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Identification of Functional Targets of the Zta Transcriptional Activator by Formation of Stable Preinitiation Complex Intermediates

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Transcriptional activator proteins stimulate the formation of a preinitiation complex that may be distinct from a basal-level transcription complex in its composition and stability. Components of the general transcription factors that form activator-dependent stable intermediates were determined by the use of Sarkosyl and oligonucleotide challenge experiments. High-level transcriptional activation by the Epstein-Barr virus-encoded Zta protein required an activity in the TFIID fraction that is distinct from the TATA-binding protein (TBP) and the TBP-associated factors. This additional activity copurifies with and is likely to be identical to the previously defined coactivator, USA (M. Meisterernst, A. L. Roy, H. M. Lieu, and R. G. Roeder, Cell 66:981–994, 1991). The formation of a stable preinitiation complex intermediate resistant to Sarkosyl required the preincubation of the promoter DNA with Zta, holo-TFIID (TBP and TBP-associated factors), TFIIB, TFIIA, and the coactivator USA. The formation of a Zta response element-resistant preinitiation complex required the preincubation of promoter DNA with Zta, holo-TFIID, TFIIB, and TFIIA. Agarose gel electrophoretic mobility shift showed that a preformed Zta–holo-TFIID–TFIIA complex was resistant to Sarkosyl and to Zta response element oligonucleotide challenge. DNase I footprinting suggests that only Zta, holo-TFIID, and TFIIA make significant contacts with the promoter DNA. These results provide functional and physical evidence that the Zta transcriptional activator influences at least two distinct steps in preinitiation complex assembly, the formation of the stable holo-TFIID-TFIIA-promoter complex and the subsequent binding of TFIIB and a USA-like coactivator.

The assembly of a preinitiation complex at the start site of RNA polymerase II (Pol II)-dependent promoters is a highly regulated multistep process (reviewed in references 45, 53, and 54). The binding of TFIID to the TATA box of the promoter constitutes an early, if not the first, step in the assembly process. TFIID is a high-molecular-weight protein complex consisting of the TATA box-binding protein (TBP) and at least seven TBP-associated factors (TAFs) (11, 55; reviewed in references 12, 13, 20, and 48). TBP binds directly to the TATA element and functions in vitro to reconstitute basal-level transcription. The TAFs are essential for activated transcription from TATA-containing and TATA-less promoters (11, 41, 42, 49, 55). TFIIA and TFIIB can assemble after TFIID binds to the promoter, and both TFIIA and TFIIB bind directly to TBP (5, 36). TFIIA stabilizes the binding of TFIID to the TATA element and interacts with DNA just upstream of the TATA box (16, 36). The cDNAs encoding two subunits of TFIIA have been isolated from yeast cells and are essential for viability (43). The cDNA encoding human TFIIB has also been isolated (14). The carboxy-terminal region of TFIIB has been shown to bind directly to TBP, and the amino-terminal region interacts with RNA Pol II-TFIIF complex (2, 15). After RNA Pol II-TFIIF binds to the TFIID-TFIIA-TFIIB (D-A-B) complex, TFIIE, TFIIJ, and TFIIH assemble (54). TFIIH has both helicase activity and a kinase activity specific for the carboxy-terminal domain of RNA Pol II (35, 46). The preinitiation complex requires at least one ATP-dependent step prior to the formation of the first dinucleotide bond (47).

The general transcription factors required for basal-level transcription from a minimal promoter containing a TATA element have been identified. A minimal transcription complex has recently been reconstituted with highly purified factors TFIIB, TBP, the small subunit of TFIIF (RAP 30), and RNA Pol II (50). In this system, the 74-kDa subunit of TFIIF and TFIIE only weakly stimulate transcription, and no requirement for TFIIA was observed (50). However, this minimal preinitiation complex cannot be regulated by most upstream regulatory factors. Regulated transcription requires that the preinitiation complex consist of several additional components. The best characterized of these components is the TAF-containing TFIID fraction, which may be partially reconstituted with recombinant TBP, drosophila TAF110, TAF250, and TAF65 to support activated transcription from Sp1 (22, 52). Several lines of evidence suggest that other activities are required to mediate the action of activators in addition to TAFs. The coactivator, USA, has been shown to stimulate activation by USF, NF-κB, and Sp1 (28, 37, 38). Several other coactivators (also referred to as mediators and adapters) have been reported to interact with upstream activators and are distinct from the basal-level transcription factors (3, 26, 27). Whether these coactivators are general factors that participate in all activated transcription or are partially specific for a certain class of activator is not clear. Moreover, how these coactivators modify the minimal basal transcription remains to be elucidated.

Promoter-specific activator proteins increase the rate of transcription presumably by recruiting one or more general transcription factors into a preinitiation complex (9, 21, 33, 34, 40, 51). The Epstein-Barr virus-encoded Zta protein is a model transcriptional activator that has been shown to bind directly to TBP (29). In addition, Zta has been found to stimulate the formation of a D-A-promoter complex (7, 30). Both the interaction with TBP and the stimulation of the D-A-promoter complex were shown to depend on the Zta activation domain. The interaction of activator proteins with general transcription factors is likely to stabilize a preinitiation complex intermediate that either is formed slowly or is unstable in the absence of

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the activator. Several experimental approaches have been used to identify stable intermediates in the assembly of the preinitiation complex. Challenge of preformed complexes with low concentrations of detergents like Sarkosyl have been successful in identifying the components of these preinitiation complex intermediates (1, 4, 18, 19, 44). Activator-specific oligonucleotide challenge experiments have also been successful in identifying components of a committed preinitiation complex (17, 23, 24). In this work, both Sarkosyl and activator-specific oligonucleotide challenge are used to identify stable preinitiation complex intermediates that are dependent upon the Zta transcriptional activator protein. We find evidence that Zta functionally interacts with a complex consisting of three general transcription factors and the coactivator, USA.

