

# A Class of Activation Domains Interacts Directly with TFIIA and Stimulates TFIIA-TFIID-Promoter Complex Assembly

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**TFIIA is a general transcription factor that interacts with the TFIID-promoter complex required for transcription initiation by RNA polymerase II. Two lines of evidence suggest that TFIIA is directly involved in the mechanism by which some activators stimulate transcription. First, binding of TFIIA to a TFIID-promoter complex is a rate-limiting step that is enhanced by transcriptional activators GAL4-AH and Zta. Second, recombinant TFIIA greatly enhances activator-dependent transcription. In this study, we found that the activation domains of Zta and VP16 bind directly to TFIIA. Both Zta and VP16 stimulated rapid assembly of a stable TFIID-TFIIA complex on promoter DNA. Analysis of deletion derivatives of the VP16 activation domain indicated that the ability to bind to TFIIA correlates with the ability to enhance TFIID-TFIIA-promoter ternary complex assembly. Thus, we propose that a class of activators stimulate transcription initiation through direct interactions with both TFIIA and TFIID, which stimulate the assembly of an activated TFIIA-TFIID-promoter complex.**

Regulated transcription initiation of protein-coding genes by RNA polymerase II requires the assembly of a large preinitiation complex composed of the polymerase and general transcription factors TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH at the transcription start site (for a review, see reference 55). The recent discovery in *Saccharomyces cerevisiae* of a specialized form of RNA polymerase involved in regulated initiation, a holoenzyme associated with an additional ~20 polypeptides (32, 33), suggests that a similarly complex holoenzyme occurs in higher eukaryotes. Thus, the preinitiation complex is an extremely complicated macromolecular assembly. Counting the additional polypeptides found in the yeast holoenzyme, it contains 60 to 70 polypeptides and has a total mass in excess of 4 MDa, roughly the size of a eukaryotic ribosome.

For promoters containing a TATA box, the binding of TFIID to this promoter element is the first step in assembly of the preinitiation complex on promoter DNA (6, 63). TFIID is a multisubunit protein of ~750 kDa, consisting of a TATA-binding polypeptide (TBP) and several additional subunits referred to as TBP-associated factors (TAFs) (19). TAFs are required for activated transcription. If the TBP subunit alone is substituted for TFIID in an *in vitro* reaction, a preinitiation complex can assemble and initiate from the correct start site but transcription is not stimulated by activator proteins (13, 58). The unregulated transcription observed in reactions in which TBP is substituted for TFIID is often referred to as basal transcription.

Activators bind to DNA tens to tens of thousands of base pairs from the transcription initiation site and increase the rate of transcription. They are often modular proteins with distinct DNA binding and activation domains (21, 43). Activators probably function by interacting with components of the preinitiation complex. Since the preinitiation complex is indeed complex, there are many potential sites of interaction. Because of its pivotal role in assembly of the preinitiation complex, TFIID has long been implicated as a site of interaction with activation

domains (1, 22, 23, 54). Many activation domains have been found to bind directly to the TBP subunit of TFIID *in vitro*, including the activation domains of herpes simplex virus VP16 (27, 56), adenovirus E1A (16, 24, 35), Epstein-Barr virus Zta (37), and p53 (25, 41, 62). In addition, E1A and p53 can bind TBP when it is part of the TFIID complex (5, 41), and mutations of VP16 and E1A that inhibit the interaction with TBP interfere with activation (16, 27). Activators have also been shown to bind *in vitro* to TFIID TAFs, and mutations that inhibit these interactions also eliminate the activator function (17, 18, 20, 60). Moreover, partial TFIID complexes composed of TBP and TAFs that bind to specific activators support *in vitro* activation by those activators (9).

Many activators have also been shown to bind to TFIIB, including VP16, c-Rel, CTF1, Epstein-Barr virus R, and some members of the steroid receptor superfamily (4, 26, 31, 40, 44, 69). As for activator-TFIID interactions, the functional significance of activator-TFIIB interactions is supported by a correlation between the effects of mutations on binding and activation (11, 53). In addition, there is evidence that binding of VP16 to TFIIB causes a conformational change in TFIIB which may be required for a subsequent step in preinitiation complex assembly (52). Recent results have also implicated TFIIF (29) and TFIIH (68) as targets for some activation domains.

TFIIA may also make functionally significant contacts with activators. In *in vitro* transcription reactions activated by GAL4-AH (64) and Zta (10), incubation of template DNA with TFIID, TFIIA, and an activator overcomes a rate-limiting step in open complex formation. TFIIA is required for this process. In binding reactions with purified proteins, Zta stimulates assembly of a stable complex of Zta, TFIID, TFIIA, and promoter DNA (Zta-D-A complex [38]). The hypothesis that this Zta activity contributes to activation was supported by the findings that, like transcriptional activation, stimulation of Zta-D-A complex assembly requires (i) Zta-binding sites on the promoter DNA, (ii) the Zta activation domain, and (iii) TFIID TAFs. In addition, it was recently reported that in a GAL4-VP16-responsive *in vitro* transcription reaction using purified general transcription factors plus HMG-2, incubation of a tem-

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plate linked to a solid support with an activator, TFIID, TFIIA, and HMG-2 yielded an activated complex when the remaining general transcription factors and RNA polymerase II were added (55a).

Human TFIIA consists of three subunits,  $\alpha$ ,  $\beta$ , and  $\gamma$  (35, 19, and 14 kDa, respectively). cDNAs encoding the subunits of human and *Drosophila* TFIAs have been cloned (12, 42, 49, 57, 70, 71). In both organisms, the two largest subunits,  $\alpha$  and  $\beta$ , are encoded by a single gene and are probably posttranslationally processed into two separate subunits from a single  $\alpha\beta$  precursor. Yeast TFIIA consists of two subunits of 32 and 13.5 kDa encoded by the TOA1 and TOA2 genes, both of which are essential for cell viability (50). The large TFIIA subunit of *S. cerevisiae* is homologous to the  $\alpha$  and  $\beta$  subunits of human and *Drosophila* TFIAs (12, 42, 70), and the small yeast TFIIA subunit is homologous to the mammalian  $\gamma$  subunit (49, 57, 71). By using recombinant TFIIA reconstituted as either  $\alpha\beta$  plus  $\gamma$  or  $\alpha$  plus  $\beta$  plus  $\gamma$  in in vitro transcription assays, TFIIA was found to enhance activated transcription by several different activators, including Zta, GAL4-VP16, GAL4-VP16C, GAL4-AH, Sp1, and NTF-1 (49, 57, 71). TFIIA can also prevent inhibition of transcription by inhibitors that interact with a TBP-promoter complex. Assembly of a TBP-TFIIA-promoter DNA complex competes with formation of inhibitor-TBP-promoter complexes that block binding of other general transcription factors (28, 34, 45–47) and stabilizes the DNA-protein complex against ATP-driven dissociation by yeast Mot1 (2, 3).

