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Requirement for Transcription Factor IIA (TFIIA)-TFIID Recruitment by an Activator Depends on Promoter Structure and Template Competition

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Different mechanisms of transcriptional activation may be required for distinct classes of promoters and cellular conditions. The Epstein-Barr virus (EBV)-encoded transcriptional activator Zta recruits the general transcription factors IID (TFIID) and IIA (TFIIA) to promoter DNA and induces a TATA box-binding protein (TBP)-associated factor-dependent footprint downstream of the transcriptional initiation site. In this study, we investigated the functional significance of TFIID-TFIIA (D-A complex) recruitment by Zta. Alanine substitution mutations in the Zta activation domain which eliminate the ability of Zta to stimulate the D-A complex were examined. These Zta mutants were defective in the ability to activate transcription from an EBV-derived promoter (BHLF1) but activated a highly responsive synthetic promoter (Z_7E4T). Both the number of activator binding sites and the core promoter region contribute to the requirement for D-A complex recruitment. These functionally distinct core promoters had significant differences in affinity for TBP and TFIID binding. The D-A complex-recruiting activity of Zta was found to be important for promoter selection in the presence of a competitor template. Conditions which limit TFIID binding to the TATA element or compromise the ability of TFIIA to bind TBP required activator stimulation of the D-A complex. These results indicate that D-A complex recruitment is one of at least two activation pathways utilized by Zta and is the essential pathway for a subset of promoters and conditions which limit TFIID binding to the TATA element.

Eukaryotic transcriptional initiation requires the formation of an active preinitiation complex at promoter core elements (38). The preinitiation complex consists of the general transcription factors, RNA polymerase II, and a poorly defined set of coactivators (19, 23, 50). Promoter-specific activators may stimulate one or more rate-limiting steps in the formation of an active preinitiation complex. Promoter structure and cellular context are likely to contribute significantly to determining which step will be rate limiting. Recognition of the core promoter by the general transcription factor IID (TFIID) is likely to be one of the first rate-limiting steps in the transcription initiation process (5). Suboptimal core promoter recognition by TFIID may become a substantial barrier to initiation, and it is likely that many activators and coactivators can overcome this rate-limiting step by interacting directly with TFIID.

Several lines of evidence suggest that TFIID binding to promoter DNA can be rate limiting for transcription in vivo (9, 16, 29, 30). TFIID is a multiprotein complex consisting of the TATA box-binding protein (TBP) and at least eight TBP-associated factors (TAFs) (7, 22, 25, 41, 51). The TAFs are essential for mediating activator-regulated transcription assayed in reconstituted transcription reaction mixtures (20, 45). Both TBP and the TAFs are direct physical targets of promoter-specific activators (20, 33, 35, 43, 49). Activator binding to TFIID is thought to stimulate transcription by stabilizing and enhancing the formation of the preinitiation complex.

The general transcription factor IIA (TFIIA) binds directly to TBP (6, 21, 44) and stabilizes TBP binding to the TATA box by making additional weak contact with sequences upstream of the TATA box (27, 32). TFIIA is essential for activator-mediated transcription in vitro (17, 40, 48) and in vivo (4). Like the TAFs, TFIIA mediates the interaction of TBP with promoter-specific activators and alters the DNA binding specificity of TFIID (15, 31, 40). The reversible association of TFIIA with TBP makes TFIIA binding an attractive candidate for regulation by activators. The formation of the TFIID-TFIIA promoter complex (D-A complex) has been shown to be the rate-limiting step enhanced by at least two mammalian transcriptional activators in vitro (10, 48). The Epstein-Barr virus (EBV)-encoded lytic activator Zta stimulates the formation of a highly stable D-A complex and alters the interaction of TFIID with sequences downstream of the transcriptional initiation site (36). Complex formation is dependent on the Zta activation domain, the TAFs in the TFIID fraction, and sequences near and downstream of the promoter start site. The Zta activation domain can interact directly with TBP (35) and both subunits of TFIIA (31, 40). The Zta-induced conformational change in TFIID binding was shown to contribute to transcriptional activation in vitro (11).

In this study we investigated the functional significance of the Zta-TFIID-TFIIA promoter complex (Z-D-A complex) by mutational analysis of the Zta activation domain. Mutations in the Zta activation domain which disrupt Z-D-A complex formation were mapped to combinations of hydrophobic aromatic residues scattered through the amino-terminal region of Zta. These mutants were used to investigate whether Zta can stimulate transcription by multiple mechanisms and whether D-A complex recruitment is the primary mechanism utilized for all promoters and transcription conditions.

MATERIALS AND METHODS

Plasmids. All Zta mutations were first generated from pPL500, which contains wild-type Zta with a hexahistidine amino-terminal tag cloned into the *Eco*RI site of pBluescript II KS$^+$ (Stratagene). Zta alanine substitution mutations were generated by overlap extension PCR, as described previously (24, 39). All mutations were confirmed by sequencing. The wild-type protein and mutants m.1 (F22A, F26A, W74A, and F75A), m.2 (L48A and W49A), m.3 (Q34A and

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D35A), and m.4 (P53A and E54A) were subcloned into the *Bam*HI-*Eco*RI site of the bacterial expression vector pQE8 (Qiagen) and in the *Eco*RI site of the eukaryotic expression vector pcDL-SRα296 (35). $pZ_7E4TCAT$, $pZ_3E4TCAT$, and pZ_7HCAT were gifts of M. Carey (12). The Z_3HCAT reporter was generated by substituting the *Bam*HI-*Hin*dIII Z_3 module from $Z_3E4TCAT$ for the Z_7 module in Z_7HCAT. The BHLF1 (H) core promoter sequence −41 to +41 was inserted into the *Bam*HI-*Kpn*I site of $pZ_7E4TCAT$. The H core promoter sequence is CCAAAAAGAGGATAAAAGAAGGCGAGCCGGCCCGGCTC GCCAGCG TCG TCCAGACGC TCGGGGGG TGCACACC TCCCAGCCGG (the TATA box is underlined). The BHLF1-CAT promoter is identical to the MinL-CAT construct described previously (35). Mutants m.1 (F22A, F26A, W74A, and F75A), m.2 (L48A and W49A), m.3 (Q34A and D35A) and m.4 (P53A and E54A) were also expressed as glutathione *S*-transferase (GST) fusions by subcloning into the *Bam*HI-*Eco*RI sites of pGEX-2T (Pharmacia). In vitro translation vectors for TBP, pTM1-TBP (33), $dTAF_{II}110$ (20), TFIIAαβ, TFIIAγ, and Zta have been described previously (40).