MATERIALS AND METHODS

Purification of transcription factors. HeLa cell nuclear extract (10) dialyzed in D buffer (20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.9], 20% glycerol, 1 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride) containing 100 mM KCl was loaded on a phosphocellulose column (Whatman P-11) at 15 mg/ml of resin (44). The 0.1 M KCl flowthrough was then chromatographed on a DE52 column at 15 mg/ml of resin, and the column was resolved with a linear gradient from 0.1 to 0.5 M KCl in D buffer. TFIIA activity eluted between 300 and 350 mM KCl and was dialyzed to 100 mM KCl D buffer. TFIIA* was further purified by chromatography over a TBP affinity column, using an epitope-tagged TBP bound to antibody-protein A-Sepharose resin. The phosphocellulose C fraction (0.3 to 0.5 M KCl step) was dialyzed to 100 mM KCl D buffer and loaded on a DEAE-Sepharose column at 15 mg/ml of resin. The column was washed with 5 column volumes of 100 mM KCl D buffer before the RNA Pol II-TFIIF-TFIIE activity was eluted with 300 mM KCl D buffer. This fraction was then dialyze to 100 mM KCl D buffer and used in transcription assays. Recombinant TFIIB and TBP were prepared from *Escherichia coli* as described previously (55). TFIID was prepared from LTR3α cells, which express an 11-amino-acid epitope-tagged TBP protein (55). Phosphocellulose D fraction was prepared from undialyzed nuclear extracts (10) loaded directly onto phosphocellulose columns at 15 mg/ml of resin and washed with 5 column volumes of 500 mM KCl D buffer. The TFIID fraction was eluted with 1.0 M KCl D buffer. Peak fractions were incubated for 6 to 12 h at 4°C with monoclonal antibody 12CA5 fixed to protein A-Sepharose (55) at 1 ml of D fraction per 100 μl of resin. The beads were washed for at least 5 min in 10 volumes of 400 mM KCl D buffer (twice) and then in 100 mM KCl D buffer (twice) before elution with 100 μl of peptide (1 mg/ml) in 0.1 M KCl D buffer (55). The eluted material was tested for purity by silver staining of sodium dodecyl sulfate (SDS)-polyacrylamide gels, and active material was referred to as holo-TFIID (hIID). The 12CA5-depleted phosphocellulose D fraction was dialyzed to 100 mM KCl D buffer and loaded onto a DEAE-Sepharose column at 15 mg/ml of resin. The fraction eluted with 0.3 M KCl was dialyzed to 100 mM KCl D buffer and referred to as DE-IID. Alternatively, the 12CA5-depleted phosphocellulose D fraction was made 0.1% Tween 40 and dialyzed to 100 mM KCl D buffer containing 0.1% Tween 40. This material was then loaded on a DEAE-Sepharose column, and the 100 mM KCl flowthrough material was loaded onto a heparin-Sepharose column at 5 mg/ml of resin. The column was washed in 100 mM D buffer and eluted with steps at 0.3 M KCl and 0.5 M KCl D buffer. The 0.5 M KCl fraction was dialyzed to 0.1 M KCl D buffer and was referred to as the USA fraction (38).

Zta protein was overexpressed in *E. coli* M15 as a fusion protein with six histidines at the amino terminus (30). Zta-expressing cells were induced with 1 mM isopropylthiogalactopyranoside (IPTG) for 45 min after the cells reached an optical density of 0.7. Cell pellets were lysed in 6 M guanidine hydrochloride lysis buffer A (Qiagen) and then purified over Ni-nitrilotriacetic acid (NTA) resin. Lysates from 1 liter of induced cells were passaged over 1 ml of Ni-NTA resin. Zta proteins were renatured directly on the Ni-NTA column by washing in 8 M urea followed by sequential reduction of urea in phosphate buffer as described previously (30). Zta was eluted in phosphate buffer at pH 5.5. Peak fractions were dialyzed into 100 mM KCl D buffer. GAL4-AH protein was purified as previously described (32).

In vitro transcription assays. Protein extracts consisting of 1.88 μg of DEAE-purified TFIIA fraction, 50 ng of recombinant TFIIB, 150 ng of recombinant Zta, 2.5 μg of the DEAE-purified Pol II-TFIIE-TFIIF fraction, 5 to 10 μl of affinity-purified hIID or 1.6 μg of DEAE-purified TFIID, and 0.5 to 1.0 μg of heparin-Sepharose USA fraction were mixed on ice in a 30-μl volume of 100 mM KCl D buffer. Twenty microliters of a solution containing $MgCl_2$, β-mercaptoethanol, 100 ng of DNA template, and ribonucleoside triphosphates (rNTPs) was added to the protein mixture to give a final concentration of 6 mM $MgCl_2$, 5 mM β-mercaptoethanol, and 0.4 mM NTP. Reaction mixtures were incubated for 60 min at 30°C, and then reactions were stopped by the addition of 50 μl of stop buffer (2% SDS, 20 mM EDTA, 200 mM NaCl) containing 100 μg of protein K and 400 μg of tRNA per ml. After 10 min of incubation at 40°C, the samples were extracted with phenol-chloroform and precipitated with 3 volumes of ethanol and 0.6 ng of radiolabeled oligonucleotide primer specific for the chloramphenicol acetyltransferase mRNA. The pellets were dried for 3 min in a Speed Vac concentrator, resuspended in 10 μl of hybridization solution containing 125 mM KCl and 25 mM Tris (pH 8.8), and incubated for 10 min at 68°C followed by 30 min at 39°C. The samples were spun in a microcentrifuge and then mixed with 25 μl of a solution containing 25 mM Tris (pH 8.8), 62.5 mM KCl, 6.25 mM $MgCl_2$, 375 mM dNTP, 50 μg of actinomycin D per ml, and 0.4 U of mouse mammary tumor virus reverse transcriptase (200 U/ml); Bethesda Research Laboratories). Reaction mixtures were incubated for 30 min at 39°C. Then, 30 μl of stop buffer was added, and mixtures were subjected to extraction with phenol-chloroform and precipitation with ethanol. Dried pellets were resuspended in 3 μl of formamide dye and loaded on 10% polyacrylamide–8 M urea gels. The chloramphenicol acetyltransferase primer had the sequence 5'-CTCAAAAT GTTCTTTACGATGCCATTGGGA-3'. The DNA template $pZ_7E4TCAT$ (6) and the $G_5E1BTCAT$ template (32) were described previously. In experiments with Sarkosyl challenge, preincubations in 25 μl were followed by addition of Sarkosyl to a final concentration of 0.035% (wt/vol) and then by addition of 25 μl of a mixture containing rNTPs and the remaining components that were also made 0.035% Sarkosyl just prior to addition. The Zta response element (ZRE) and ΔZRE oligonucleotides were described previously as ZRE 5 and ΔZRE5 (31).