To better understand how TFIIA participates in transcriptional activation mechanisms, we analyzed protein-protein interactions between TFIIA and the activation domains of Zta and VP16. We found a correlation between the ability of the Zta activation domain and the VP16 C-terminal activation subdomain to bind TFIIA in vitro and their ability to stimulate activator-TFIID-TFIIA-promoter DNA complex assembly in vitro. We also found that the VP16 N-terminal activation subdomain does not bind to TFIIA or stimulate this complex assembly, suggesting that activators can stimulate transcription by alternative mechanisms involving distinct interactions with different components of the preinitiation complex.

## MATERIALS AND METHODS

**Plasmid constructions.** A *myc* epitope (EQKLISEEDL [14]) was appended to the N terminus of TFIIA $\alpha\beta$  by PCR amplification of the cDNA with a primer encoding the epitope flanked by a *NcoI* site at the 5' end of the gene and a primer complementary to the other strand at the 3' end of the gene. The second primer contained an *EcoRI* site immediately following the termination codon, and the amplified fragment was cloned into pET21d (Novagen) between the *NcoI* and *EcoRI* sites to create pET-mycTFIIA $\alpha\beta$ . pTMGAL4-VP16 was constructed by cloning the 600-bp *HpaI*-*BamHI* restriction fragment of pJL2 (8) between the *HpaI* and *BamHI* sites of pTMGAL4, a plasmid containing the DNA sequence encoding GAL4 amino acid residues 1 to 147, between the *NcoI* and *EcoRI* sites of pTM1 (48). pTMGAL4-VP16N and pTMGAL4-VP16C were constructed by inserting PCR fragments encoding VP16 amino acid residues 413 to 456 and 452 to 490, respectively, between the *EcoRI* and *BamHI* sites of pTMGAL4. All of the protein-coding DNA sequences prepared by PCR were confirmed by dideoxy sequencing to ensure that no mutations were introduced during amplification.

**Preparation of proteins.** The TFIIA $\alpha\beta$  and TFIIA $\gamma$  subunits were expressed in *Escherichia coli* BL21 from plasmids pET-mycTFIIA $\alpha\beta$  and pQIIA- $\gamma$  (49), respectively, by inducing the cells with 1 mM isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) for 2 h after the cells reached an optical density of 0.8 at 600 nm. Cell pellets were resuspended in buffer A (6 M guanidine hydrochloride, 0.1 M sodium phosphate, 0.01 M Tris-HCl, pH 8.0) and stirred for 1 h at room temperature. Lysate from a 1-liter culture was incubated with 1 ml of Ni-nitrilotriacetic acid beads (Qiagen) for 60 min at room temperature. The resin was washed in a batch twice, with 20 volumes of buffer A and 10 volumes of buffer B (8 M urea, 0.1 M sodium phosphate, 0.01 M Tris-HCl, pH 8.0), and packed in a column in buffer B. Following washing with 30 column volumes of buffer B with 10 mM imidazole, the proteins were eluted from the Ni-nitrilotriacetic acid column with buffer B containing 200 mM imidazole. The purified mycTFIIA $\alpha\beta$

subunit was renatured by itself or with an equimolar amount of the  $\gamma$  subunit by dialysis first against D100 buffer (100 mM KCl, 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES]-KOH [pH 7.9], 0.2 mM EDTA, 10 mM  $\beta$ -mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 20% glycerol) containing 1 M urea overnight and then against D100 buffer with 500 mM urea for 4 h with three subsequent changes of D100 buffer without urea every 4 h. Dialysis was conducted at a protein concentration of 0.5 mg/ml at 4°C.

The TFIIA used in electrophoretic mobility shift assay (EMSA) and DNase I footprinting experiments was prepared as previously described (42), with some modifications. HeLa cell nuclear extract dialyzed in D buffer containing 100 mM KCl was loaded onto a phosphocellulose column (Whatman P-11) at 15 mg/ml. The 100 mM KCl flowthrough was incubated with Ni-nitrilotriacetic acid beads for 2 h at 4°C in the presence of 1 mM imidazole. The Ni-nitrilotriacetic acid beads were washed once with D100 buffer containing 1 mM imidazole and once with D100 buffer containing 5 mM imidazole. The resin was packed in a column and eluted by a linear gradient of 5 to 60 mM imidazole in D100 buffer. The peak fractions were pooled and loaded onto a fast protein liquid chromatography mono Q column and eluted with a 0.1 to 1 M KCl gradient in D buffer. The peak fractions (~0.7 M KCl) were dialyzed against D100 buffer.

TFIID was prepared as described previously (72). Zta was expressed from pPL122 in *E. coli* N5151, and the bacterial lysate was prepared as previously described (36). The insoluble pellet was dissolved in 8 M urea–50 mM Tris-HCl (pH 7.5)–10 mM EDTA–1 mM phenylmethylsulfonyl fluoride–10 mM  $\beta$ -mercaptoethanol and centrifuged at 45,000 rpm for 90 min in a Beckman 45 Ti rotor, and the cleared supernatant was subjected to electrophoresis on a sodium dodecyl sulfate (SDS)–11% polyacrylamide gel. The region of the gel containing the 33-kDa Zta protein (as judged by Coomassie blue staining of a strip of the preparative gel) was electroeluted and precipitated with 5 volumes of cold acetone. The precipitate was resuspended in D100 buffer containing 8 M urea and renatured by consecutive dialysis against D buffer containing 8, 4, and 1 M urea and two subsequent changes of D buffer without urea at 4°C, as described for recombinant TFIIA subunits.

GAL4-VP16 was prepared as previously described (8), and GAL4-VP16N and GAL4-VP16C were gifts of Steven Triezenberg.

In vitro-translated proteins were prepared with rabbit reticulocyte lysate (Promega) in the presence of [<sup>35</sup>S]methionine with messages in vitro transcribed by T7 RNA polymerase from plasmid pTMeTBP (35), pET11-Zta, pET11- $\Delta$ Z(131–245) (10), pTMGAL4-VP16, pTMGAL4-VP16N, or pTMGAL4-VP16C. RNA for in vitro translation of luciferase was supplied by Promega.