Proteins. Zta-derived proteins were all expressed to high levels from the pQE8 expression vector in the M15 strain. Proteins were purified by guanidinium lysis, Ni-nitrilotriacetic acid (NTA) purification, and renaturation into D100 buffer as described previously (39). Renatured Zta derivatives were examined for activity by gel mobility shift assay and for concentration by Coomassie staining of sodium dodecyl sulfate-polyacrylamide gels. Zta proteins were estimated to be greater than 70% pure. Recombinant human TFIIAαβ + γ subunits were generated as previously described (40). Immunoaffinity-purified flu-tagged TFIID was generated as described previously (51). One footprinting unit of TFIID was equivalent to 10 μl. The TFIIA-γW72A mutation has also been described previously (39). Recombinant human TBP was generated as a hexa-His fusion, purified on Ni-NTA agarose, and renatured as described previously (39).

Transfections and CAT assays. Plasmid DNAs were prepared by the Qiagen column purification procedure. HeLa cells were maintained in Dulbecco minimal essential medium supplemented with 10% fetal bovine serum, 0.292 mg of L-glutamine, 100 U of penicillin, and 100 μg of streptomycin per liter. HeLa cells were transfected with 1 μg of each input plasmid with lipofectase as suggested by the manufacturer (Gibco BRL). The DNA-lipofectase mixture was overlaid on cell monolayers for 14 h, followed by a change of medium. At 48 h posttransfection, the cells were harvested and lysed by three freeze-thaw cycles. Chloramphenicol acetyltransferase (CAT) assays were conducted by using the direct liquid scintillation assay, as described previously (8). Cell extracts were heat inactivated for 20 min at 65°C to inactivate endogenous CAT activity. After pelleting of the cellular debris, equal amounts of extract were incubated with [^{14}C]butyryl-coenzyme A and chloramphenicol for 2 h. Acetylated chloramphenicol was selectively extracted with 0.5 ml of xylene and then mixed with Econofluor-2 (NEN) for liquid scintillation counting. Zta expression levels were determined to be similar by Western blotting.

In vitro transcription and DNA binding assays. All transcription reactions were assayed by primer extension analysis with the CAT primer as previously described (34). Zta derivatives (~150 ng) were mixed with 50 μg of HeLa cell nuclear extract (18), 150 ng of supercoiled template DNA, and 500 μM ribonucleoside-5′-triphosphates. Transcription mixtures reconstituted with partially purified components included recombinant TFIIA (40), immunopurified TFIID (51), recombinant TFIIB, and partially purified TFIIE-TFIIF-RNA polymerase II and USA coactivator fractions as described previously (34, 37). Transcription reaction mixtures reconstituted with the TFIIA-γW72A mutation used TFIIA-depleted nuclear extracts as described previously (40). DNase I footprinting and Mg-agarose electrophoretic mobility shift assays (EMSAs) were also described previously (35, 36). Mg-acrylamide EMSA was performed in 4% polyacrylamide (30:1, bisacrylamide) gels cast and run in 5 mM MgOAc, 45 mM Tris-Cl, and 45 mM boric acid. Probes were end labeled with Klenow polymerase under identical conditions to generate similar specific activities.

GST binding assays. Purified GST fusion proteins (200 ng) were incubated with 2×10^4 cpm of ^{35}S-labeled protein generated from a rabbit reticulocyte-coupled transcription-translation system (Promega). Binding reactions and washing conditions were essentially identical to those described previously, except that the protein-binding buffer (PBB 300) was modified as follows: 20 mM HEPES (pH 7.9), 20% glycerol, 0.5 mM EDTA, 6 mM $MgCl_2$, 300 mM KCl, 0.2% Tween 20, 5 mM β-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride (40).

RESULTS

Generation of activation domain mutants with two distinct phenotypes. A series of deletion mutations and 17 alanine substitution mutations were generated in the Zta activation domain to identify residues critical for transcriptional activation function and the formation of the Z-D-A complex (unpublished results). Two alanine substitution mutations (m.1 and m.2) which were defective in formation of the Z-D-A complex were examined further in this study. These mutations resulted from the replacement of a combination of aromatic residues with alanine. A second class of Zta mutations (m.3 and m.4) were capable of stimulating the Z-D-A complex but were found to be generally defective in stimulation of transcriptional activation. Zta mutants m.3 and m.4 resulted from the substitution of alanine for acidic residues in hydrophobic patches of the Zta activation domain. These Zta mutants were purified and assayed together for the ability to stimulate formation of the Z-D-A complex by DNase I footprinting and Mg-agarose EMSA (Fig. 1). Zta and the four mutants were compared for the ability to stimulate TATA box protection and formation of the downstream hypersensitive sites on the Z_7E4T promoter with rTFIIA and TFIID (Fig. 1). DNase I footprinting analysis indicated that m.1 and m.2 were incapable of stimulating the downstream hypersensitive sites at positions +2 and +13 (Fig. 1A). Furthermore, m.1 and m.2 did not stimulate the TFIID footprint over the TATA box. In contrast, m.3 and m.4 functioned indistinguishably from wild-type Zta in the ability to stimulate the hypersensitive sites downstream of the start site and protection over the TATA box (Fig. 1A, lanes 10 and 12). Mg-agarose EMSA clearly confirmed that m.1 and m.2 were incapable of forming the Z-D-A complex, while m.3 and m.4 were essentially equivalent to the wild type in Z-D-A complex formation (Fig. 1B).

Promoter-specific requirement for D-A complex recruitment. Previous work has suggested that Z-D-A complex formation correlates with general transcription activation function (11, 12, 36). To directly test the requirement for Z-D-A complex formation on transcriptional activation function, we compared the ability of wild-type and mutant Zta to stimulate transcription from two classes of promoters in vivo (Fig. 2A) and in vitro (Fig. 2B). Zta and the mutants were examined for transcriptional activity from the highly responsive synthetic Z_7E4T promoter and from the EBV-derived BHLF1 promoter (35). The Z_7E4T promoter contains seven Zta bindings sites fused upstream of the adenovirus E4 TATA element. The BHLF1 promoter used in these experiments consists of four Zta binding sites upstream of a noncanonical TATA element (GATAAA). In transfection experiments, we found that Zta mutants m.1 and m.2 were significantly reduced in the ability to activate the BHLF1 promoter but were more active than the wild type in the stimulation of the Z_7E4T promoter (Fig. 2A). Mutants m.3 and m.4 were reduced in transcription from both promoters (Fig. 2A). These results were confirmed by in vitro transcription reactions with each template alone or with the two templates mixed in the same reaction (Fig. 2B). Zta proteins used for in vitro transcription reactions were normalized for DNA binding activity, thus eliminating any variation in activator protein levels (Fig. 2C). In transcription reactions with single templates, m.1 and m.2 stimulated the Z_7E4T promoter better than the wild type but were reduced to less than 30% of wild-type activity on the BHLF1 promoter (Fig. 2B, compare lanes 9 and 10 with 3 and 4). The defect in m.1 and m.2 was particularly dramatic when the two templates were included in the same reaction mixture (lanes 13 to 18). In these reactions, m.1 and m.2 stimulated almost undetectable levels of transcription from the BHLF1 promoter and were only slightly reduced in stimulation of the Z_7E4T promoter relative to wild-type Zta (lanes 15 and 16). Interestingly, we found that m.3 and m.4 were slightly more defective on the Z_7E4T promoter than they were on BHLF1 (lanes 17 and 18). These results suggest that Z-D-A complex formation is essential for transcriptional activation of the BHLF1 promoter but not the Z_7E4T promoter. Results with m.3 and m.4 suggest that Z-D-A complex formation is not sufficient for high-level transcriptional activation of the Z_7E4T promoter.