DNA binding assays. The promoter fragment from the $Z_7E4TCAT$ template was cut at the 5' end with *Hin*dIII, filled in with [α-^{32}P]dATP and Klenow polymerase, and then digested with *Eco*RI to produce a 224-bp promoter from −170 to +54 relative to the transcriptional start site. Reaction volumes for electrophoretic mobility shift assay (EMSA) were 12.5 μl in

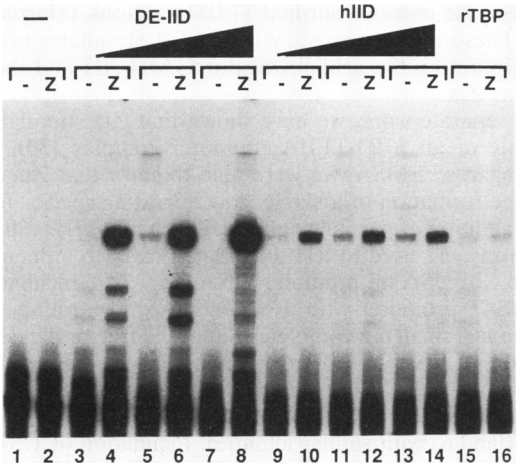

FIG. 1. High levels of transcriptional activation require additional activities in the crude TFIID fraction. Primer extension assays of in vitro transcription reactions with 100 ng of Z_7E4TCAT promoter template and partially purified general transcription factors, with or without 150 ng of Zta (Z) and different sources of TFIID. Reactions shown in lanes 1 and 2 have no TFIID. Reactions in lanes 3 and 4 have 1.25 μg, those in lanes 5 and 6 have 2.5 μg, and those in lanes 7 and 8 have 5.0 μg of DE-IID. Reactions in lanes 9 and 10 have 2.5 μl, those in lanes 11 and 12 have 5.0 μl, and those in lanes 13 and 14 have 10 μl of immunopurified hIID. Lanes 15 and 16 have 20 ng of rTBP. The amounts of TBP in 5 μg of DE-IID and 10 μl of hIID were approximately equal to 5 ng of TBP by Western blotting.

conditions identical to those described for transcription or with reduced protein concentrations as described in the legend to Fig. 6. EMSA reaction mixtures were analyzed in 1.4% agarose gels containing 40 mM Tris, 40 mM boric acid, and 5 mM magnesium acetate. The running buffer was identical to the gel buffer, and electrophoresis was conducted for 4 to 8 h at 50 V. DNase I footprinting was performed in 25-μl reaction volumes containing 10 μl hIID, 1.5 μg of TFIIA, 50 ng of TFIIB, 1 μg of USA, 75 ng of Zta, and ~6 fmol of radiolabeled DNA probe as described previously (29).

RESULTS

Previous work demonstrated that immunopurified TFIID, referred to here as hIID, which consists of TBP and tightly associated TAFs, was sufficient for the reconstitution of activated transcription with several different activator proteins. With certain preparations of general transcription factors, I observed that partially purified TFIID had higher activity than did hIID in reconstitution experiments with activator proteins. In particular, TFIID prepared from fractions that were partially immunodepleted for epitope-tagged TBP and then concentrated by chromatography on DEAE were found to have significantly higher levels of activity than immunopurified hIID (Fig. 1). In transcription reactions reconstituted with partially purified general transcription factors TFIIA, RNA Pol II-TFIIF-TFIIE, and recombinant TFIIB, I detected no transcription in the absence of any D fraction, without or with the Zta transcriptional activator (Fig. 1, lanes 1 and 2). Addition of the DE-IID fraction produced high levels of transcription in the presence of Zta (lanes 4, 6, and 8), and low but detectable basal levels in the absence of Zta (lane 3, 5, and 7). Immunopurified hIID produced relatively weak levels of activation compared with the DE-IID fraction that had been normalized for TBP concentration by Western blot (immunoblot) analysis (compare lanes 3 to 8 and lanes 9 to 14). This difference in activity was true over a fourfold range in concentration. Increasing DE-IID from 1.25 to 5.0 μg resulted in a corresponding increase in Zta-activated transcription levels (lanes 4, 6, and 8). In contrast, a similar increase in hIID concentration did not produce any higher levels of Zta-activated transcription (lanes 10, 12, and 14). Increasing DE-IID resulted in a slight decrease in basal-level transcription (lacking Zta), while increasing hIID did not effect basal level transcription significantly (compare lanes 7 and 13). As expected, Zta did not activate transcription with recombinant TBP (rTBP) (lanes 15 and 16). These results demonstrate that Zta-mediated activation requires TAFs in the hIID preparation and, moreover, that high levels of activated transcription require additional components in the phosphocellulose D fraction that do not copurify with the immunopurified hIID fraction.

High levels of transcription require TAFs and USA. Meisterernst et al. (38) reported that high levels of transcriptional activity could be observed when an activity in the phosphocellulose D fraction was added back to partially purified TFIID. This activity was referred to as USA because of its ability to stimulate USF-activated transcription, although USA has been shown to stimulate transcription from Sp1 and NF-κB as well (28). The DE-IID fraction that contained higher transcriptional activity than hIID was fractionated by the procedures described for USA. This USA activity when added back to the immunopurified TFIID could reconstitute high levels of Zta-activated transcription (Fig. 2). In vitro transcription reactions reconstituted without any TFIID fraction resulted in undetectable levels of transcription (lanes 1 and 2). Reactions containing the DE-IID fraction resulted in high levels of Zta-activated transcription (lane 4) and moderate levels of basal transcription (lane 3). In the presence of hIID, Zta weakly stimulated transcription (compare lanes 6 and 5), but activated levels were considerably lower than that observed for reactions containing DE-IID (compare lanes 6 and 4). Addition of the USA fraction in the absence of any additional TFIID activity resulted in a low level of transcription in the presence of Zta but no transcription in the absence of Zta (lanes 8 and 7). The combination of USA and hIID together resulted in Zta-activated transcription levels similar to that observed for the DE-IID fraction (compare lanes 10 and 4). To rule out the possibility that USA was merely contaminated with TFIID activity, USA was heat treated for 15 min at 48°C, conditions known to inactivate TFIID (39). Heat treatment did not reduce the activity of USA significantly, suggesting that USA was not contaminated with TFIID (lanes 11 and 12). Furthermore, TBP was not detected in the USA fraction by Western blotting (data not shown).