**Binding experiments with glutathione S-transferase (GST)–TFIIA fusion proteins.** GST, GST-TBP, GST-TFIIA $\alpha\beta$ , and GST-TFIIA $\gamma$  were expressed in *E. coli* BL21 containing either plasmid pGEX-2T (Pharmacia), pGEX-TBP (35), pGEX-TFIIA $\alpha\beta$  (42), or pGEX-TFIIA $\gamma$  (49), respectively. After induction with 0.1 mM IPTG at 30°C for 60 min, cells were harvested; resuspended in 100 ml of phosphate-buffered saline (PBS) containing 1 mg of lysozyme per ml, 10 mM  $\beta$ -mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride; and disrupted by sonication. Nonidet P-40 was added to a 0.1% final concentration, and a cleared lysate was prepared after 30 min of incubation at 4°C with gentle mixing of the sonicate. Glutathione-Sepharose 4B beads (20  $\mu$ l; Pharmacia) were bound with 2  $\mu$ g of GST or GST fusion proteins and washed three times with PBS containing 0.1% Nonidet P-40, 10 mM  $\beta$ -mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride and twice with D100 buffer. The purified GST or GST fusion proteins on glutathione beads were incubated with in vitro-translated, <sup>35</sup>S-labeled proteins ( $1.5 \times 10^5$  to  $2 \times 10^5$  cpm) in 200  $\mu$ l of D100 buffer for 75 min at room temperature and washed four times with D100 buffer. Samples were eluted by incubation with 15  $\mu$ l of glutathione buffer (20 mM glutathione, 100 mM Tris-HCl [pH 8.0], 120 mM NaCl) for 15 min at room temperature and separated by SDS–12% polyacrylamide gel electrophoresis (PAGE). Gels were fixed in 50% methanol–10% acetic acid, soaked in 1 M Na salicylate for 30 min, dried, and visualized by fluorography. Binding of recombinant GAL4-VP16 to GST or GST fusion proteins was done essentially as described above, except that 200 ng of recombinant GAL4-VP16 instead of <sup>35</sup>S-labeled protein was incubated with GST fusion proteins immobilized on glutathione beads. Bound GAL4-VP16 was electrophoresed on SDS–12% polyacrylamide gels, Western blotted (immunoblotted) with a monoclonal antibody against the DNA-binding domain of GAL4 (Santa Cruz Biotechnology), and visualized by enhanced chemiluminescence detection (ECL; Amersham).

**Coimmunoprecipitation assays.** Recombinant eTFIIA $\alpha\beta$  or eTFIIA $\alpha\beta$ + $\gamma$  (800 ng) was incubated with  $5 \times 10^4$  cpm of in vitro-translated, <sup>35</sup>S-labeled protein in 40  $\mu$ l of D100 buffer at 30°C for 30 min. Protein A-Sepharose beads (25  $\mu$ l) which had been incubated for 2 h at room temperature with 2  $\mu$ l of mouse ascites containing monoclonal antibody 9E10 in 100  $\mu$ l of PBS was added to the reaction mixture. After 60 min of incubation at 25°C with gentle mixing, the beads were washed three times with 10S buffer (250 mM NaCl, 50 mM HEPES [pH 7.2]), 0.3% Nonidet P-40, 0.1% Triton X-100, 0.005% SDS, 0.5 mM dithiothreitol, 10 mM sodium phosphate [pH 7.0], 1 mM NaF, 1 mM Na<sub>2</sub>P<sub>2</sub>O<sub>7</sub>), once with 0.8 M LiCl, and once with PBS. The beads were incubated at 100°C in 15  $\mu$ l of 2 $\times$  Laemmli SDS-loading buffer, and the eluted samples were analyzed by SDS–12% PAGE followed by fluorography with sodium salicylate.

**DNA binding assays.** Mg<sup>2+</sup>-containing agarose gel EMSA was performed as previously described (38). The binding reaction mixture (15  $\mu$ l) contained 12.5 mM HEPES (pH 7.8), 60 mM KCl, 12.5% glycerol, 5 mM MgCl<sub>2</sub>, 0.5 mg of

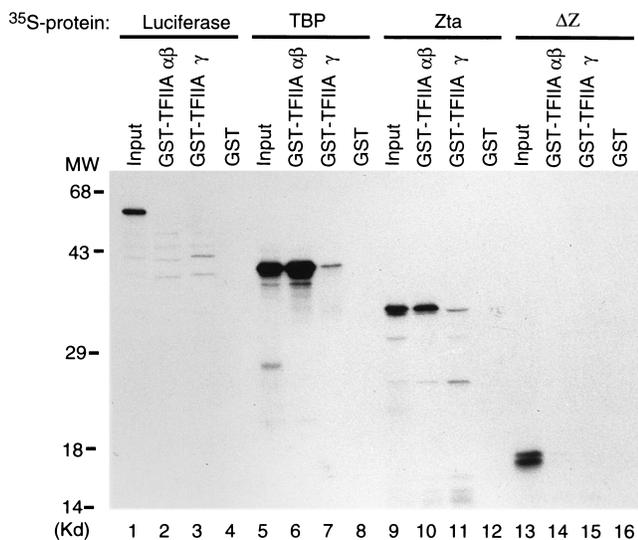


FIG. 1. Zta interacts with TFIIIA $\alpha\beta$  through its activation domain. In vitro-transcribed and translated,  $^{35}\text{S}$ -labeled luciferase (lanes 1 to 4), TBP (lanes 5 to 8), Zta (lanes 9 to 12), or  $\Delta\text{Z}$  (lanes 13 to 16) was incubated with GST (lanes 4, 8, 12, and 16), GST-TFIIIA $\alpha\beta$  (lanes 2, 6, 10, and 14), or GST-TFIIIA $\gamma$  (lanes 3, 7, 11, and 15) immobilized on glutathione-Sepharose beads for 75 min at room temperature. After the beads were washed, bound proteins were eluted with glutathione and analyzed by SDS-12% PAGE and fluorography. The input lanes contained 5% of the material used in the binding reactions. MW, molecular mass in kilodaltons (Kd).

bovine serum albumin per ml, 40  $\mu\text{g}$  of poly(dI-dC)  $\cdot$  poly(dI-dC) per ml, 10 mM  $\beta$ -mercaptoethanol, and the DNA probe ( $10^4$  cpm) and was incubated with protein samples at 30°C for the times indicated in the figure (see Fig. 9). The  $\text{Z}_7\text{E4T}$  probe was prepared by PCR amplification with plasmid pG $_3\text{E4T}$  (72) as the template and oligonucleotide T35570 (5'-ACACCACTCGACACGGCA-3') and the SP6 primer (72), which was labeled with [ $\gamma$ - $^{32}\text{P}$ ]ATP and T4 polynucleotide kinase.

DNase I protection assays were conducted in a 25- $\mu\text{l}$  reaction volume in the same buffer as that used for EMSA with  $5 \times 10^4$  cpm of a radiolabeled probe at 30°C for 30 min. A 4- $\mu\text{l}$  volume of 0.4  $\mu\text{g}$  of DNase I per ml in 60 mM  $\text{CaCl}_2$  was added, incubation was continued for 1 min at 30°C, the reaction was terminated, and samples were processed as previously described (36). The  $\text{Z}_7\text{E4T}$  probe used for DNase I footprinting assays was the 224-bp, *Hind*III-*Eco*RI fragment which was end labeled at the *Hind*III site with the Klenow fragment and [ $\alpha$ - $^{32}\text{P}$ ]dATP as previously described (38).