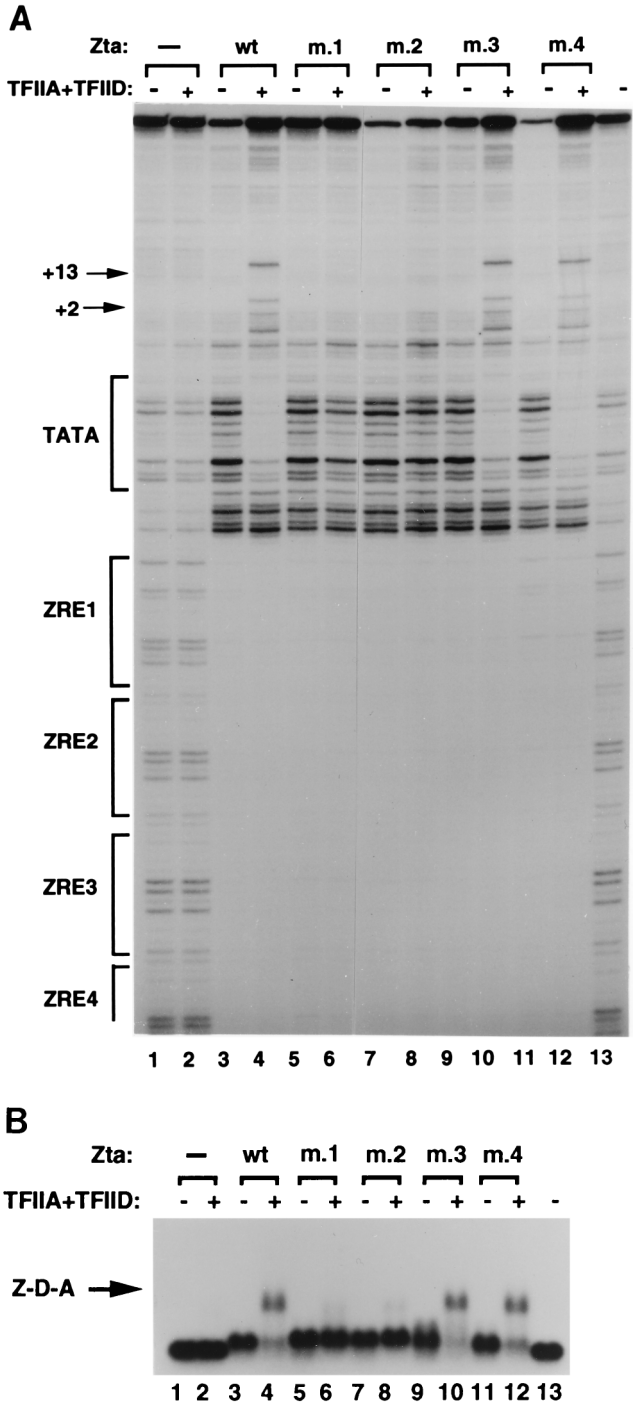

FIG. 1. Mutations in the Zta activation domain disrupt Z-D-A complex formation. (A) DNase I footprinting of the Z-D-A complex formed with Zta mutants m.1 (F22A, F26A, W74A, and F75A), m.2 (L48A and W49A), m.3 (Q34A and D35A), and m.4 (P53A and E54A) on the Z_7E4T promoter with affinity-purified TFIID and recombinant TFIIA. The TATA box and DNase I hypersensitive sites at the +2 and +13 positions are indicated. The Z_7E4T promoter was labeled at the *Hin*dIII position 5′ to the TATA box. wt, wild type. (B) An aliquot of the DNase I footprinting reaction mixture described for panel A was analyzed by Mg-agarose EMSA. The stable Z-D-A complex is indicated by the arrow.

Promoter structure determines the requirement for D-A complex recruitment. To determine the relative contributions of the core promoter and number of activator binding sites to the requirement for D-A recruitment, we compared the activity of Zta mutants on a set of promoters which vary the number of Zta binding sites (Z7 versus Z3) or the core promoter (E4 versus H) (Fig. 3). The H core promoter was derived from BHLF1. The effect of the Zta mutants on the various promoters was plotted as a percentage of wild-type activity. The Z_7H and Z_7E4T promoters behaved similarly, with m.1 and m.2 producing higher levels of transcription than wild-type Zta. On the Z_3E4T promoter, we found that the m.1 mutant was reduced to 60% of wild-type Zta activity, but the activity of the m.2 mutant was greater than the wild-type (Fig. 3A). On the Z_3H promoter, m.1 and m.2 were reduced to 38 and 44% of wild-type activity. Zta mutants m.3 and m.4 were reduced for all promoters relative to the wild type but most significantly for promoters with seven Zta binding sites. These results were corroborated by in vitro transcription reactions (Fig. 3B). The in vitro activation levels were similar to those observed for in vivo transfection. Zta mutants m.1 and m.2 were more defective on promoters with three binding sites than on those with seven binding sites. In contrast, Zta mutants m.3 and m.4 were more defective on promoters with seven Zta binding sites than on those with three Zta binding sites, suggesting that these mutants define an activation function that is mechanistically distinct from m.1 and m.2. Taken together, these results indicate that multiple activator binding sites can compensate for a weak core promoter and an activator defective in D-A complex recruitment.

Functionally distinct core promoters differ in their affinity for TFIID. Core promoter structure may determine which step in the formation of an active preinitiation complex is rate limiting. To determine if the BHLF1 and Z_7E4T core promoters had different affinities for TFIID or TBP, we compared the two core promoters' abilities to form complexes with TBP (Fig. 4A) or TFIID (Fig. 4B) in the absence and presence of TFIIA. The H core promoter (−41 to +41) was assayed in the same background as the E4T promoter by deriving probe from the Z_7H construct. Mg-acrylamide EMSA analysis revealed that TBP bound to the E4 TATA box with as little as 25 ng of TBP, while similar concentrations of TBP had no detectable binding on the BHLF1 core promoter (Fig. 4A, compare lanes 2 to 4 and 11 to 13). Addition of TFIIA stimulated the binding of TBP to both promoters by almost 10-fold. The TFIIA-TBP complex bound to the E4T promoter with almost fourfold greater activity than its binding to the BHLF1 promoter (Fig. 4A, compare lanes 5 to 7 and 14 to 16). These results were confirmed by DNase I footprinting, which clearly showed specific interaction of TBP with the respective TATA element of each promoter (data not shown). It is also worth noting that TFIIA-TBP migrates faster than TBP in this gel system, as was previously reported (39).