These results demonstrate that high levels of Zta-activated transcription require USA activity in addition to TBP and the TAFs. While others have shown that USA is required for Sp1, USF, and NF-κB, USA as prepared in this study was also tested for its ability to stimulate transcription from the strong synthetic acidic activator GAL4-AH (Fig. 2B). As observed with Zta, high levels of activated transcription were produced in reactions reconstituted with DE-IID (Fig. 2B, lanes 3 and 4). Very low levels of activated transcription were observed when hIID alone was the source of TFIID activity (lanes 5 and 6). Reactions reconstituted with USA in the absence of any TFIID resulted in undetectable levels of transcription (lanes 7 and 8). The combination of both USA and hIID restored activated transcription to high levels (lanes 9 and 10), similar to that observed for the DE-IID fraction (compare lane 10 and 4). Transcription reconstituted with recombinant TBP at concentrations comparable to the levels of TBP found in the DE-IID

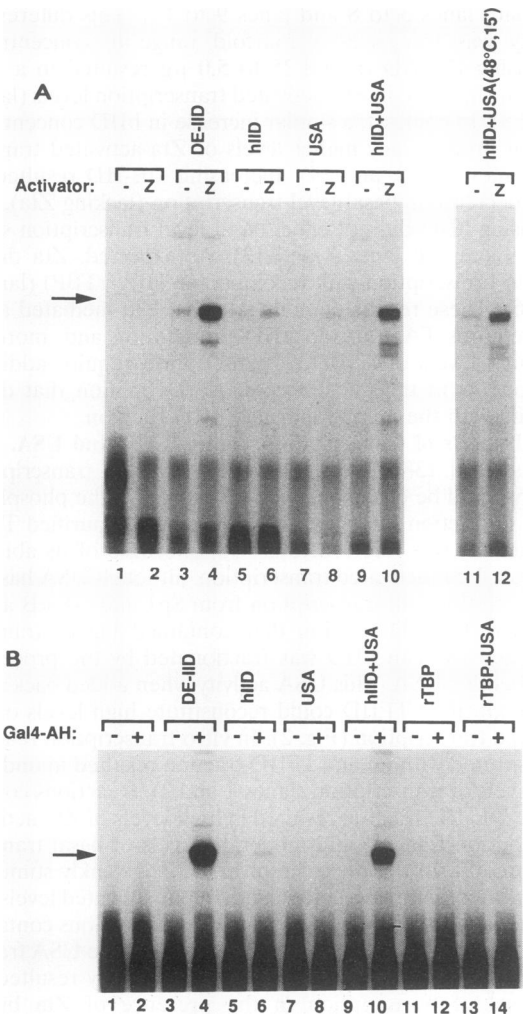

FIG. 2. The immunopurified hIID complex requires addition of the USA coactivator to produce high levels of activated transcription. (A) Primer extension assay of in vitro transcription reactions in the absence (odd-numbered lanes) or presence (even-numbered lanes) of Zta (Z). The general transcription factors TFIIA, TFIIB, and RNA Pol II-TFIIF-TFIIE were incubated with no TFIID (lanes 1 and 2), 2.5 μg of DE-IID (lanes 3 and 4), 5 μl of hIID (lanes 5 and 6), 1 μg of USA (lanes 7 and 8), or hIID plus USA (lanes 9 and 10). USA was heat treated prior to transcription reactions for 15 min at 48°C in lanes 11 and 12. (B) GAL4-AH also requires USA for high levels of transcription in reactions complemented with immunopurified hIID. Primer extension assay of in vitro transcription reactions with (even-numbered lanes) or without (odd-numbered lanes) the GAL4-AH activator. Partially purified general transcription factors were complemented as in panel A with no TFIID (lanes 1 and 2), DE-IID (lanes 3 and 4), hIID (lanes 5 and 6), USA (lanes 7 and 8), hIID plus USA (lanes 9 and 10), 20 ng of rTBP (lanes 11 and 12), or rTBP plus USA (lanes 13 and 14).

fraction resulted in undetectable transcription (lanes 11 and 12). Addition of USA to reactions containing rTBP failed to result in high levels of transcription observed for reactions reconstituted with hIID and USA (compare lanes 14 and 10). Since USA does not significantly stimulate basal levels of transcription in the presence of TBP, it seems unlikely that USA is merely a basal-level general transcription factor. These results demonstrate that high levels of activated transcription require both the TAFs in the hIID fraction and an additional activity in the partially purified TFIID fractions, referred to as USA. These results also show that USA stimulates activated transcription by the acidic activator GAL4-AH and the viral activator Zta.

In a separate work, we have shown that Zta stimulates the assembly of an hIID-TFIIA promoter complex (30). Using Mg^{2+} agarose EMSA, we were able to show that Zta stimulated the formation of a stable slow migrating species formed by the addition of partially purified TFIIA and hIID (30). This same assay was used to test the ability of Zta to influence the assembly of USA on promoter DNA (Fig. 3A). Incubation of the Z_7E4T promoter with a saturating concentration of Zta resulted in a small but complete shift of the free probe (lane 2). Addition of USA to binding reactions had no effect on the probe alone (lane 3) or to Zta-bound probe (lane 4). hIID bound to the promoter probe alone (lane 5) and to Zta-bound probe (lane 6) with similar affinities. Incubation of USA with hIID had no effect on the mobility of the hIID-promoter complex (lane 7). However, addition of USA to the Zta-hIID (Z-D)-promoter DNA complex resulted in the formation of a slower-mobility complex (lane 8). This slow-mobility Z-D-USA complex may be unstable to electrophoresis, since a significant portion of the probe appears as a smear and not a distinct complex (lane 8). The effect of USA on the complex mobility was clearly dependent upon the presence of Zta and hIID, supporting the functional significance of this binding assay. As reported previously (30), addition of partially purified TFIIA also resulted in a slow-mobility complex that was dependent upon the presence of TAFs in the hIID fraction and the activation domain of Zta (lane 10). The combination of TFIIA and USA in the absence of Zta stimulated hIID binding slightly (lane 9), but in the presence of Zta, the majority of the probe did not enter the gel (lane 10), suggesting that a large complex was formed.

To determine if the smearing pattern in the Z-D-USA complex was merely a result of trace TFIIA contamination in the USA fraction, EMSA was performed at reduced concentration of TFIIA (Fig. 3B). When TFIIA was reduced by twofold relative to the amounts used in Fig. 3A, it had a negligible effect on hIID binding (Fig. 3B; compare lanes 1 and 4). In the presence of Zta, TFIIA still stimulated the formation of a discrete Z-D-A complex at concentrations 0.125 that used in Fig. 3A (Fig. 3B, lane 6). In addition, increasing USA did not result in an EMSA pattern that resembled the Z-D-A complex (data not shown). To further eliminate the possibility that USA was contaminated with TFIIA or TFIIB, the USA fraction was analyzed by Western blotting (Fig. 3C and D). Ten transcription units of USA (10 μg) was compared with four transcription units of TFIIA (7.5 μg) by probing with rabbit polyclonal antisera raised against the large subunit of human TFIIA (Fig. 3A). The 33-kDa α subunit and 19-kDa β subunit of TFIIA were clearly detected in the TFIIA fraction (lane 2) but were not detected in the USA fraction (lane 1). The blot was then reprobed with antisera directed against TFIIB (Fig. 3D), which could detect 0.1 transcription unit of TFIIB (5 ng) (lane 3) but detected no TFIIB in the USA fraction (Fig. 3D, lane 1). These results suggest that USA contains an activity that interats with the Z-D-promoter complex and is distinct from TFIIA and TFIIB.

