed but that they represent 10% of the flowthrough and 5% of the eluate, respectively.

substitution of Phe to Pro or Ala at position 442 also moderately reduced the transactivation potency of VP16 with an ICP4 reporter gene (88). These mutations had a similar effect on activation by GAL4-VP16 in *S. cerevisiae* cells (3) and a stronger effect with reporters bearing single GAL4-binding sites in mammalian cells (111). These mutations in the amino-terminal portion of the activation domain of VP16 also substantially reduced the binding of TFIIH to the activation domain of VP16 (Fig. 2b, lanes 4 and 5). Mutations of Phe residues at positions 473 and 475 within the carboxy-terminal portion of the activation domain by themselves have little effect on transactivation by VP16 (43) and only slightly reduce the binding of TFIIH to VP16 (Fig. 2b, lane 7). However, mutations at Phe-473 and Phe-475, when combined with a mutation at the amino-terminal 442 position, significantly reduced transactivation by VP16 (88) or GAL4-VP16 (111) and markedly reduced the ability of VP16 to bind to TFIIH (compare lanes 4 and 5 with lanes 6, 8, and 9). In summary, our data show that both the amino-terminal (amino acids 412 to 456) and carboxy-terminal (amino acids 457 to 490) portions of the VP16 activation domain are very important for binding TFIIH. This correlation between transactivation activity in vivo and binding of VP16 to TFIIH in vitro implies that TFIIH is a target of VP16 for the purpose of transcriptional activation.

The VP16 activation domain binds directly to the 62-kDa subunit (p62) of TFIIH. Since the activation domain of VP16 binds directly to TFIID (30, 103), TFIIB (66), and DNA replication factor A (40, 63), it was possible that one of these proteins acts as a bridge between VP16 and TFIIH. To test this possibility, the TFIIH activity eluted from a GST-VP16 column was further purified by phosphocellulose chromatography (Fig. 3a). The resulting phosphocellulose fractions had TFIIH activity (Fig. 3a, lanes 6 to 8) but undetectable amounts of the other general transcription factors as judged by the in vitro transcription assay and by Western blotting analysis with antibodies against TBP and TFIIB (data not shown). Furthermore, this fraction did not contain detectable amounts of replication factor A as judged by Western blotting analysis with monoclonal antibodies against the 70- and 32-kDa subunits of replication factor A (data not shown). When this highly purified preparation of TFIIH was tested for binding to VP16, 75 to 80% of the TFIIH activity could still bind to a GST-VP16 column (Fig. 3b, compare lane 4 with lane 2), while virtually all the activity again flowed through a control GST column (compare lane 1 with lane 3). Therefore, we concluded that interaction of the VP16 activation domain with TFIIH might not require the presence of any other general transcription factor or replication factor A and could be direct.

Mammalian TFIIH and its yeast equivalent, factor b, have at least five subunits (12, 21, 22, 27). Since human cDNAs encoding the 62-kDa subunit have been cloned (25), we tested the possibility that p62 mediates the interaction between VP16 and other subunits of TFIIH. For this purpose, agarose beads containing immobilized GST or GST-VP16 were incubated with ^{35}S-labeled p62 that had been synthesized in vitro in a rabbit reticulocyte lysate. The beads were washed with low-salt buffer, and bound proteins were eluted successively with buffers containing 1 M NaCl and 1% SDS. The eluates were then analyzed by SDS-PAGE followed by autoradiography. As shown in Fig. 4a, full-length p62 made in vitro (lane 1) bound efficiently to the GST-VP16 beads (lanes 3 and 5) but only weakly to the control GST beads (lanes 2 and 4). About half of the p62 was eluted with 1 M NaCl (lane 3), and the rest was only eluted with buffer containing 1% SDS (lane 5). As was the case for binding of TFIIH activity from a HeLa extract (Fig. 2b), the F442P mutation in VP16 greatly reduced the ability of GST-VP16 to bind p62 made in vitro (Fig. 4b, compare lane 3 with lane 4), indicating that p62 contains a biologically important site in TFIIH for binding VP16. To rule out the possibility that mammalian proteins present in reticulocyte lysate mediate the interaction between VP16 and p62, we also used a bacteriophage T7 RNA polymerase-based system to produce p62 in *E. coli*. In this case, soluble bacterial extract that was partially purified by being passed through a DEAE-Sepharose column was loaded onto affinity columns containing immobilized GST or GST-VP16. The bound proteins were eluted and analyzed by SDS-PAGE followed by Western blotting with M.Ab3c9. As shown in Fig. 4c (lane 1), the input fraction used for affinity chromatography contained a small amount of apparently intact bacterially produced p62 and large amounts of proteolytic fragments of p62 that were detected with M.Ab3c9. All of the polypeptides detected by M.Ab3c9 were derived from p62, because none were present in an extract derived from a control strain containing a plasmid vector lacking p62 sequences (data not shown). None of these p62-derived polypeptides bound to the GST column (lane 2), but a 30-kDa proteolytic fragment of p62 bound to the GST-VP16 column (lane 3). Therefore, VP16 binds directly to the 62-kDa subunit of TFIIH. Curiously, the largest form of p62 made in *E. coli* did not bind VP16 in these experiments, possibly because full-length p62 made in *E. coli*, unlike full-length p62 made in a reticulocyte lysate, is not properly folded or modified for binding VP16.