Since TBP exists primarily as a multiprotein TFIID complex, we examined whether TFIID also had a similar greater affinity for the E4T promoter than for the H promoter. In acrylamide EMSA we found that TFIIA and TFIID could form a complex on the E4 but not the H promoter (Fig. 4A, lanes 9 and 18). To confirm this result, we compared the ability of TFIID to bind to the two promoters in the sensitive Mg-agarose gel system. As was observed with TBP, TFIID also bound to the E4 TATA better than to the H promoter (Fig. 4B, compare lanes 2 and 3 with 11 and 12). The addition of TFIIA stimulated binding modestly, but little was detected on the BHLF1 promoter. Addition of Zta and TFIIA stimulated a massive increase in complex formation for both the E4T and H promoters (Fig.

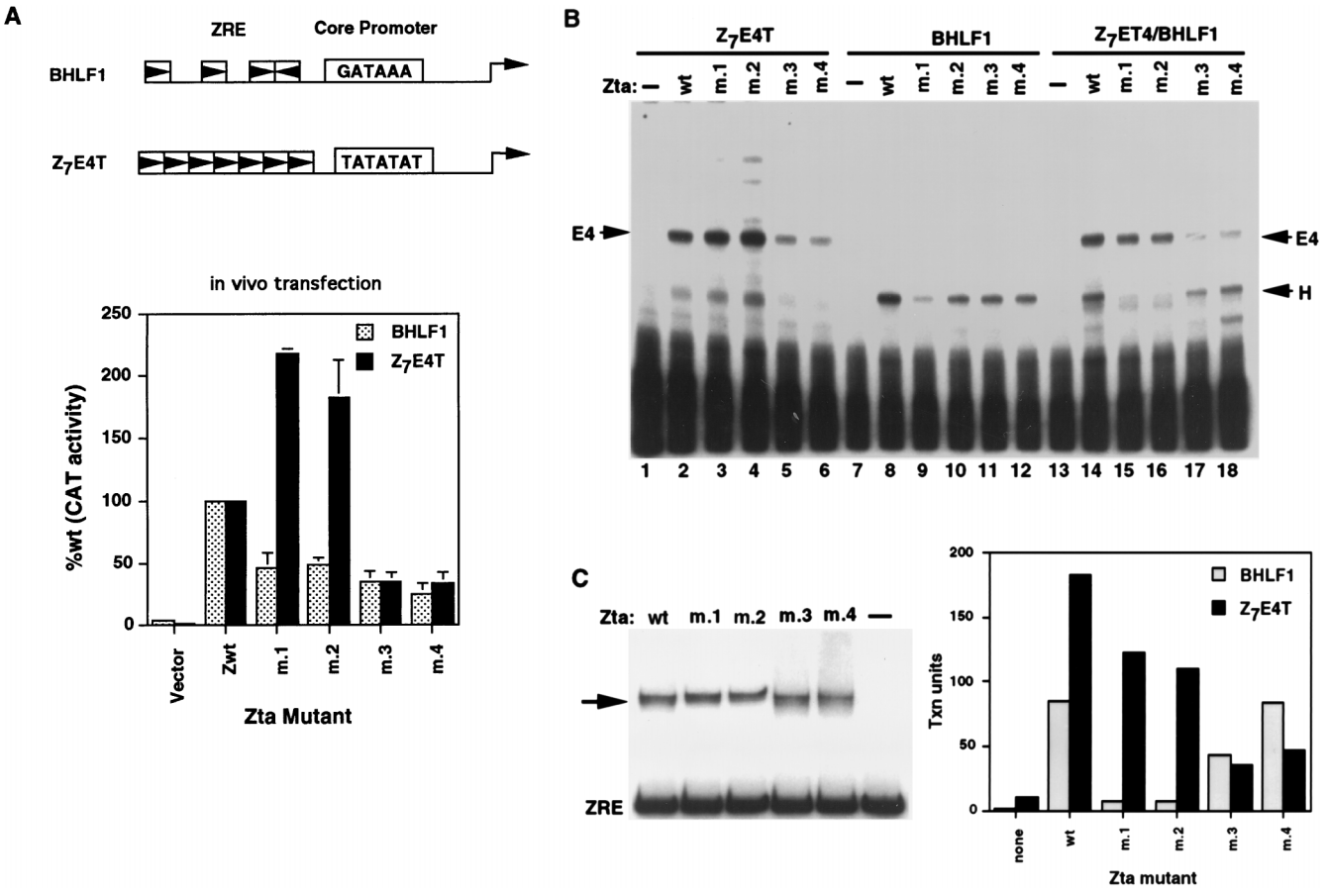

FIG. 2. Promoter specificity of Zta activation domain mutants. Zta mutants were assayed for transcriptional activation of two distinct promoters. (A) The BHLF1CAT and Z_7E4TCAT constructs were transfected in HeLa cells with the indicated Zta mutant or vector (pSRα). CAT activity is presented as a percentage of wild-type (wt) Zta activity. Values are averages from at least three independent experiments. (B) The Z_7E4T promoter (lanes 1 to 6), the BHLF1 promoter (lanes 7 to 12), or the two promoters together (lanes 13 to 18) were assayed for responsiveness to Zta derivatives in in vitro transcription reactions with HeLa cell nuclear extracts. (C) Quantitation of in vitro transcription reactions for lanes 13 to 18 in panel B with a PhosphorImager (Molecular Dynamics) (right) and by normalization of Zta mutants by EMSA (left).

4B, compare lanes 8 and 9 with 17 and 18). The Z-D-A complex formed on the E4T and H promoters was further examined by DNase I footprinting. In the presence of Zta, TFIIA, and TFIID, both the E4 TATA and the BHLF1 GATA elements are completely protected (Fig. 4D). No protection was generated in the absence of Zta or TFIIA (data not shown). Both promoters reveal extensive interactions with sequences near and downstream of the transcriptional initiation site, indicating that the Z-D-A complex is qualitatively similar on both promoters.

D-A complex recruitment is required for template commitment. Recruitment and stabilization of the D-A complex by Zta may be essential for promoter selection and commitment. To test the contribution of the Z-D-A complex to promoter selection, we compared the abilities of Zta, m.1, and m.2 to stimulate transcription from the Z_7E4T promoter in the presence or absence of an equimolar competing template containing the adenovirus E1B promoter (51) (Fig. 5A). The addition of the E1B promoter reduced the ability of mutants m.1 and m.2 to stimulate transcription while having only a small effect on wild-type Zta. Thus, promoter competition reveals the selective advantage of Zta relative to m.1 and m.2. Presumably this advantage results from the ability of wild-type Zta to recruit and stabilize the D-A complex.