Factors required for the formation of a Sarkosyl-resistant preinitiation complex activated by Zta. Several methods have been used to identify stable intermediates in the preinitiation complex assembly pathway. Sensitivity to different concentrations of Sarkosyl have been used to define functional steps in basal preinitiation complex assembly (19, 36, 44) as well as characterization of steps in which activators may function in

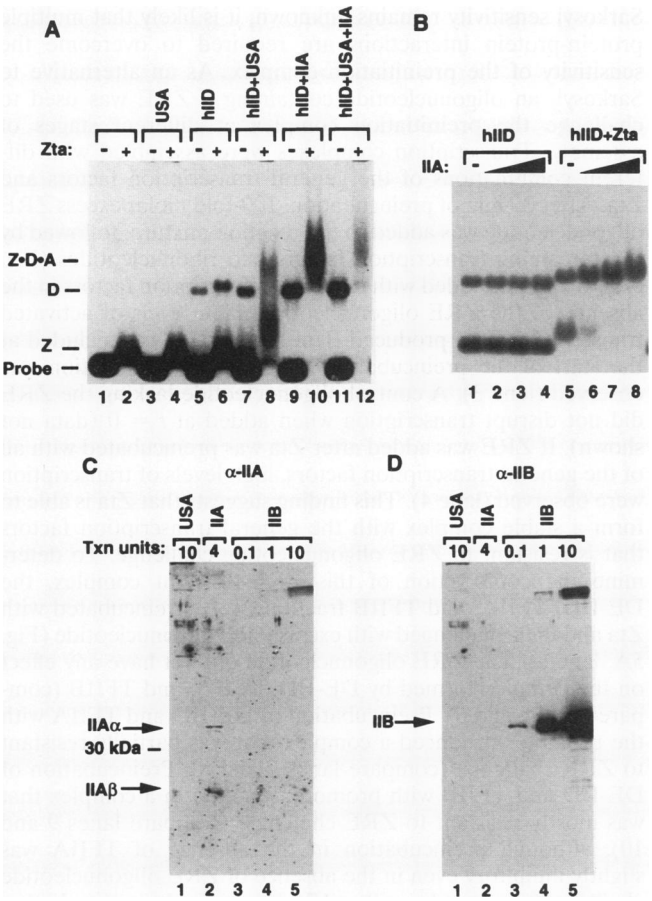

FIG. 3. (A) Zta stimulates the assembly of USA and TFIIA with the hIID-promoter complex in Mg^{2+} agarose gel EMSA. Promoter DNA probe was incubated with (even-numbered lanes) or without (odd-numbered lanes) 37.5 ng of Zta in a reaction volume of 12.5 μl and partially purified general transcription factors at a concentration equivalent to that of in vitro transcription. Probe with or without Zta was incubated with 0.25 μg of USA (lanes 3 and 4), 2.5 μl of hIID (lanes 5 and 6), hIID plus USA (lanes 7 and 8), hIID plus 0.75 μg of TFIIA (lanes 9 and 10), or hIID plus 0.75 μg of TFIIA and 0.25 μg of USA (lanes 11 and 12) for 40 min at 30°C and electrophoresed for 6 h at 50 V. (B) Mg agarose EMSA analysis of TFIIA concentration-dependent formation of the Z-D-A complex. Promoter DNA was incubated with 2.5 μl of hIID in the absence (lanes 1 to 4) or presence (lanes 4 to 8) of 37.5 ng of Zta. TFIIA was added to reactions in lanes 2 and 6 (0.1 μg), in lanes 3 and 7 (0.19 μg), and lanes 4 and 8 (0.38 μg). (C) Western blot analysis of USA with rabbit polyclonal antisera raised against the large subunit of TFIIA (αβ). Ten micrograms of USA (lane 1), 7.5 μg of TFIIA (lanes 2), 5 ng of TFIIB (lane 3), 50 ng of TFIIB (lane 4), or 500 ng of TFIIB (lane 5) was fractionated on SDS–12.5% polyacrylamide gels, transferred to nitrocellulose, and visualized by chemiluminescence after antibody probing. The 33-kDa TFIIA-α polypeptide and the TFIIA-β polypeptide are indicated by the arrows at the left. Txn, transcription. (D) Western blotting analysis of USA with antisera raised against TFIIB. The blot is identical to the one shown in panel C but reprobed with antisera against TFIIB. Mobility of TFIIB is indicated by the arrow.