The activation domain of p53 also binds TFIIH. The p53 activation domain (amino acids 1 to 73) is similar in size, net negative charge, and potency to that of VP16 (23, 74, 87). Previous experiments have shown that the p53 activation domain binds TBP (67, 72, 84, 97, 108) but not TFIIB (67). To test whether the binding of VP16 to TFIIH might represent a phenomenon with general validity for acidic activation domains, we investigated whether p53 could also bind TFIIH. First, HeLa whole-cell extracts were chromatographed on GST and GST-p53 (amino acids 1 to 73) columns (108). Bound proteins were eluted with buffer containing 0.5 M NaCl and

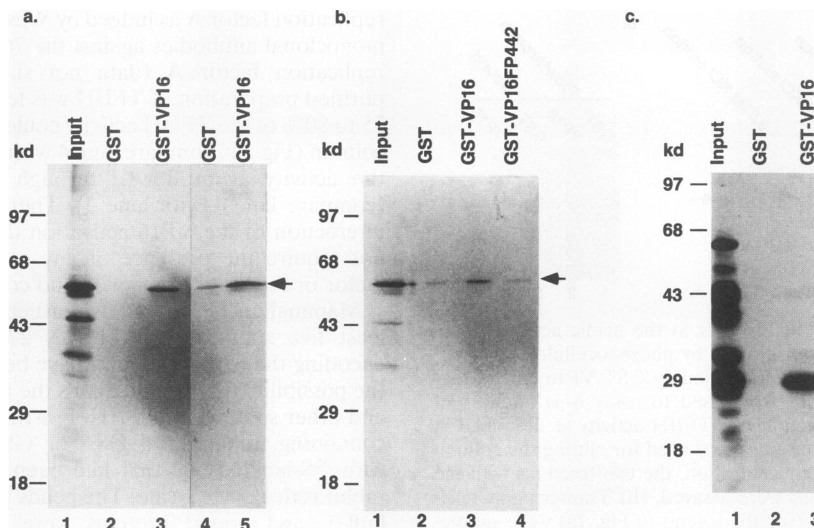

FIG. 4. The 62-kDa subunit of TFIIH mediates the interaction between TFIIH and VP16. (a) An autoradiogram of an SDS-polyacrylamide gel shows the binding of VP16 to p62. Lane 1 shows the input p62. The arrow indicates the position of full-length [^{35}S]methionine-labeled p62 made in vitro. The bound proteins were eluted from GST beads and GST-VP16 beads first by treatment with 1 M NaCl in ACB buffer (lanes 2 and 3) and then by boiling in SDS-PAGE sample buffer (lanes 4 and 5). (b) An autoradiogram of an SDS-polyacrylamide gel shows the specific binding of p62 to VP16. Lane 1 is an aliquot of the in vitro translated p62 used as input. Salt eluates (1 M NaCl) from GST, GST-VP16, and GST-VP16 FP442 beads are shown (lanes 2, 3, and 4, respectively). (c) Binding of VP16 to p62 is direct. Soluble bacterial extract containing proteolyzed p62 was loaded on GST and GST-VP16 affinity columns. Aliquots of the input fraction (lane 1) and proteins eluted with salt (1 M NaCl) from the GST column (lane 2) or the GST-VP16 column (lane 3) were analyzed on an SDS-polyacrylamide gel and immunoblotted with the p62 monoclonal antibody M.Ab3c9.