D-A complex recruitment is required when TATA binding is made rate limiting. Conditions which limit TFIID binding to the TATA element are likely to require activator stimulation of the D-A promoter complex. The Z-D-A complex-defective mutants m.1 and m.2 were compared with wild-type Zta for in vitro transcription activity with the Z_7E4T promoter (Fig. 5B). In unfractionated nuclear extracts, we found that m.1 and m.2 were more active than wild-type Zta, which is consistent with our previous results in vitro and in vivo (Fig. 5B, lanes 1 to 4). This suggests that TFIID-TFIIA binding is not rate limiting for this promoter in HeLa cell nuclear extracts. Since TFIIA may be preassembled with TFIID in unfractionated extracts, we tested the ability of these mutants to activate transcription in reaction mixtures reconstituted with recombinant TFIIA and affinity-purified TFIID (Fig. 5B, lanes 5 to 8). Reconstitution of transcription mixtures with TFIIA and TFIID did not reveal a defect in Zta mutants m.1 and m.2. We next tested whether these Zta mutants had a phenotype when TFIID binding was made artificially rate limiting by the addition of a molar excess of TATA oligonucleotide to transcription reaction mixtures during preinitiation complex assembly. Transcription reaction mixtures were preassembled with TFIIA, TFIID, and Zta in the presence of a 100-fold molar excess of TATA oligonucleotide. After a brief preincubation period, the remaining gen-

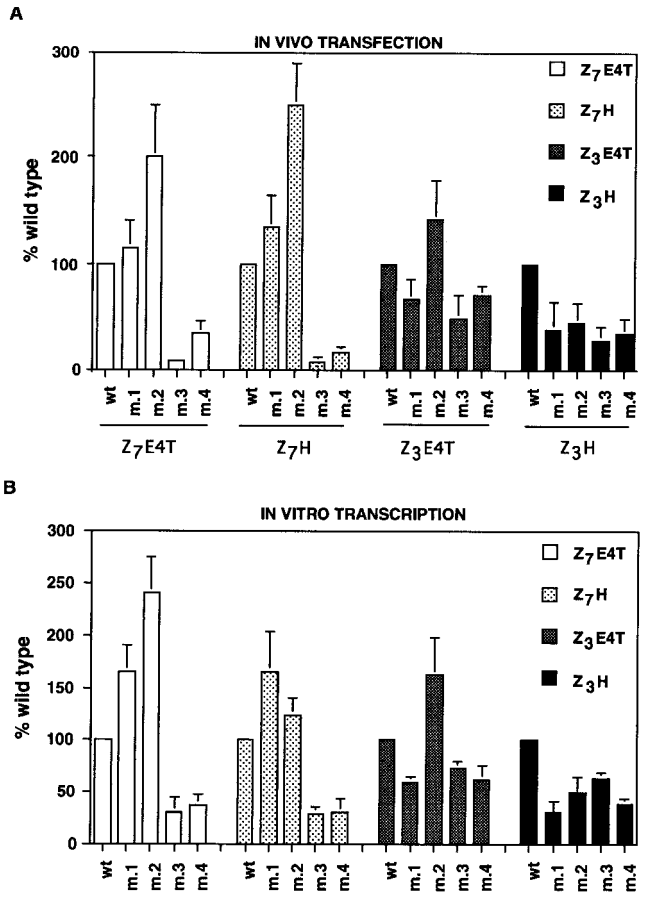

FIG. 3. The number of activator binding sites and the core promoter determine the requirement for D-A complex recruitment. (A) The Z_7E4T, Z_7H, Z_3E4T, and Z_3H promoter CAT constructs were contransfected with wild-type (wt) or mutant Zta in HeLa cells. CAT activity was determined 48 h posttransfection. Values of the average of at least seven independent experiments are plotted as the percentage of wt Zta activity for each promoter. (B) In vitro transcription analysis of wt and mutant Zta proteins on the Z_7E4T, Z_7H, Z_3E4T, and Z_3H promoter templates with HeLa nuclear extract. Transcription activity from primer extension reactions is plotted as the percentage of wt Zta activity for each promoter.

eral transcription factors and nucleotides were added to the reaction mixture. Under these conditions, m.1 and m.2 revealed a significant transcriptional defect (Fig. 5B, lanes 10 to 12). Thus, the requirement for the D-A complex-recruiting activity of Zta is important when the stability of the D-A promoter complex is challenged.

D-A complex recruitment is required when TFIIA binding to TBP is made rate limiting. Conditions which limit TFIIA binding to TBP should also require activator stimulation of the D-A promoter complex. To test if Zta mutants defective for D-A complex recruitment were sensitive to conditions which limit TFIIA binding to TBP, we utilized a mutant of TFIIA (TFIIA-γW72A) that was compromised in the ability to stimulate TBP-DNA binding (39). Crystal structures reveal that γW72 makes direct contact with TBP and predict that mutations of this residue will compromise the interaction between TFIIA and TBP (21, 44). TFIIA-γW72A was shown to be defective in the ability to stimulate TBP binding to DNA (Fig. 6, left panel). The Zta wild type and Z-D-A complex-defective mutants (m.1 and m.2) were compared for the ability to stimulate transcription in reaction mixtures reconstituted with TFIIA-γW72A (Fig. 6, right panel). In unfractionated HeLa nuclear extracts, m.1 and m.2 stimulated transcription better than wild-type Zta (Fig. 6, lanes 1 to 4). In TFIIA-depleted nuclear extracts, no transcription was detectable (Fig. 6, lanes 5 to 8). TFIIA-depleted extracts reconstituted with wild-type TFIIA resulted in a pattern of expression identical to those of unfractionated nuclear extracts (Fig. 6, lanes 9 to 12). When TFIIA-depleted extracts were reconstituted with TFIIAγ-W72A, only wild-type Zta produced high levels of transcription (Fig. 6, lanes 13 to 16). Zta mutants m.1 and m.2 were significantly reduced in the ability to activate transcription in reaction mixtures reconstituted with TFIIA-γW72A (Fig. 6, lanes 15 and 16). This biochemical synthetic lethality strongly suggests that m.1 and m.2 are defective in stimulating TFIIA-TBP binding. They also support the conclusion that recruitment of the D-A complex by an activator is required when the interaction of TFIIA with TBP is made rate limiting.

Direct binding of Zta to TFIIA and TFIID is important for activation function. To determine whether mutations in the Zta activation domain were capable of disrupting physical interaction with general transcription factors, derivatives of Zta were expressed as GST fusion proteins and assayed for binding to in vitro-translated general factor polypeptides (Fig. 7). We compared several general transcription factors for the ability to bind to GST-Zta; to GST-ΔZta, which lacks the amino-terminal activation domain; or to GST-m.1, -m.2, -m.3, and -m.4. In general we found that TFIIAαβ, TFIIAγ, TBP, and $dTAF_{II}110$ interacted with the activation domain of Zta. We could not identify a specific interaction between Zta and TFIIB, $dTAF_{II}40$, $dTAF_{II}80$, $dTAF_{II}30\alpha$, $dTAF_{II}30\beta$, $dTAF_{II}150$, PC4, TFIIE, or TFIIF (data not shown). Zta was included as a positive control for GST-Zta proteins since Zta forms homodimers through its zipper domain. These experiments revealed that Zta mutant m.1 was defective in binding to TFIIAαβ and TBP. This mutation was also slightly reduced in binding to $dTAF_{II}110$ but was not affected for binding to TFIIAγ or Zta. No binding defects could be found for the other Zta mutations, even though the transcription and Z-D-A complex phenotype of m.2 was similar to that of m.1. These results indicate that some of the hydrophobic aromatic residues (F22, F26, W74, and F75) in the Zta activation domain are essential for direct physical interaction between Zta and the general factors TFIIA and TFIID.