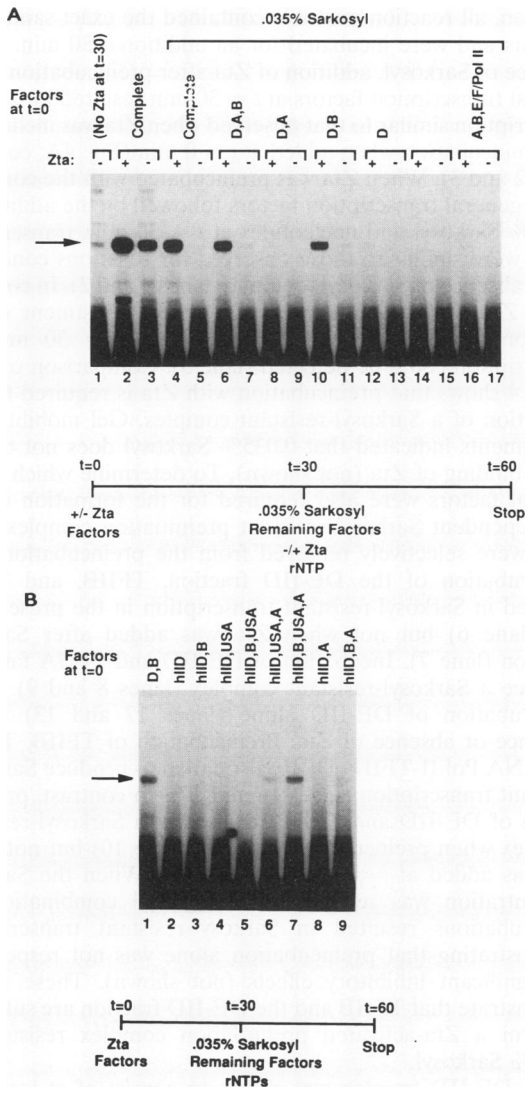

FIG. 4. Factors required for Sarkosyl-resistant Zta-activated preinitiation complex. (A) Primer extension assay of in vitro transcription reactions reconstituted with the partially purified DE-IID fraction. The Z_7E4TCAT template DNA was preincubated with general factors as indicated above each set of lanes in the presence of Zta (+) or with Zta added at t = 30 min (−). No Zta was added at t = 30 in the reaction shown in lane 1. Complete sets of general transcription factors were preincubated with Zta (lane 2 and 4) or with Zta added at t = 30 min (lane 3 and 5). Reactions were made 0.035% Sarkosyl at t = 30 in lanes 4 to 17. (B) Factors required for Sarkosyl resistance in reactions reconstituted with immunopurified hIID. Transcription reactions were set up similar to those in panel A except that hIID and USA were used in place of DE-IID in lanes 2 to 9. Factors preincubated with Zta are indicated above the lanes. The DE-IID fraction and TFIIB were preincubated with Zta in lane 1.

complex assembly (1, 4, 24). In this work, Sarkosyl was used to determine the transcription factor composition of a Zta-activated stable complex. Preliminary experiments showed that 0.035% Sarkosyl inhibited transcription of a preinitiation complex preincubated in the absence of Zta but did not inhibit a preinitiation complex preincubated with Zta (not shown). Consequently, an experiment was designed to test which components of the general transcription factors were required to produce this Zta-activated Sarkosyl resistant complex (Fig. 4). Factors were preincubated with or without Zta for 30 min, and then Sarkosyl was added to a final concentration of 0.035%. The remaining factors and nucleotides were then added and incubated for an additional 30 min. For reactions in which Zta was not included in the preincubation, Zta was added with the remaining general factors. After Sarkosyl

addition, all reaction mixtures contained the exact same components and were incubated for an additional 30 min. In the absence of Sarkosyl, addition of Zta after preincubation of the general transcription factors at $t = 30$ min resulted in levels of transcription similar to that observed when Zta was included in the preincubation when added at $t = 0$ min (Fig. 4A; compare lanes 2 and 3). When Zta was preincubated with the complete set of general transcription factors followed by the addition of 0.035% Sarkosyl and nucleotides at $t = 30$ min, transcription levels were similar to those observed for reactions conducted in the absence of Sarkosyl (compare lanes 4 and 2). In contrast, when Zta was added immediately after adjustment of the reaction mixture to 0.035% Sarkosyl at $t = 30$ min, no transcription could be detected (lane 5). Comparison of lanes 5 and 4 shows that preincubation with Zta is required for the formation of a Sarkosyl-resistant complex. Gel mobility shift experiments indicated that 0.035% Sarkosyl does not disrupt DNA binding of Zta (not shown). To determine which of the general factors were also required for the formation of this Zta-dependent Sarkosyl-resistant preinitiation complex, fractions were selectively removed from the preincubation step. Preincubation of the DE-IID fraction, TFIIB, and TFIIA resulted in Sarkosyl-resistant transcription in the presence of Zta (lane 6) but not when Zta was added after Sarkosyl addition (lane 7). Incubation of DE-IID and TFIIA failed to produce a Sarkosyl-resistant complex (lanes 8 and 9) as did preincubation of DE-IID alone (lanes 12 and 13) in the presence or absence of Zta. Preincubation of TFIIB, TFIIA, and RNA Pol II-TFIIF-TFIIE also failed to produce Sarkosyl-resistant transcription (lanes 16 and 17). In contrast, preincubation of DE-IID and TFIIB resulted in a Sarkosyl-resistant complex when preincubated with Zta (lane 10) but not when Zta was added at $t = 30$ min (lane 11). When the Sarkosyl concentration was reduced to 0.02%, all combinations of preincubations resulted in Sarkosyl-resistant transcription, demonstrating that preincubation alone was not responsible for significant inhibitory effects (not shown). These results demonstrate that TFIIB and the DE-IID fraction are sufficient to form a Zta-activated preinitiation complex resistant to 0.035% Sarkosyl.

The DE-IID fraction was shown to consist of at least two active fractions, the TAF-containing hIID and USA (Fig. 2). The Sarkosyl resistance assay (Fig. 4A) was used to determine which components in the DE-IID fraction were required to produce the stable preinitiation complex (Fig. 4B). As observed previously, preincubation of DE-IID, TFIIB, and Zta resulted in a Sarkosyl-resistant transcription complex (Fig. 4B, lane 1). Preincubation of hIID with Zta did not produce a Sarkosyl-resistant complex (lane 2). Interestingly, preincubation of Zta with hIID and TFIIB also did not result in a Sarkosyl-resistant complex (lane 3). This finding shows that additional components in the DE-IID fraction were required to produce the stable preinitiation complex. Addition of USA to the preincubation of Zta and hIID also failed to produce a Sarkosyl-resistant complex (lane 4). Surprisingly, preincubation of USA, TFIIB, hIID, and Zta also failed to result in Sarkosyl-resistant transcription (lane 5). However, preincubation of TFIIA with USA, TFIIB, hIID, and Zta did restore the Sarkosyl-resistant transcription complex (lane 7). No other combination of factors in the preincubation could completely restore transcription (Fig. 4B), suggesting that at least four separate activities were required to produce the Sarkosyl-resistant preinitiation complex and that at least three of these activities, hIID, USA, and TFIIA, were present in the DE-IID fraction.