assayed for the various general initiation factors by using the reconstituted in vitro transcription system described for Fig. 1. As shown in Fig. 5a, eluate from the GST-p53 column (lane 10) but not the GST control column (lane 9) contained an activity that could substitute for TFIIH (lane 1). Consistent with this, Western blotting with M.Ab3c9 (Fig. 5b) showed that only the GST-p53 column (lane 3) and not the GST column (lane 2) bound the immunoreactive p62 found in the HeLa extract (lane 1). In agreement with results of previous experiments, the GST-p53 column but not the GST control column also bound TFIID activity (Fig. 5a, lanes 1 to 4) but relatively little TFIIB activity (lanes 5 to 8). The trace amount of TFIIB bound by p53 may represent an indirect association mediated by TFIID, since we and others have not detected binding of recombinant TFIIB to p53 (67).

We also used recombinant p62 made in reticulocyte lysates or in *E. coli* in p53-binding experiments. As shown in Fig. 5c, intact ^{35}S-labeled p62 made in a reticulocyte lysate (lane 1) bound to GST-p53 beads (lane 3) but not to GST beads (lane 2). Similarly, as was the case for VP16 (Fig. 4c), only a 30-kDa fragment of p62 made in *E. coli* bound to a GST-p53 column (Fig. 5d, lane 3) and also, as before, it did not bind to a GST column (lane 2). Taken together, these data imply that there are similar binding sites for VP16 and p53 in the p62 subunit of TFIIH.

The VP16 and p53 activation domains also interact with yeast factor b. Since acidic activation domains, including those of VP16 and p53, generally function well in *S. cerevisiae*, interactions of activators with the yeast analog of TFIIH provide a critical test for the biological importance of activator-TFIIH interactions. As a first test of whether VP16 can bind factor b, yeast whole-cell extract was chromatographed on affinity columns containing immobilized GST or GST-VP16. Bound proteins were eluted with buffer containing 1 M NaCl and analyzed by Western blotting with a rabbit antiserum against TFB1, a 73-kDa subunit of factor b (29). As shown in Fig. 6a, the GST-VP16 column (lane 2) but not the GST column (lane 1) bound some of the TFB1 in the yeast whole-cell extract. Also, as expected from the results of previous experiments showing that VP16 directly binds yeast TBP (103), the GST-VP16 column bound a substantial amount of the TBP in the extract (data not shown).

TFB1 is the yeast homolog of human p62 (25, 29), and SSL1 (120) and RAD3 (44) have also been identified as subunits of yeast TFIIH (factor b) (22). As well, SSL2 (RAD25) (33, 83) is the yeast homolog of human XPB (ERCC3) (see reference 113 and references therein), which is associated with purified human TFIIH (BTF2) (95). Therefore, to identify the subunit of yeast TFIIH which binds to VP16, ^{35}S-labeled TFB1, SSL1, RAD3, and SSL2 were synthesized in vitro in a rabbit reticulocyte lysate and subjected to chromatography on GST and GST-VP16 columns. Bound proteins were eluted with buffer containing 0.5 M NaCl and analyzed by SDS-PAGE and autoradiography. As shown in Fig. 6b, the full-length TFB1 made in vitro, but not shorter fragments of TFB1 (see lane 1), bound to a GST-VP16 column (lane 3) but not a GST column (lane 2), while SSL1, RAD3, and SSL2 did not bind to either kind of column (lanes 4 to 12). Similarly, a GST-p53 (amino acids 1 to 73) column bound full-length ^{35}S-labeled TFB1 (Fig. 6c, lane 5), while a control GST column did not (lane 2). Therefore, TFB1, the yeast counterpart of human p62, contains a binding site for VP16 and p53. The inability of shorter forms of TFB1 made in vitro, probably as a consequence of transcriptional initiation at internal AUG codons, to bind VP16 or p53 suggested that the amino-terminal region of TFB1 might be important for binding acidic activators. Consistent with this, an amino-terminal fragment containing residues 1 to 526 of TFB1 (Fig. 6d, lane 1) also bound to GST-VP16 (lane 3) but not GST (lane 2), indicating that the carboxy-terminal portion of TFB1 is not needed for binding