DISCUSSION

Eukaryotic activators stimulate transcription by several distinct mechanisms. Zta is representative of a class of activators that can stimulate D-A promoter complex formation (31). In this work, point mutations in the Zta activation domain which abolish D-A complex-stimulatory activity were used to examine the function of D-A complex recruitment in transcriptional activation. Combinations of aromatic residues in the Zta activation domain were essential for recruitment of the D-A complex (Fig. 1). D-A complex-recruiting activity was required for the full activation of the EBV-derived BHLF1 promoter but not for the Z_7E4T synthetic promoter (Fig. 2A). The requirement for D-A complex recruitment was determined by the number of activator binding sites and the core promoter structure (Fig. 3). The BHLF1 and E4 core promoters had different affinities for TBP and TFIID, and this difference could be compensated for by the addition of TFIIA and Zta (Fig. 4). Activation of the synthetic Z_7E4T promoter was made dependent on D-A complex-recruiting activity when transcription was challenged with a competitor template (Fig. 5A) or excess TATA oligonucleotide (Fig. 5B), or when transcription was

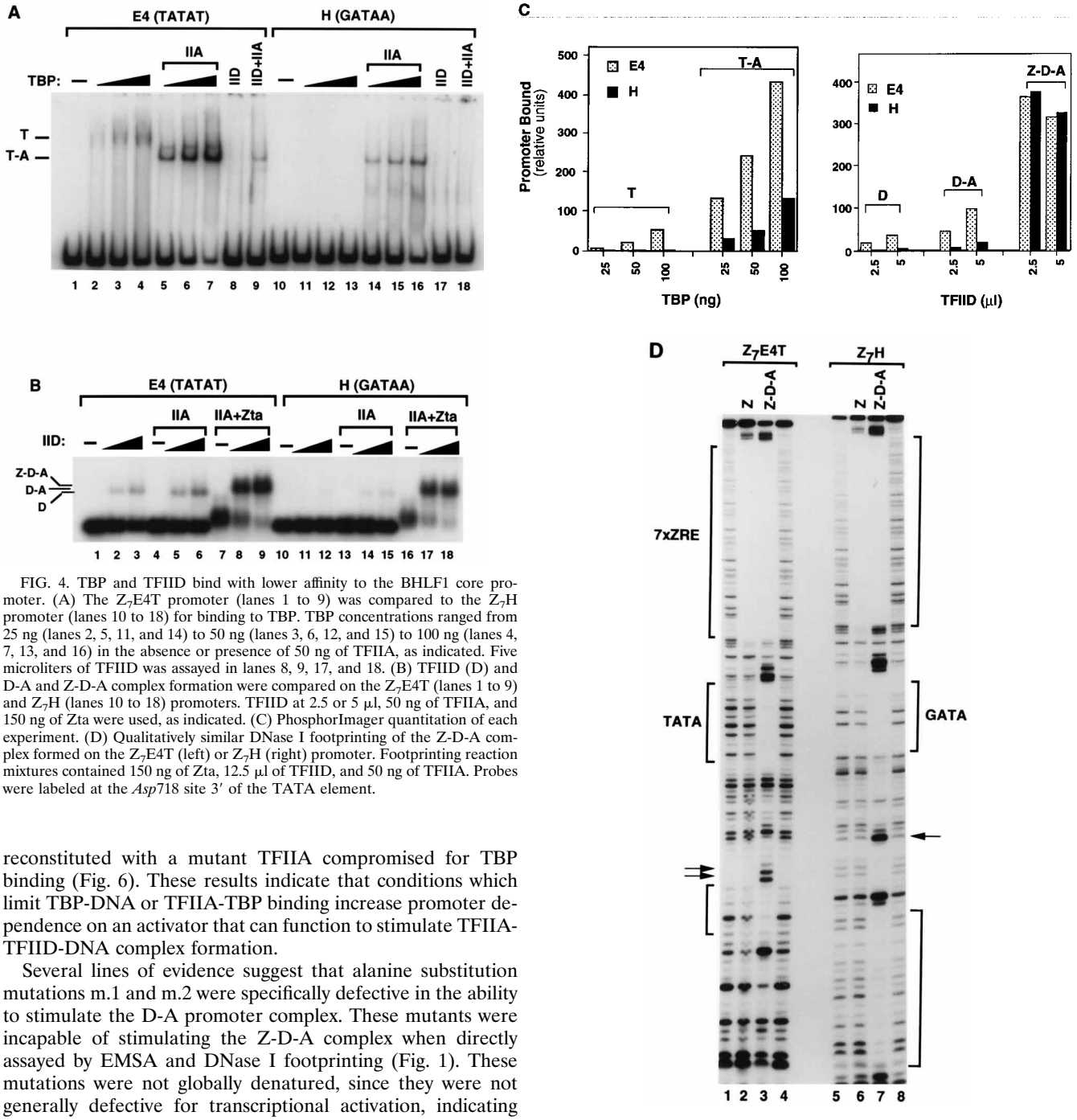

FIG. 4. TBP and TFIID bind with lower affinity to the BHLF1 core promoter. (A) The Z_7E4T promoter (lanes 1 to 9) was compared to the Z_7H promoter (lanes 10 to 18) for binding to TBP. TBP concentrations ranged from 25 ng (lanes 2, 5, 11, and 14) to 50 ng (lanes 3, 6, 12, and 15) to 100 ng (lanes 4, 7, 13, and 16) in the absence or presence of 50 ng of TFIIA, as indicated. Five microliters of TFIID was assayed in lanes 8, 9, 17, and 18. (B) TFIID (D) and D-A and Z-D-A complex formation were compared on the Z_7E4T (lanes 1 to 9) and Z_7H (lanes 10 to 18) promoters. TFIID at 2.5 or 5 μl, 50 ng of TFIIA, and 150 ng of Zta were used, as indicated. (C) PhosphorImager quantitation of each experiment. (D) Qualitatively similar DNase I footprinting of the Z-D-A complex formed on the Z_7E4T (left) or Z_7H (right) promoter. Footprinting reaction mixtures contained 150 ng of Zta, 12.5 μl of TFIID, and 50 ng of TFIIA. Probes were labeled at the *Asp*718 site 3′ of the TATA element.

reconstituted with a mutant TFIIA compromised for TBP binding (Fig. 6). These results indicate that conditions which limit TBP-DNA or TFIIA-TBP binding increase promoter dependence on an activator that can function to stimulate TFIIA-TFIID-DNA complex formation.