Factors required for the formation of a ZRE-resistant preinitiation complex. Although the precise physical basis for Sarkosyl sensitivity remains unknown, it is likely that multiple protein-protein interactions are required to overcome the sensitivity of the preinitiation complex. As an alternative to Sarkosyl, an oligonucleotide containing a ZRE was used to challenge the preinitiation complex at different stages of assembly. Transcription complexes were assembled with different combinations of the general transcription factors and Zta. After 30 min of preincubation, 100-fold molar excess ZRE oligonucleotide was added to the reaction mixture, followed by the remaining transcription factors and ribonucleotides (Fig. 5A). If Zta was added with all of the transcription factors in the absence of the ZRE oligonucleotide, high levels of activated transcription were produced (lane 2). If ZRE was included at the start of the preincubation ($t = 0$), no transcription was observed (lane 3). A control oligonucleotide lacking the ZRE did not disrupt transcription when added at $t = 0$ (data not shown). If ZRE was added after Zta was preincubated with all of the general transcription factors, high levels of transcription were observed (lane 4). This finding suggests that Zta is able to form a stable complex with the general transcription factors that is resistant to ZRE oligonucleotide challenge. To determine the composition of this ZRE-resistant complex, the DE-IID, TFIIA, and TFIIB fractions were preincubated with Zta and then challenged with excess ZRE oligonucleotide (Fig. 5A, lane 6). The ZRE oligonucleotide did not have any effect on the complex formed by DE-IID, TFIIA, and TFIIB (compare lanes 5 and 6). Preincubation of DE-IID and TFIIA with the promoter produced a complex that was partially resistant to ZRE challenge (compare lanes 7 and 8). Preincubation of DE-IID and TFIIB with promoter resulted in a complex that was mostly resistant to ZRE challenge (compare lanes 9 and 10), although preincubation in the absence of TFIIA was slightly inhibitory even in the absence of ZRE oligonucleotide challenge (compare lanes 9 and 7). Interestingly, preincubation of Zta and DE-IID with promoter resulted in a complex that was highly sensitive to ZRE oligonucleotide challenge (lanes 11 and 12). These results were similar to those for the Sarkosyl challenge experiments in demonstrating that Zta must interact with DE-IID fraction and TFIIB before a resistant preinitiation complex is formed.

As with the Sarkosyl experiment, the ZRE challenge experiment was used to determine which components in the DE-IID fraction were required to produce the ZRE-resistant complex (Fig. 5B). Preincubation of hIID, USA, TFIIB, TFIIA, and RNA Pol II-TFIIE-TFIIF fraction with Zta produced a complex resistant to ZRE oligonucleotide challenge (Fig. 5B; compare lanes 2 and 3). Preincubation of Zta with hIID, USA, TFIIA, and TFIIB resulted in a complex mostly resistant to ZRE challenge (compare lanes 4 and 5). Interestingly, preincubation of Zta with hIID, TFIIA, and TFIIB resulted in a complex also partly resistant to ZRE challenge (compare lanes 6 and 7). However, elimination of TFIIA (lane 9) or TFIIB (lane 11) from the preincubation resulted in complexes highly sensitive to ZRE challenge. As expected, preincubation of hIID alone with Zta also failed to produce ZRE-resistant transcription (compare lanes 12 and 13). In contrast to the Sarkosyl-resistant preinitiation complex which required USA, ZRE-resistant transcription was only slightly increased by the addition of USA to the stable D-B-A complex (compare lanes 5 and 7 in Fig. 5B). These results suggest that hIID, TFIIA, and TFIIB are critical factors required for the establishment of a preinitiation complex that is resistant to ZRE oligonucleotide challenge.

Zta has been shown to stimulate the assembly of the D-A complex when assayed by Mg^{2+} agarose EMSA (30). This assay can now be used to test whether Sarkosyl or ZRE

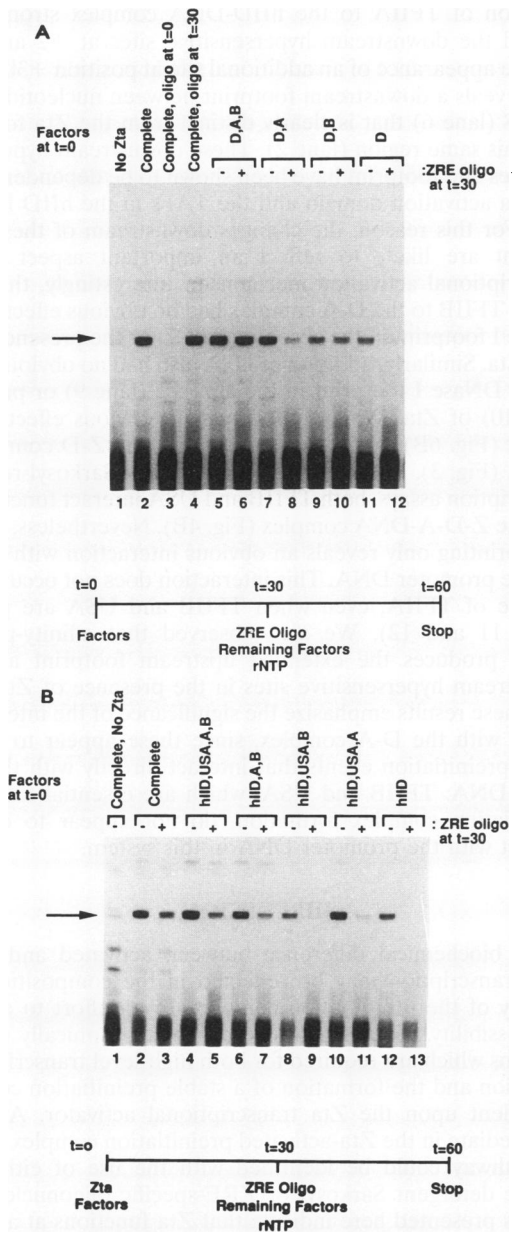

FIG. 5. Factors required for ZRE oligonucleotide-resistant preinitiation complex. (A) Transcription reactions were reconstituted with the DE-IID fraction as the source of TFIID. All reactions except that in lane 1 have Zta present at $t = 0$ min. The ZRE oligonucleotide (oligo; 0.4 μM) was added at $t = 0$ min in lane 3 and $t = 30$ min in lanes 4, 6, 8, 10, and 12. No ZRE oligonucleotide was added to reactions in lanes 5, 7, 9, and 11. Factors preincubated with Zta and DNA are indicated above the lanes. (B) Transcription reactions were reconstituted with immunopurified hIID and USA in place of DE-IID. Factors preincubated with Zta and DNA are indicated above the lanes. The ZRE oligonucleotide was added at $t = 30$ min to reactions in lanes 3, 5, 7, 9, 11, and 13.