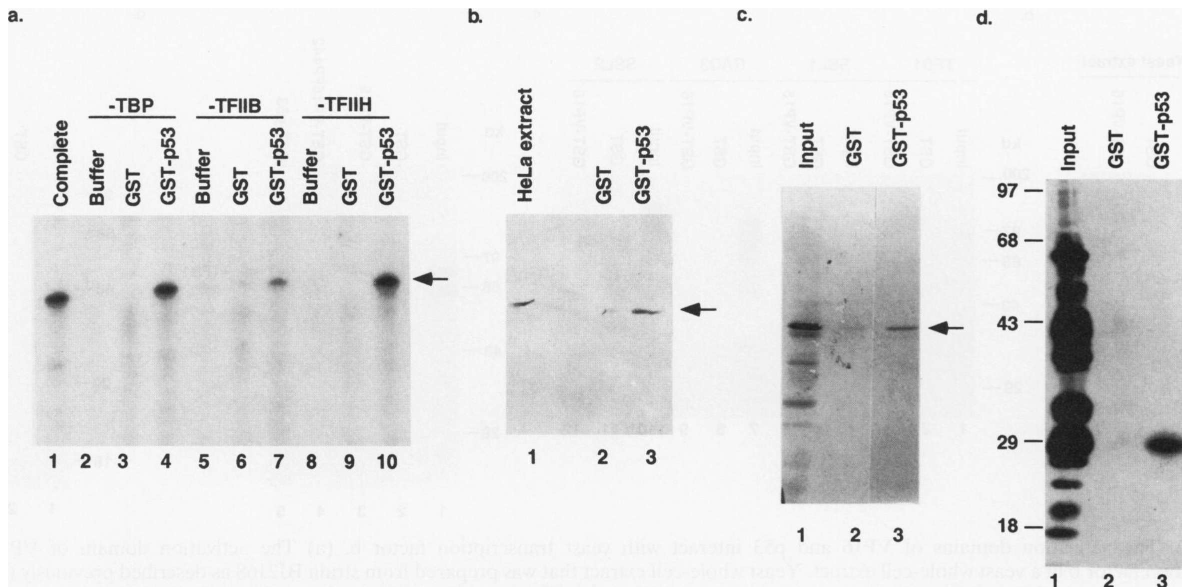

FIG. 5. The activation domain of p53 binds TFIIH. (a) TFIIH activity is retained by a p53 affinity column. Affinity chromatography with HeLa whole-cell extract and transcription assays were performed as described in the legend to Fig. 1a. Reaction mixtures contained all the basal transcription factors (lane 1) or were missing either recombinant TBP, TFIIB, or TFIIH, as indicated at the top of the autoradiogram. Reaction mixtures were supplemented with 4 μl of buffer D (lanes 2, 5, and 8) or HeLa proteins that were eluted with salt (0.5 M NaCl) from a GST column (lanes 3, 6, and 9) or from a GST-p53 column (lanes 4, 7, and 10). An arrow indicates the position of the specific transcript. (b) Western blotting analysis shows that p53 binds authentic TFIIH. HeLa whole-cell extract (lane 1) and bound proteins from the GST column (lane 2) or the GST-p53 column (lane 3) were analyzed by SDS-PAGE and immunoblotted with anti-p62 (M.Ab3c9). (c) The activation domain of p53 binds p62. The binding-assay mixtures contained [^{35}S]methionine-labeled p62 made in vitro. Lanes: 1, input fraction; 2, salt eluate (1 M NaCl) from GST beads; 3, eluate from GST-p53 beads. (d) The binding of p62 to p53 is direct. Affinity chromatography with p62 made in *E. coli* and Western blotting analysis with M.Ab3c9 were performed as for Fig. 4c. Lanes: 1, input fraction; 2, eluted proteins from a GST affinity column; 3, eluted proteins from a GST-p53 column.

acidic activators. Moreover, the F442P mutation in VP16 affects activation by GAL4-VP16 in *S. cerevisiae* (3), as in mammalian cells (111), and greatly reduces binding to VP16 of ^{35}S-labeled yeast TFB1 (Fig. 6c, compare lanes 3 and 4) just as it reduces binding to VP16 of p62 (Fig. 4b). Therefore, we conclude that acidic activators bind in very similar ways to human p62 and yeast TFB1.