## RESULTS

### The Zta activation domain binds the $\alpha\beta$ subunits of TFIIIA.

Zta stimulates assembly of a stable complex of TFIIIA, TFIID, and promoter DNA, and the resulting Zta-D-A complex forms an activated preinitiation complex when the remaining general transcription factors, RNA polymerase II and factors in the USA fraction are added (38). This activity of Zta probably involves the previously characterized interaction between the Zta activation domain and the TBP subunit of TFIID (37). To gain further understanding of the mechanism by which Zta stimulates Zta-D-A complex assembly, we determined whether Zta also binds directly to TFIIIA.

First, we tested the interaction of Zta with TFIIIA by using GST fusions to the TFIIIA  $\alpha\beta$  and  $\gamma$  subunits. Equal amounts of GST-TFIIIA $\alpha\beta$ , GST-TFIIIA $\gamma$ , and GST were bound to glutathione-Sepharose beads and incubated with in vitro-translated,  $^{35}\text{S}$ -labeled Zta. After extensive washing, the samples were eluted with glutathione and analyzed by SDS-PAGE. Approximately 5% of the input Zta bound to GST-TFIIIA $\alpha\beta$ , while a much smaller fraction bound to GST-TFIIIA $\gamma$  (Fig. 1). No detectable Zta bound to GST. As a positive control for a specific protein-protein interaction with TFIIIA, we tested the

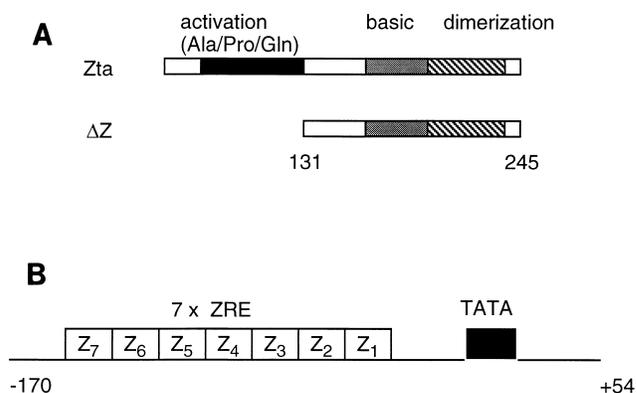


FIG. 2. The Zta activator (A) and the  $\text{Z}_7\text{E4T}$  DNA probe (B). Schematic presentation of the 245-amino-acid residue full-length Zta protein and  $\Delta\text{Z}$  lacking amino-terminal residues 2 to 131, including the activation domain. The  $\text{Z}_7\text{E4T}$  DNA probe used in DNase I protection experiments was 224 bp long and contained seven consecutive Zta binding sites and the TATA element of the adenovirus type 2 E4 promoter.

binding of TBP to the GST-TFIIIA affinity resins. A similar fraction of the input TBP protein bound specifically to GST-TFIIIA $\alpha\beta$  as did that of Zta (Fig. 1, compare lanes 5 and 6 to 9 and 10). The in vitro-translated free TFIIIA $\gamma$  subunit bound to the GST-TFIIIA $\alpha\beta$  affinity matrix efficiently, and in vitro-translated TFIIIA $\alpha\beta$  bound to the GST-TFIIIA $\gamma$  affinity matrix with similar efficiency (data not shown), indicating that the TFIIIA subunits expressed as GST fusion proteins retained their proper functional conformations. Firefly luciferase, used as a negative control, did not bind to GST-TFIIIA $\alpha\beta$  or to GST-TFIIIA $\gamma$ , further supporting the specificity of the interaction between Zta and TFIIIA $\alpha\beta$ . (However, it should be noted that truncated luciferase in vitro translation products did bind to both GST-TFIIIA $\alpha\beta$  and GST-TFIIIA $\gamma$ , demonstrating that mechanistically insignificant protein-protein interactions can be observed by using this assay.) Binding of Zta to the GST-TFIIIA $\alpha\beta$  matrix was not disrupted when the matrix was washed in buffer containing 0.5 M KCl (data not shown). Taken together, these results suggest that Zta interacts specifically with TFIIIA $\alpha\beta$ .

The deletion mutant of Zta,  $\Delta\text{Z}$ , lacks the N-terminal 130 amino acids (Fig. 2). Previous studies have shown that this N-terminal domain is required for transcriptional activation by Zta (10, 15, 36), as well as for stimulation of Zta-D-A complex formation (38). Significantly, the interaction between TFIIIA $\alpha\beta$  and Zta also required the activation domain of Zta because  $\Delta\text{Z}$  failed to bind to the GST-TFIIIA affinity resins (Fig. 1, lanes 13 to 16). Ozer et al. (49) have also reported a specific, activation domain-dependent interaction between GST-Zta and TFIIIA $\gamma$ .

**Zta interacts with holo-TFIIIA.** To determine whether Zta binds to native TFIIIA containing all three subunits, we performed coimmunoprecipitation experiments with holo-TFIIIA. The N terminus of the TFIIIA $\alpha\beta$  subunit was fused to the *myc* epitope (eTFIIIA $\alpha\beta$ ). Recombinant holo-eTFIIIA was prepared by associating an equimolar amount of the epitope-tagged  $\alpha\beta$  subunit with the  $\gamma$  subunit (see Materials and Methods). The activity of this recombinant holo-eTFIIIA used in the coimmunoprecipitation experiments was similar to that of isolated HeLa cell TFIIIA when we examined its activity for (i) stimulation of Zta-D-A complex assembly on the  $\text{Z}_7\text{E4T}$  probe, (ii) induction of TFIID-Zta dependent-DNase I footprinting-hypersensitive sites at the initiator region (38), and (iii) activation of transcription in in vitro reactions with GAL4-AH (data

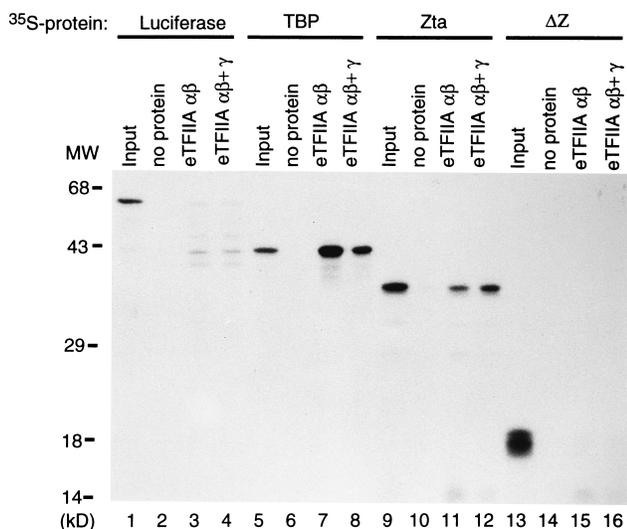


FIG. 3. Coimmunoprecipitation assays detect an interaction between holo-TFIIA and Zta. <sup>35</sup>S-labeled luciferase (lanes 1 to 4), TBP (lanes 5 to 8), Zta (lanes 9 to 12), or ΔZ (lanes 13 to 16) was incubated with the *myc* epitope-tagged TFIIAαβ subunit or holo-eTFIIA, composed of eTFIIAαβ plus TFIIAγ, as indicated above each lane. After 30 min of incubation at 30°C, the reactions were subjected to immunoprecipitation with monoclonal antibody 9E10, which is directed against the *myc* epitope, and analyzed by SDS–12% PAGE. The input lanes contained 5% of the input labeled protein. Lanes marked “no protein” show the amount of labeled proteins immunoprecipitated from binding reactions without the TFIIA protein. MW, molecular mass in kilodaltons (kD).