Several lines of evidence suggest that alanine substitution mutations m.1 and m.2 were specifically defective in the ability to stimulate the D-A promoter complex. These mutants were incapable of stimulating the Z-D-A complex when directly assayed by EMSA and DNase I footprinting (Fig. 1). These mutations were not globally denatured, since they were not generally defective for transcriptional activation, indicating that interaction with other general transcription factors was less likely to be compromised. These mutants were defective in activating transcription reconstituted with TFIIA mutations compromised in the ability to bind TBP, indicating a sensitivity to further defects in D-A complex formation. Similarly, these mutations were sensitive to competition with TATA oligonucleotide, suggesting a defect in the ability to stimulate TBP binding. Finally, mutant m.1 was shown to be defective in direct interaction with TFIIAαβ, TBP, and $dTAF_{II}110$ (Fig. 7). Formally, we can not rule out the possibility that these mutants are also defective in interacting with an additional general transcription factor or coactivator which may contribute to the observed transcriptional phenotype. Nevertheless, it seems likely that the transcriptional defects of m.1 and m.2 are at least partly a result of the inability to recruit the D-A complex.

Mutations in Zta that abolish D-A complex formation were still capable of activating transcription from the Z_7E4T promoter. This suggests that Zta can activate transcription by at least two distinct mechanisms. The alanine substitution mutations m.3 and m.4 had no effect on D-A complex formation but were reduced in transcription from the Z_7E4T and Z_7H promoters. Both m.3 and m.4 substitute a single acidic residue isolated within a large stretch of hydrophobic amino acids in the Zta activation domain. We consider it probable that these acidic residues are essential for the interaction of Zta with

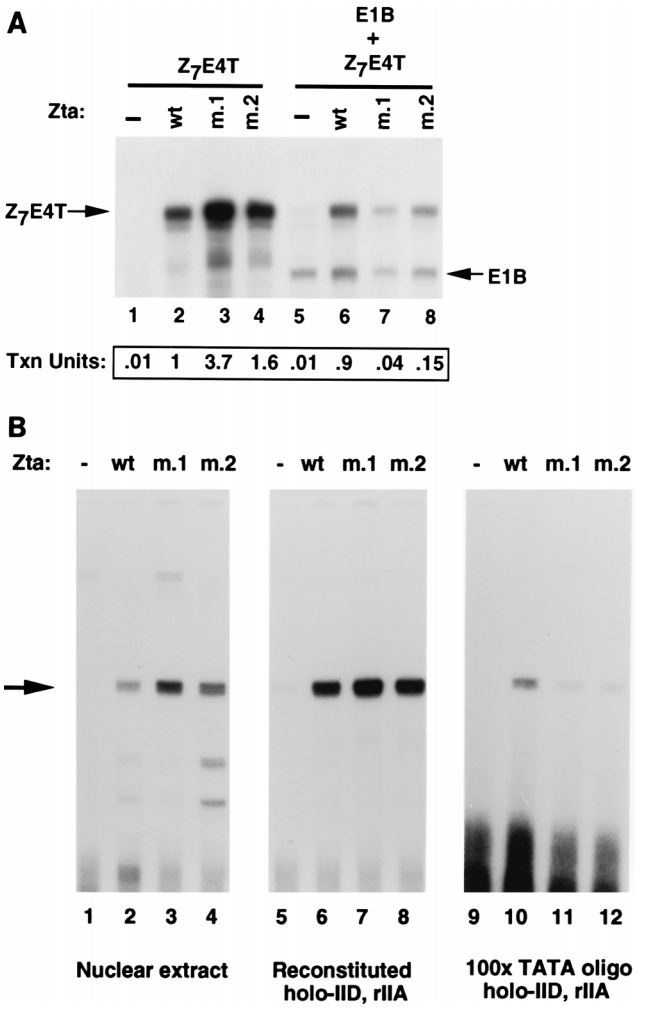

FIG. 5. D-A complex recruitment is required to overcome template and TATA box competition. (A) Transcription reaction mixtures were reconstituted with HeLa nuclear extracts, purified Zta, m.1, or m.2 and with $pZ_7E4TCAT$ in the absence (lanes 1 to 4) or presence (lanes 5 to 8) of an equimolar concentration of the pE1BCAT plasmid. Zta, m.1, and m.2 were added as indicated above each lane. Txn, transcription. (B) TFIIA recruitment is essential when TATA binding is made rate limiting. Purified wild-type (wt) Zta, m.1, or m.2 was added to transcription reaction mixtures reconstituted with the Z_7E4T promoter and unfractionated HeLa cell nuclear extracts (lanes 1 to 4) or with affinity-purified TFIID (holo-IID), TFIIA, and partially purified general transcription factors (lanes 5 to 12). A 100-fold molar excess of TATA oligonucleotide was added during a preincubation step with promoter DNA, TFIID, TFIIA, and either wt Zta (lane 10), m.1 (lane 11), or m.2 (lane 12).

general factors and coactivators distinct from the D-A complex. One attractive candidate would be holo-RNA polymerase II, but we have not yet demonstrated an interaction between wild-type Zta and any known components in the holoenzyme. It is also possible that m.3 and m.4 make essential contacts with coactivators that are not yet described but are important for activation functions in vitro and in vivo. The target of m.3 and m.4 is likely to represent an activation pathway that is distinct from the recruitment of TFIID and TFIIA. Both activation mechanisms may be required for promoters with few Zta binding sites and nonconsensus TATA elements. TFIID binding to the promoter has been shown to be a rate-limiting step that activators could target to stimulate transcription (9, 16, 29, 30). However, TFIIB and components of holo-RNA polymerase II have also been identified as rate-limiting targets of activator recruitment (3, 14). The most consistent interpretation of these contrasting results is that activators stimulate multiple distinct steps in the assembly of an active preinitiation complex and that the rate-limiting steps may be determined by promoter structure (e.g., the number of activator binding sites), cellular conditions (e.g., relative concentration of factors), or experimental design (e.g., addition of competitor template).

In addition to the recruitment of TFIIA and TFIID to promoter DNA, Zta induces a conformational change in TAF interaction with downstream promoter elements (11). This isomerization of TFIID induced by Zta was reported to be necessary and sufficient for transcriptional activation from the E4T core promoter (11). Our results suggest that Zta can also activate transcription by an alternative pathway. We found that Zta mutants m.1 and m.2 were incapable of recruiting TFIIA and TFIID but were capable of activating transcription from the Z_7E4T promoter. We found no evidence that these mutants were able to induce the downstream hypersensitive sites at concentrations of TFIID that we were able to generate. This implies that activator-mediated TFIID isomerization may not be necessary for all promoters, which would be consistent with the observation that some activators have no direct effect on TFIID-TFIIA binding. It seems more likely that TFIID isomerization can be induced either by direct interaction with activator and TFIIA or, indirectly, by recruitment of other general factors and coactivators. The direct recruitment and stimulation of the D-A complex is essential for a subset of promoters, like BHLF1, which have weak core promoters and limited activator binding sites. Our data also suggests that TFIID isomerization is not sufficient for high-level transcriptional activation. Zta mutants m.3 and m.4 clearly induce the downstream hypersensitive sites indicative of a TFIID conformational change, yet these mutants fail to stimulate transcription, especially from the Z_7E4T promoter. This result is consistent with previous observations, which suggested that Zta must functionally interact with TFIIB and a coactivator fraction to achieve high-level transcription activation (34).