DISCUSSION

Our data demonstrate that transcriptional activation domains can interact with the general transcription factor TFIIH. Binding of TFIIH by the VP16 activation domain was not detected previously (65, 103) because TFIIH activity had not yet been separated from fractions containing TFIID, TFIIE, and TFIIF, and so TFIIH activity could not be assayed. Many binding studies with other activator proteins have been published since then, but these have generally focused on TBP, TFIIB, and TAFs, and no systematic effort was made to examine the binding to activators of other general transcription factors. Depending on the salt and ligand concentrations and perhaps on the particular extract used in the experiments, immobilized VP16 quantitatively removed either the TFIID activity (103) or the TFIIB activity (65) from a HeLa nuclear extract but apparently did not quantitatively remove the TFIIH activity. This may mean that there is an excess of TFIIH in an extract or that there is more than one form of TFIIH in an extract, only one of which contains its 62-kDa subunit and binds to VP16. Alternatively, since transcriptional initiation in vitro on supercoiled DNA does not require TFIIH (76, 109), transcription may not have been TFIIH dependent in some of these experiments.

The correlation between the effects of the F442P mutation and other VP16 point mutations on transactivation (3, 17, 88, 111) and their effects on binding to VP16 of human and yeast TFIIH (Fig. 2, 4, and 6) is striking. Therefore, these interactions are likely to be important for the activation of transcription. Two other observations strengthen this conclusion: first, another acidic activation domain, that of p53, also binds to human p62 and its yeast homolog, TFB1; and second, the ability to bind both VP16 and p53 has been evolutionarily conserved even through p62 and TFB1 are only 21% identical in overall amino acid sequence (25, 29).

The effects of TFIIB mutations on activation by GAL4-VP16 in vitro (90) suggest that an activator-TFIIB interaction is also important for transactivation. One experiment with the F442P mutation in VP16 led to the same conclusion (66). In other studies, neither the F442P mutation in the amino-terminal portion of the VP16 activation domain nor mutations in important phenylalanine residues in the carboxy-terminal portion of the VP16 activation domain, which all strongly reduce transactivation in vivo (17, 88, 111), affected the binding to VP16 of TFIIB (30, 34, 111). These mutations do, however, reduce the binding to VP16 of TFIIH (Figures 2, 4, and 6), human TFIID activity (34), and yeast TBP (50). Since the critical F442 residue of VP16 is outside the portion of VP16 that binds TAF40 and since point mutations in VP16 that affect both transactivation by VP16 and the binding to VP16 of TAF40 have yet to be described (30), the effects of VP16 point mutations on transactivation seem to correlate best