not shown). Monoclonal antibody 9E10, against the *myc* epitope, was used to precipitate eTFIIAαβ or holo-eTFIIA (αβ+γ) which had been incubated with the in vitro-translated, <sup>35</sup>S-labeled Zta protein (Fig. 3). Zta was coprecipitated with both eTFIIAαβ and holo-eTFIIA(αβ+γ). Quantitation of the Zta protein bound to holo-eTFIIA and to eTFIIAαβ in three independent experiments indicated that the γ subunit contributed little to the interaction between Zta and holo-TFIIA. Importantly, ΔZ did not bind to either eTFIIAαβ or eTFIIAαβ+γ, indicating that the activation domain of the Zta protein is required for the interaction with holo-TFIIA (Fig. 3, lanes 13 to 16). These results indicate that the activation domain of Zta specifically interacts with native TFIIA.

**The Zta-TFIIA interaction is direct.** DNase I footprinting experiments with Zta and TFIIA also indicated that Zta interacts with native TFIIA. When a DNA probe containing seven consecutive Zta binding sites upstream of the adenovirus E4 TATA box (Fig. 2) was incubated with the purified Zta protein prepared from *E. coli*, the Zta sites were protected from DNase I digestion (Fig. 4A, lane 3, and 4B, lane 2). When highly purified HeLa cell TFIIA was added to the binding reaction with Zta, new DNase I-hypersensitive sites were observed between the Zta binding sites (Fig. 4A, lane 7, and 3B, lanes 3 to 6), even though TFIIA alone did not affect the DNase I footprint (Fig. 4A, compare lanes 2 and 4). When increasing amounts of TFIIA were added to the binding reaction with a constant amount of Zta, the hypersensitive sites increased in intensity (Fig. 4B). Similar hypersensitive sites were not observed when TFIID and TFIIA were bound to the probe in the absence of Zta (data not shown). Although we do not know how TFIIA induces the DNase I-hypersensitive sites that punctuate the Zta binding sites, their appearance demonstrates that TFIIA can interact with DNA-bound Zta under transcription reaction conditions. These results also show that reticulocyte lysate is not necessary for the TFIIA-Zta interac-

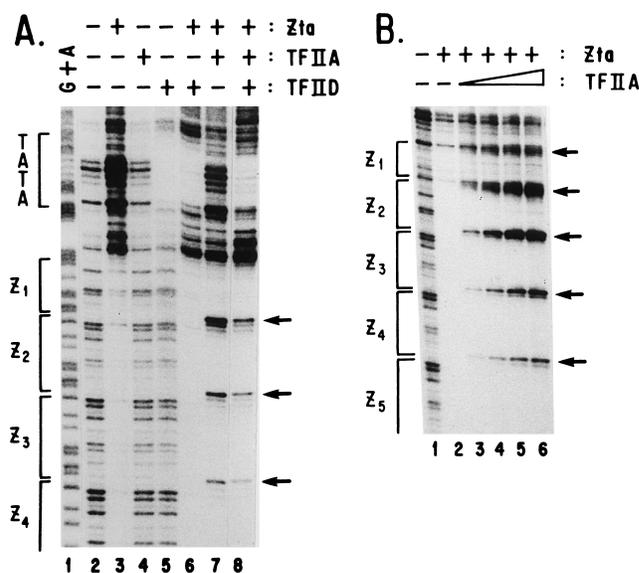


FIG. 4. DNase I footprinting of Zta in the absence or presence of TFIIA. (A) Coincubation of Zta and TFIIA with the Z<sub>7</sub>E4T DNA probe produced distinct hypersensitive sites (arrows) which punctuate each Zta binding site. The Z<sub>7</sub>E4T DNA probe was incubated with TFIIA alone (lane 4) or with Zta in the absence (lane 3) or presence of TFIIA (lane 7), as indicated above each lane. After 30 min of incubation at 30°C, samples were subjected to DNase I footprinting and analyzed on a 6% denaturing polyacrylamide gel. The control lane 2 shows DNase I digestion of the free probe. Lane 1 (G+A) contained Maxam and Gilbert G+A sequencing reaction mixtures used as molecular weight markers. (B) The Z<sub>7</sub>E4T DNA probe was incubated with a constant amount of Zta and increasing amounts of TFIIA at 30°C for 30 min and then digested with DNase I (lanes 3 to 6). In lane 2, only Zta was added to the reaction mixture. In lane 1, the binding reaction mixture contained no added protein.

tion. We also observed binding between purified recombinant TFIIA αβ subunit and purified recombinant Zta by GST-TFIIA binding assays (data not shown). Addition of TFIID to the binding reaction did not change the hypersensitive sites significantly (Fig. 4A, lane 8), indicating that the Zta-TFIIA interaction is maintained in the Zta-D-A complex, which can interact with other factors required for transcription to form an activated preinitiation complex (38).

#### The activation domain of VP16 also interacts with TFIIA.

To explore the generality of TFIIA activation domain interactions, we analyzed the interaction of TFIIA with the well-characterized activator herpes simplex virus type I VP16 by using coimmunoprecipitation assays with *myc* epitope-tagged TFIIA. Following coincubation, in vitro-translated GAL4-VP16 coprecipitated with holo-eTFIIA (Fig. 5, lane 4). Unlike the situation with Zta, less GAL4-VP16 interacted with TFIIAαβ in the absence of the γ subunit.

The N-terminal half (amino acid residues 413 to 456) and the C-terminal half (residues 452 to 490) of the VP16 activation domain can independently activate transcription when fused to the GAL4 DNA binding domain (Fig. 6 shows the structures; 18, 51, 61). The coimmunoprecipitation assay showed that, like GAL4-VP16, the C-terminal domain of VP16 (GAL4-VP16C) interacted with holo-eTFIIA (Fig. 5, lanes 9 to 12). Again, the interaction with TFIIAαβ was much weaker than that with holo-eTFIIA (Fig. 5, lanes 11 and 12). In contrast, GAL4-VP16N did not bind significantly to either holo-eTFIIA or eTFIIAαβ. Thus, VP16 interacts with TFIIA through the C-terminal subdomain of its activation domain.