The importance of the TFIIA-TBP and TAF-TBP interactions in transcriptional activation in vivo is strongly supported by mutations in TBP which specifically disrupt activated but not basal transcription (4, 42, 46). Furthermore, TFIIA-depleted extracts failed to support activation from all activators and promoters tested, clearly indicating that TFIIA is essential for activated transcription in general (40). While TFIIA-induced conformational change of TFIID may be essential for most activated transcription, the D-A complex may not be the direct target of all activators. TAFs and TFIIA make direct contact with DNA elements downstream and upstream, respectively, of the TATA box element (21, 32, 44, 47). The TAFs may interact with downstream sequences of some promoters, like the adenovirus major late promoter, in the absence of activator stimulation (13, 51). Other promoters, like E4 and BHLF1, require activator stimulation to generate TFIID interaction with downstream sequences. Thus, activators provide functions to compensate for low-affinity interactions between the promoter and the TAFs. It is likely that amplification of additional low-affinity interactions with other general factors and coactivators, as occurs on promoters with reiterated activator binding sites, can overcome the requirement for direct recruitment of the D-A complex by the activator. Our findings support the general model that direct activator recruitment of the D-A complex is essential under conditions which limit TFIID binding to the TATA element or TFIIA interaction with TBP (Fig. 5 and 6). These conditions are likely to exist in vivo where promoter competition and TBP-specific repressors, like Mot1p/ADI or DR1/NC2, are

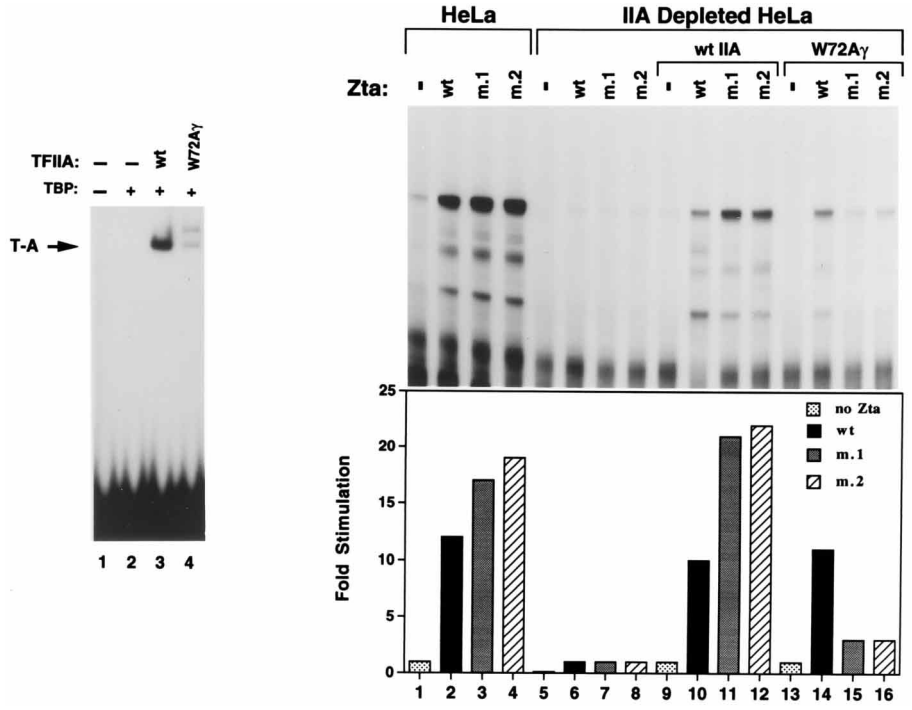

FIG. 6. TFIIA recruitment is essential when TFIIA binding to TBP is made rate limiting. A mutation in the TFIIA small subunit (γW72A) results in a defective TBP-TFIIA complex (left panel). Wild-type (wt) Zta, m.1, and m.2 were incubated with untreated nuclear extracts (right panel, lanes 1 to 4) or with nuclear extracts depleted of TFIIA by Ni-NTA resin (lanes 5 to 8). TFIIA-depleted extracts were reconstituted with wt recombinant TFIIA (lanes 9 to 12) or with TFIIA-γW72A (lanes 13 to 16). PhosphorImager quantitation of primer extension reactions is presented as fold stimulation relative to comparable reactions without activator (lower right panel).

present in high concentrations (1, 28). D-A complex recruitment may also be essential to overcome chromatin-associated repression of some promoters (26).

Why has Zta evolved the capacity to efficiently stimulate a D-A promoter complex? In the reactivation of latent EBV, Zta stimulates transcription from multiple viral genes necessary to initiate lytic-cycle replication. Both promoter selectivity and transcriptional derepression may govern the regulation of lytic-cycle gene expression. The lytic-cycle viral genes must be kept transcriptionally silent in the absence of Zta. Although we have examined only one lytic promoter in this study, inspection of the EBV sequence reveals that many viral lytic promoters have noncanonical TATA elements (2). Interestingly, the human T-cell leukemia virus type 1 (HTLV-1) Tax protein also stimulates D-A complex formation and is required for the reactivation of a latent virus (15). Thus, the coordinate activation of lytic-cycle promoters may require the specific recruitment of TFIIA by activators like Zta to overcome rate-limiting conditions imposed by weak core promoters and TBP-specific repressors.

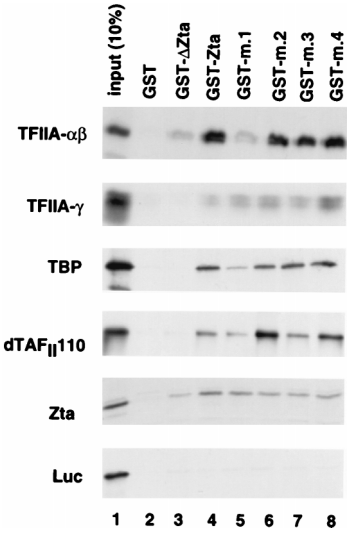

FIG. 7. Activation domain mutations disrupt binding to TFIIA and TBP. GST and GST fusion proteins of Zta and mutants were compared for the ability to bind to ^{35}S-labeled in vitro-translated proteins. Lane 1 shows 10% input of each labeled protein, as indicated on the left.

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