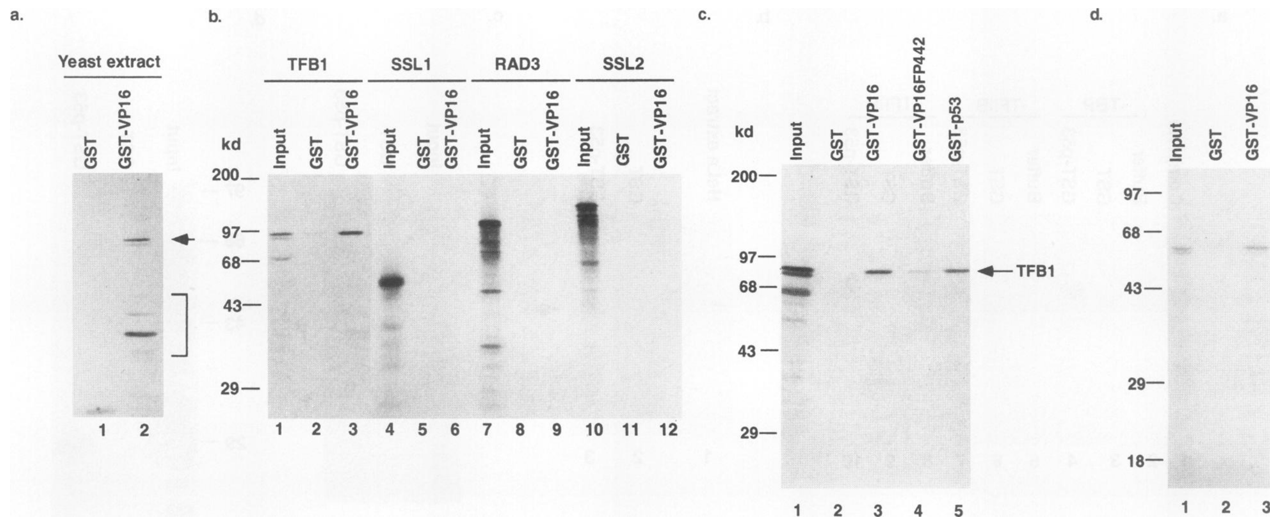

FIG. 6. The activation domains of VP16 and p53 interact with yeast transcription factor b. (a) The activation domain of VP16 binds transcription factor b in a yeast whole-cell extract. Yeast whole-cell extract that was prepared from strain BJ2168 as described previously (115) was chromatographed on a 20-μl GST affinity column and a 20-μl GST-VP16 affinity column. After being washed with 10 column volumes of ACB buffer containing 0.1 M NaCl, the columns were eluted with ACB buffer containing 1 M NaCl. Equal volumes of eluates from the GST column (lane 1) and the GST-VP16 column (lane 2) were resolved by SDS-PAGE and immunoblotted with a rabbit polyclonal antiserum against a recombinant GST-TFB1 fusion protein. The arrow indicates the position of TFB1. The bracket indicates some GST-VP16 column ligand that was eluted from the column with salt and also reacted with the antibody against GST-TFB1. (b) The activation domain of VP16 interacts with TFB1. ^{35}S-labeled TFB1 (lane 1), SSL1 (lane 4), RAD3 (lane 7), and SSL2 (lane 10) made in vitro were chromatographed on 10-μl GST or GST-VP16 affinity columns. Bound proteins from GST columns (lanes 2, 5, 8, and 11) or GST-VP16 columns (lanes 3, 6, 9, and 12) eluted with ACB buffer containing 0.5 M NaCl were resolved by SDS-PAGE followed by autoradiography. (c) Mutant VP16 is deficient in binding TFB1, and p53 also interacts with TFB1. Binding assays used ^{35}S-labeled TFB1 made in vitro (lane 1), as described for panel b. Other lanes: 2, GST eluate; 3, GST-VP16 eluate; 4, GST-VP16^{FP442} eluate; 5, GST-p53 eluate. (d) The carboxy-terminal of TFB1 is not needed for the binding of VP16. A truncated ^{35}S-labeled version of TFB1 was synthesized in vitro after cleavage of the TFB1 plasmid DNA with *Sal*I within codon 527 of TFB1. The binding assays were done as for panel b. Lanes: 1, input; 2 and 3, eluates from the GST and GST-VP16 columns, respectively.

so far with their effects on the binding to VP16 of TFIID (TBP) and TFIIH (p62). It is unclear whether VP16 point mutations that affect activation have similar effects on the binding to VP16 of TBP and TFIIH because these general factors bind to VP16 in similar ways or because the VP16 point mutations affect the folding of the VP16 activation domain.

Like VP16, the proline-rich activation domain of CTF binds TBP (117) and TFIIB (57) and recruits TFIIB into the initiation complex (57). However, since binding of p53 to human TFIIB is very weak (Fig. 5) (67), a strong interaction of an activator with TFIIB may not always be important for transactivation. Indeed, the glutamine-rich activation domains of Sp1 bind TBP (20) and recruit TFIIB into the preinitiation complex (11) even though they have not yet been shown to bind TFIIB. One reasonable possibility is that even a weak interaction of an activator with TFIIB is sufficient to enable that activator to recruit TFIIB into the assembling preinitiation complex. Alternatively, it is possible that interaction of activators with TBP causes TBP to recruit TFIIB into the preinitiation complex, while interaction of activators with TFIIB and/or TAFs leads to subsequent recruitment of RNA polymerase II and other factors (11). Consistent with this latter possibility, Kim et al. (56) recently described a mutation in TBP which prevents the binding to TBP of VP16 and results in a failure of GAL4-VP16 to recruit TFIIB into the preinitiation complex. Furthermore, in view of the recent discovery that *S. cerevisiae* contains an RNA polymerase II holoenzyme containing most of the general initiation factors and susceptible to transcriptional activation in the absence of TAFs (58, 59), it is also possible that an activator simultaneously contacts both TBP and TFIIB to assemble a preinitiation complex.