DNase I footprinting analysis with highly purified TFIIA and recombinant GAL4-VP16 failed to produce hypersensitive

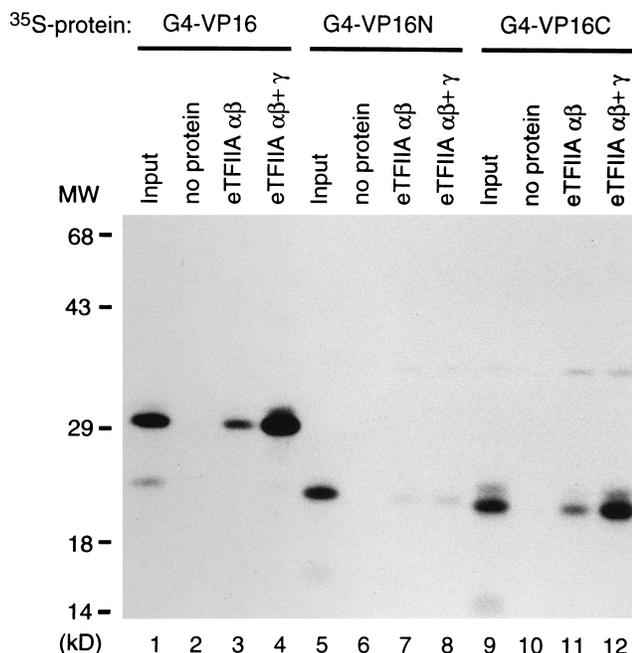


FIG. 5. Acidic activator VP16 interacts with holo-TFIIA. Coprecipitation of  $^{35}\text{S}$ -labeled GAL4-VP16, GAL4-VP16N, or GAL4-VP16C with eTFIIA $\alpha\beta$ , holo-eTFIIA( $\alpha\beta+\gamma$ ), or a no-protein control by monoclonal antibody 9E10. Immunoprecipitates were analyzed by SDS-12% PAGE. Input lanes contained 5% of the input labeled protein used in the binding reactions. MW, molecular mass in kilodaltons (kD).

sites on a probe with GAL4 binding sites similar to those observed with Zta and TFIIA. To determine whether VP16 interacts with TFIIA directly, we analyzed the ability of recombinant protein GAL4-VP16 produced in *E. coli* to bind to GST-TFIIA affinity beads prepared from recombinant proteins. Bound protein was analyzed by SDS-PAGE and Western

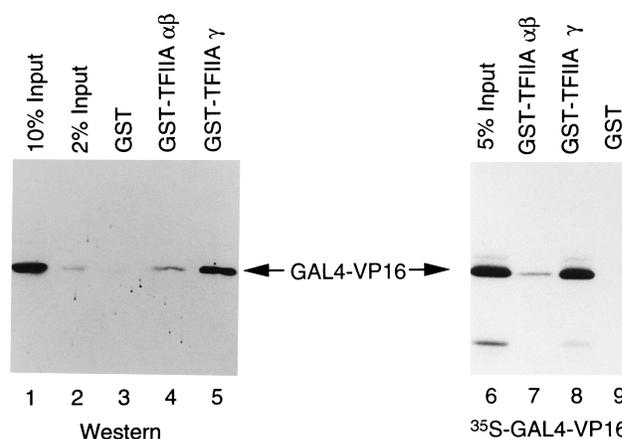


FIG. 7. GAL4-VP16 directly interacts with TFIIA subunits. The recombinant GAL4-VP16 protein (200 ng) was incubated with 2  $\mu\text{g}$  of GST (lane 3), GST-TFIIA $\alpha\beta$  (lane 4), or GST-TFIIA $\gamma$  (lane 5) linked to 20  $\mu\text{l}$  of glutathione-Sepharose beads. After extensive washing, the bound protein was eluted with glutathione and loaded onto an SDS-12% PAGE gel along with 20 and 4 ng of GAL4-VP16 in lanes 1 and 2, respectively, and transferred to a nitrocellulose membrane. A monoclonal antibody against the DNA binding domain of GAL4 was used for Western blotting analysis. Binding of  $^{35}\text{S}$ -labeled GAL4-VP16 to GST, GST-TFIIA $\alpha\beta$ , or GST-TFIIA $\gamma$  was also examined (lanes 6 to 9). Bound protein was visualized by fluorography. Lane 6 contained 5% of the input labeled protein.

blotting (Fig. 7). Approximately 10% of the input GAL4-VP16 was retained on a GST-TFIIA $\gamma$  matrix (Fig. 7, lane 5). From 1 to 2.5% of the input GAL4-VP16 protein bound to GST-TFIIA $\alpha\beta$ , and very little bound to GST alone (Fig. 7, lanes 4 and 3, respectively). Similar results were observed in parallel binding assays with *in vitro*-translated,  $^{35}\text{S}$ -labeled GAL4-VP16 (Fig. 7, lanes 6 to 9). Thus, VP16 can directly interact with TFIIA, largely through the  $\gamma$  subunit.

#### VP16 stimulates TFIIID-TFIIA-promoter complex assembly.

The activation domain of Zta is required both for stimulation of D-A complex formation and for interaction with TFIIA. Since we observed an interaction between GAL4-VP16 and TFIIA, we determined whether GAL4-VP16, like Zta, could stimulate the assembly of a TFIIA-TFIIID complex on a promoter with GAL4 binding sites. Complex formation was assayed by Mg-agarose gel EMSA. A radiolabeled DNA probe containing five GAL4 binding sites and the adenovirus E4 TATA box ( $G_5E4T$ ; Fig. 6) was incubated with amounts of purified TFIIID and TFIIA that led to retardation of only a small fraction of the probe in a complex with TFIIID and TFIIA (Fig. 8, lane 3). Addition of sufficient GAL4-VP16 to saturate the GAL4 binding sites (data not shown) resulted in a dramatic increase in the amount of a slow-mobility complex (Fig. 8, lane 6). As for Zta, TFIIA was required for GAL4-VP16 to stimulate assembly of a stable complex of TFIIID and promoter DNA (compare lanes 5 and 6). In other experiments, sufficient TFIIID was used to observe a TFIIID complex in the absence of TFIIA. Under these conditions as well, GAL4-VP16 did not stimulate complex assembly unless TFIIA was added to the reaction mixture (data not shown).

In contrast to GAL4-VP16, GAL4-VP16N, which did not bind to TFIIA (Fig. 5), did not increase TFIIID binding to the promoter, even in the presence of TFIIA (Fig. 8, lane 9). However, GAL4-VP16C, which did bind to TFIIA (Fig. 5), did stimulate complex assembly (Fig. 8, lane 12), although to a lesser extent than the GAL4 fusion to the full-length VP16 activation domain (compare lanes 6 and 12). Zta stimulated the rate of D-A complex formation, as well as the amount of

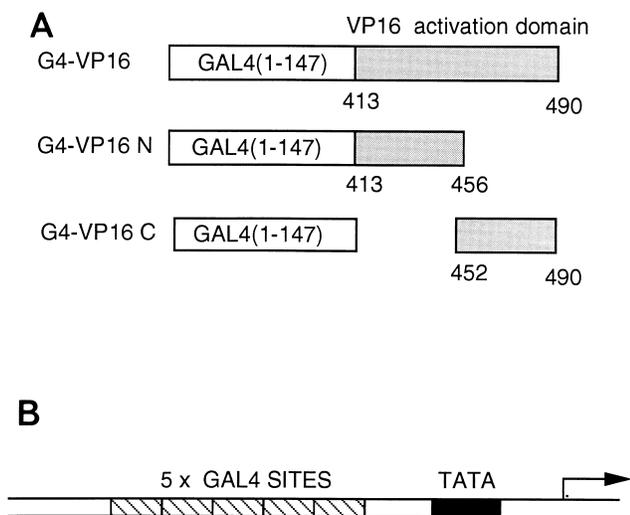


FIG. 6. Structures of GAL4-VP16, GAL4-VP16N, and GAL4-VP16C (A) and the  $G_5E4T$  promoter (B). The activation domain of VP16, spanning the carboxyl-terminal 78 amino acids (amino acid residues 413 to 490), was fused to the DNA binding domain (residues 1 to 147) of the GAL4 protein. GAL4-VP16N and GAL4-VP16C contained the amino-terminal half (residues 413 to 456) and the carboxyl-terminal half (residues 452 to 490) of the VP16 activation domain, respectively.

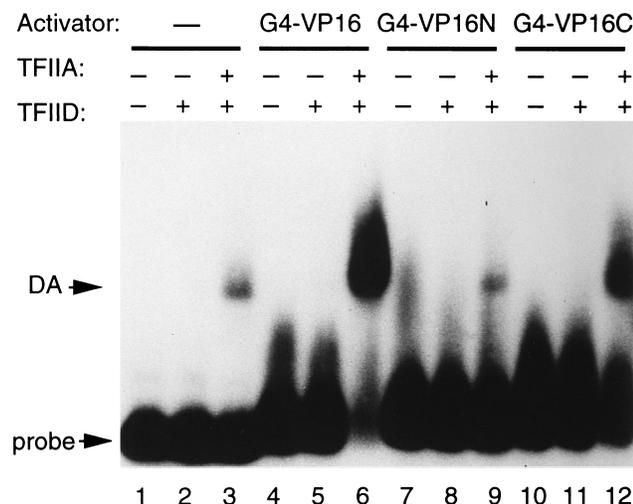


FIG. 8. GAL4-VP16 stimulates TFIID-TFIIA-promoter complex assembly. The effect of GAL4-VP16, GAL4-VP16N, and GAL4-VP16C on TFIID-TFIIA-promoter complex formation was examined by Mg-agarose EMSA. The  $G_5E4T$  probe was incubated with TFIID and TFIIA as indicated by the plus signs and with GAL4-VP16 (lanes 4 to 6), GAL4-VP16N (lanes 7 to 9), or GAL4-VP16C (lanes 10 to 12). DA, D-A complex.

the stable D-A complex formed (38). Experiments that assayed the kinetics of complex formation showed that GAL4-VP16 and GAL4-VP16C also accelerated the rate of stable complex assembly (Fig. 9A and B). The D-A complex continued to assemble for at least 20 min when the probe was incubated with TFIID and TFIIA alone. However, when GAL4-VP16 or GAL4-VP16C was included in the binding reaction mixture, complex assembly was complete in 1 to 2 min.

These results obtained with Zta,  $\Delta Z$ , GAL4-VP16, GAL4-VP16N, and GAL4-VP16C, indicate a correlation between the ability of an activation domain to bind to TFIIA and its ability to stimulate assembly of a stable activator-TFIID-TFIIA complex on promoter DNA. Taken together, these results support a model in which interaction between a class of transcriptional activators and TFIIA stimulates TFIID-TFIIA-promoter complex assembly.

## DISCUSSION

To understand how activators stimulate transcription, it is necessary to determine with which macromolecules they interact and how these interactions affect the assembly and activity of the preinitiation complex. We report here that the activation domains of strong viral activators Zta and VP16 can directly and specifically interact with general transcription factor TFIIA. The interaction requires the activation domain and correlates with the previously reported ability of Zta to stimulate the rapid assembly of a stable TFIID-TFIIA-promoter complex (i.e., a D-A complex). The 78-amino-acid activation domain of VP16 can be further dissected into two subdomains, VP16N and VP16C, both of which can activate transcription (18, 51, 61). However, only the VP16C subdomain, and not the VP16N subdomain, interacts with TFIIA directly and stimulates D-A complex assembly. VP16N may require an additional cofactor to stimulate D-A complex assembly, or VP16N might stimulate transcription by a mechanism significantly different from that used by Zta and VP16C. These results lead to a model in which a subset of activation domains activate transcription in part by stimulating assembly of a D-A complex.

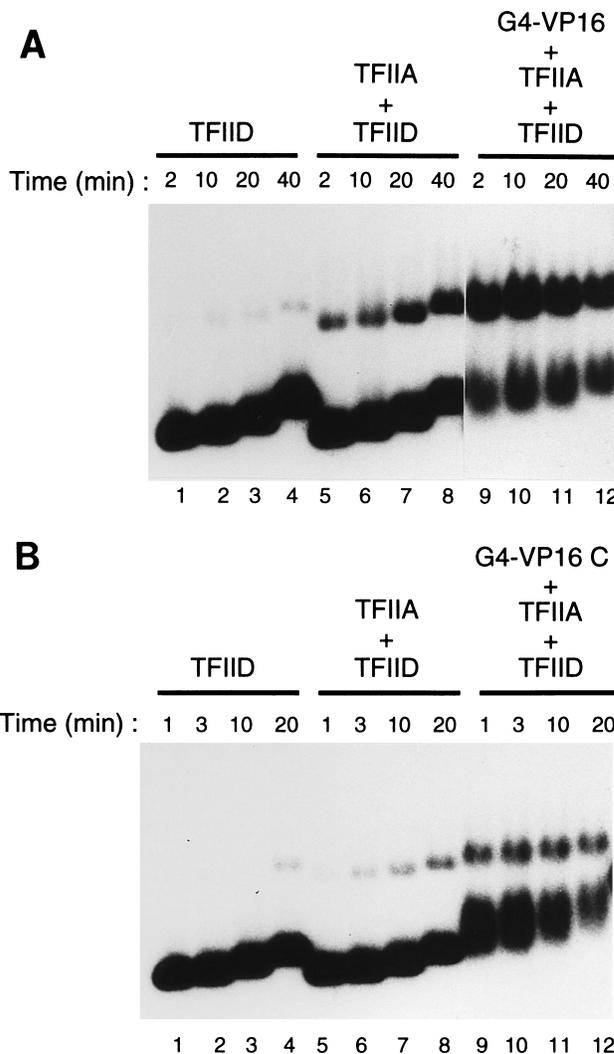


FIG. 9. Kinetics of D-A complex formation with GAL4-VP16. TFIID binding to the  $G_5E4T$  probe as a function of time in the absence (lanes 1 to 4) or presence (lanes 5 to 9) of TFIIA was examined. GAL4-VP16 (A, lanes 9 to 12) or GAL4-VP16C (B, lanes 9 to 12) was added to the reaction mixture containing TFIID and TFIIA. Binding reactions were incubated at 30°C, and an aliquot was removed at the time indicated above each lane and loaded onto a running gel.