The interaction of acidic activators with multiple general initiation factors (e.g., TFIID, TFIIB, and TFIIH) is compatible with the high degree of synergy that is observed in transcriptional activation (7, 11) as discussed by Greenblatt (32) and Herchlag and Johnson (41). Formation of at least a partial open complex and transcriptional initiation on linear DNA do not require TFIIE, TFIIH, or ATP, since RNA polymerase II can synthesize a promoter-specific trinucleotide in the absence of these factors (31). However, promoter escape by RNA polymerase II in the absence of stress generated by negative supercoiling does require TFIIE, TFIIH, and ATP (31). TFIIH can assemble late into the preinitiation complex (12, 26) and could be involved in unwinding the DNA to convert a partial open complex to a complete open complex. Consistent with this, TFIIH has an associated ATP-dependent DNA helicase activity (95, 96). In particular, the yeast TFIIH subunit RAD3 (22) and its human counterpart XPD (ERCC2) (94) are DNA helicases (19, 106). Moreover, human XPB (ERCC3) is a helicase (19, 95), and XPB and its yeast homolog, SSL2, have helicase motifs in their amino acid sequences (33, 75, 113). One could therefore explain part of the synergism in transcriptional activation if interaction of acidic activators with TFIIH stimulates melting of the DNA at the promoter. This phenomenon would have been difficult to detect in vitro because acidic activators also stimulate formation of the closed preinitiation complex (65, 112, 114). However, such a model is consistent with observations that mutations which affect transcriptional activation by VP16 also affect open-complex formation (51).

Phosphorylation of the CTD on RNA polymerase II occurs in the transition from an initiation complex to an elongation

complex (6, 77). Since human and yeast TFIIH have associated protein kinase activities that can phosphorylate the CTD (12, 21, 68), interaction of activators with TFIIH may have a role in this process. Phosphorylation of the CTD reduces the affinity of the CTD for TBP (110) and might facilitate the escape of RNA polymerase II from the promoter region following transcriptional initiation. Indeed, this could explain how heat shock factor enables RNA polymerase II to escape a pause site about 20 to 40 nucleotides downstream from the *Drosophila* hsp70 promoter (86). No direct stimulation of the CTD kinase or DNA helicase activity of TFIIH has yet been reported, however, for any transcriptional activators.

Interaction of activators with TFIIH may also lead to more processive transcription by RNA polymerase II. For example, the transactivator protein Tat of HIV-1 binds TBP (53) and activates HIV-1 transcription by both stimulating initiation in the HIV-1 long terminal repeat (61; reviewed in reference 18) and increasing the processivity of chain elongation by RNA polymerase II (52, 54, 61, 71). Similarly, other typical activators, including VP16, may also generally stimulate chain elongation by RNA polymerase II (119). In recent preliminary experiments we have observed that Tat, like VP16, also binds TFIIH (i.e., p62) (116). Effects of activators like Tat on chain elongation might be mediated, for example, via phosphorylation of the CTD on RNA polymerase II. Alternatively, although the XPD and XPB DNA helicases have not been found in association with elongating RNA polymerase II (19), interaction of activators with p62 may stabilize the association of p62 with the elongation complex. In that case, p62 might recruit XPB or XPD to unwind the DNA at pause sites ahead of the RNA polymerase II. Interaction of Tat with TFIIH might also indirectly stabilize the association with the elongation complex of TFIIF (54), which increases the rate of elongation by RNA polymerase II (2).

An additional possibility, which we cannot dismiss, is that interaction of activators with TFIIH also has a role in DNA repair. The *RAD3* and *SSL2* (*RAD25*) genes encoding subunits of yeast TFIIH are essential genes required for transcription by RNA polymerase II in *S. cerevisiae* (35, 83). However, just as mutations in the human *XPB* gene lead to the DNA repair disorders xeroderma pigmentosum and Cockayne's syndrome (113), subunits of yeast TFIIH are involved in DNA repair in *S. cerevisiae* (22, 44, 75). Therefore, interaction of activators with TFIIH might help ensure that TFIIH is still associated with RNA polymerase II when it stalls at a site of DNA damage (discussed in reference 4). Sorting out whether interaction of activators with TFIIH is important for DNA repair as well as transcription will require further experimentation.

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