The stimulation of D-A complex assembly provides a straightforward mechanism for transcriptional activation by Zta and VP16C. The D-A complex can serve as a platform for assembly of the complete preinitiation complex (6, 63). Consequently, stimulation of D-A complex formation could drive assembly of the complete preinitiation complex. Also, transcriptional inhibitors have been described that interact with TBP-promoter complexes, blocking the binding of additional general transcription factors (2, 3, 28, 34, 45-47). In *S. cerevisiae*, the essential *MOT1* gene encodes such a generalized inhibitor of transcription that clearly functions in vivo (3). TFIIA competes with these inhibitors for binding to TBP-promoter complexes. Zta and VP16 stimulation of D-A complex formation should counteract the influence of such inhibitors. In addition, nucleosomes are another type of inhibitor that can repress transcription by binding to template DNA. TFIID binding to promoter DNA counteracts the inhibitory influence of nucleosomes (66). Consequently, stimulation of

D-A complex assembly could account for the ability of the VP16 activation domain to counteract nucleosomal inhibition *in vitro* (30, 67) and might counteract the repressive influence of nucleosomes *in vivo* (59). Thus, stimulation of D-A complex formation would both increase the assembly of preinitiation complexes and counteract the influence of inhibitors that act on the TFIID-promoter complex.

In a recent report analyzing the mechanism of VP16 activation in an *in vitro* transcription system using purified general transcription factors and HMG-2, binding of GAL4-VP16 to the template stimulated the formation of an "activated TFIID-TFIIA-promoter complex" without increasing the amount of D-A complex formed (55a). These studies were performed with the adenovirus major late promoter, a promoter with which TFIID makes downstream contacts in the absence of an activator. In light of these results, it may be that the key step in activation is the induction of a conformational change in the TFIID-TFIIA-promoter complex. In the E4 promoter used in our studies, which is bound less stably by isolated TFIID than the major late promoter, the conformational change may stabilize the D-A complex, as observed in an agarose gel EMSA. But it may be that the postulated GAL4-VP16-induced conformational change in the D-A complex makes a greater contribution to activation than the GAL4-VP16-induced increase in the amount of the D-A complex assembled.

Stimulation of the assembly of an activated D-A complex does not preclude other activation mechanisms. For example, several activators stimulate TFIIB binding to TFIID-promoter (11, 39) and TBP-promoter (11, 31) complexes. This would be expected to further stimulate preinitiation complex assembly after initial stimulation of D-A complex formation. Indeed, the VP16 activation domain has been shown to bind to TBP (56), *Drosophila* TAF<sub>II</sub>40 (18, 20), TFIIB (53), and now TFIIA (this report and reference 49). Do all of these interactions demonstrated *in vitro* actually contribute to VP16 activation *in vivo*? There is support for the functional significance of each of these VP16 interactions. Mutants defective in TBP binding are defective for activation *in vivo* (27). Antiserum against TAF<sub>II</sub>40 inhibits VP16 activation *in vitro* (18). Mutants of TFIIB that interfere with the interaction with VP16 inhibit VP16-activated, but not basal, transcription (53). The VP16 interactions with both TFIID and TFIIA probably each contribute to the stimulation of D-A complex assembly. Interactions between VP16 and TFIIB could contribute to a second step in the overall VP16 activation mechanism. VP16 may stimulate transcription at yet another step in the initiation process through an interaction with TFIID (68). It appears that in VP16, herpes simplex virus has evolved an extremely effective protein for activating transcription of its immediate-early genes. The protein is brought into infected cells in the inner portion of the complex virion particle. Following infection, it is transported into the nucleus, where it activates viral immediate-early genes (65). Clearly, there is a strong selective pressure to rapidly induce these genes immediately following infection. This strong selective pressure appears to have driven the evolution of a protein that activates transcription through multiple contacts with different polypeptides in the preinitiation complex. This multiplicity of interactions with the preinitiation complex can account for VP16's ability to activate transcription synergistically (7).

We observed that the activation domain of Zta interacted with the  $\alpha$  and  $\beta$  subunits of TFIIA more strongly than with the  $\gamma$  subunit, while VP16 interacted more strongly with the  $\gamma$  subunit. The activation domain of VP16 is rich in acidic amino acids, while Zta is not an acidic activator. Its activation domain is rich in glutamine, proline, and alanine but does not share

obvious characteristics with other activation domain motifs (10, 15). It appears that Zta and VP16 each stimulate D-A complex assembly through interactions with different subunits of TFIIA. This scenario is similar to the interactions of distinct activators with different TAFs in the TFIID complex (9). While this study was in progress, Ozer et al. (49) reported that Zta interacts with the  $\gamma$  subunit of TFIIA. In their study, GST-Zta was incubated with the *in vitro*-translated  $\gamma$  subunit. Since it is the activation domain of Zta that interacts with TFIIA, and the Zta activation domain is near its N terminus, it is possible that some Zta-TFIIA contacts were sterically blocked by the fusion to GST, blocking the interaction with TFIIA $\alpha\beta$ . In our study, both GST-TFIIA binding assays and coimmunoprecipitation assays independently identified an interaction between the Zta activation domain and the TFIIA  $\alpha\beta$  subunit. Most importantly, our studies showed that the Zta activation domain interacts with holo-TFIIA, which consists of all three subunits. All three subunits are necessary for TFIIA binding to a promoter-DNA complex and stimulation of activator-dependent transcription (49, 57, 71).

How does the activator-TFIIA interaction stimulate D-A complex formation? The interaction probably contributes to the greater stability of the Zta-D-A complex than the D-A complex alone. Previous studies have shown that the activation domain of Zta interacts with TBP (37). Both TBP and *Drosophila* TAF<sub>II</sub>110 interact directly with TFIIA (49, 57, 70, 71). In addition, when the Zta activation domain is present in the complex, DNase I footprinting reveals TAF-DNA interactions that are not observed in the D-A complex (38). The multiple protein-protein interactions between TFIID, Zta, and TFIIA and the multiple DNA-protein interactions between promoter DNA, Zta, TFIID, and TFIIA could result in highly cooperative interactions of the three proteins and promoter DNA that stabilize the complex to a greater extent than the smaller number of interactions present in the D-A complex without Zta. In fact, the Zta-D-A complex is much more stable during the dilution that occurs during EMSA than is the D-A complex (38). Zta also stimulates the kinetics of D-A complex formation (38), as does the C-terminal domain of VP16 (Fig. 9). Kinetic measurements of open complex formation also indicate that Zta stimulates the formation of active preinitiation complexes by increasing the kinetics of TFIIA binding (10). Thus, Zta and VP16 appear to increase preinitiation complex formation by increasing both the stability of the D-A complex and the kinetics of its assembly.

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