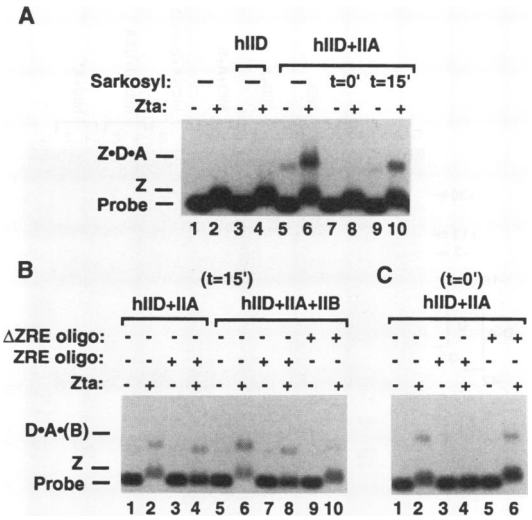

FIG. 6. Effects of Sarkosyl and ZRE on hIID-TFIIA-DNA complex formation. (A) Mg^{2+} agarose EMSA analysis of complex formation with 0.035% Sarkosyl added at the start of the reaction ($t = 0$ min; lanes 7 and 8) or after a 15-min preincubation ($t = 15$ min; lanes 9 and 10). TFIIA (0.375 μg) and hIID (1 μl) were used at concentrations which produce only Zta (20 ng)-dependent complexes. Samples were loaded on gels after a 60-min incubation. (B) Mg^{2+} agarose EMSA analysis of complex formation when the ZRE oligonucleotide (oligo; 0.4 μM, final concentration) was added at 15 min (lanes 3, 4, 7, and 8) or control oligonucleotide ΔZRE (0.4 μM, final concentration) was added at $t = 15$ (lanes 9 and 10). TFIIB (50 ng) was included in reactions shown in lanes 5 to 10. (C) EMSA analysis of the Z-D-A complex with the ZRE (lanes 3 and 4) or ΔZRE (lanes 5 and 6) oligonucleotide added at $t = 0$. Factors in each reaction are indicated in the legend above the lanes, and major species are indicated at the left.

oligonucleotide challenge affects this preinitiation complex assembly step. To clearly distinguish between the Zta-dependent Z-D-A complex and the D-A complex formed in the absence of Zta, concentrations of TFIIA and hIID were reduced to levels that produce a stable D-A complex only in the presence of Zta (Fig. 6A, lanes 1 to 6). The stable Z-D-A complex did not form if 0.035% Sarkosyl was included at the start of the DNA binding reaction ($t = 0$) (Fig. 6A, lane 7 and 8). However, if reaction mixtures were preincubated for 15 min, allowing the Z-D-A complex to form before the addition of 0.035% Sarkosyl, the complex was largely resistant to Sarkosyl disruption (Fig. 6A, lane 10). Zta binding was unaffected by the presence of Sarkosyl (compare lanes 2 and 8). EMSA experiments with polyacrylamide gels showed more clearly that 0.035% Sarkosyl had little effect on Zta binding (not shown). These results provide physical evidence that Sarkosyl does not disrupt a preformed Z-D-A complex and suggest that Sarkosyl also interferes with a subsequent Zta-dependent step, like the binding of TFIIB or USA, since these are both required for the formation of a transcriptionally competent Sarkosyl-resistant complex (Fig. 4B).

DNA binding experiments with the addition of excess ZRE oligonucleotide revealed a finding similar to that observed for Sarkosyl (Fig. 6B). The Z-D-A complex was allowed to form for 15 min and then challenged with ZRE oligonucleotide. Addition of ZRE oligonucleotide at 15 min after complex formation eliminated most of the Zta binding (Fig. 6B [compare mobility marked Z in lanes 2 and 4] and data not shown). However, the ZRE oligonucleotide did not disrupt the D-A complex that had been formed by the preincubation of Zta, hIID, and TFIIA for 15 min before addition of oligonucleotide (compare mobility marked D-A in lanes 2 and 4). Addition of TFIIB had little detectable effect on the extent of DNA binding (lane 6). The complex formed in the presence of TFIIB had a mobility identical to that of the D-A complex and a

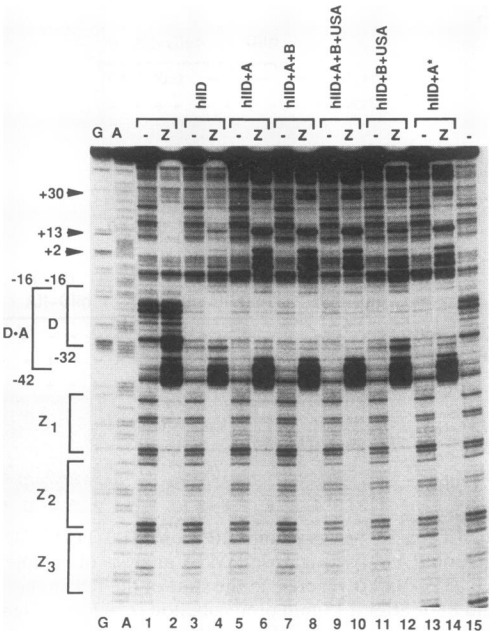

FIG. 7. DNase I footprinting of the interaction of transcription factors with the Z_7E4T promoter in the presence (even-numbered lanes) or absence (odd-numbered lanes) of Zta (Z). Factors incubated in reaction mixtures are indicated above the lanes. A* represents a TBP affinity-purified TFIIA fraction. G and A represent the G- and G+A-specific sequencing marker lanes, respectively. Arrows at positions +30, +13, and +2 at the left indicate DNase I-hypersensitive sites induced by the addition of Zta-hIID and enhanced by TFIIA. The bracket labeled D represents the footprint resulting from hIID alone, and D-A represents the extended footprint observed with hIID and TFIIA. The first three ZRE binding sites are indicated by the brackets labeled Z1, Z2, and Z3.

similar resistance to ZRE oligonucleotide challenge (lane 8). When the ZRE oligonucleotide was added at the start of the DNA binding reaction, the D-A complex did not form (Fig. 6C, lane 4). The effect of the ZRE oligonucleotide was shown to be specific, since a control oligonucleotide (ΔZRE) which lacks the ZRE-binding element failed to disrupt the D-A complex when added after 15 min of preincubation (Fig. 6B, lane 10) or at the start of the binding reaction (Fig. 6C, lane 6). Since Sarkosyl and ZRE challenge do not abolish a preformed D-A complex, they presumably inhibit transcription by interfering with a subsequent step(s) in the assembly of the preinitiation complex that is also Zta dependent.

We have previously shown that Zta stimulates the binding of TFIIA to the hIID-DNA complex by Mg^{2+} agarose gel EMSA as well as by DNase I footprinting (30). In Fig. 3, Mg^{2+} agarose EMSA was used to demonstrate that USA also formed a complex with Zta and hIID but not with Zta alone or hIID alone. Transcription factor complexes similar to those formed in the transcription reactions were now examined for their ability to alter the DNase I cleavage pattern on promoter DNA (Fig. 7). As observed previously, hIID bound to the TATA element between −16 and −32 (lane 3). Zta alone binds to the ZREs at positions −70 to −140 and also interacts with a downstream sequence between −2 to +20 of this promoter when present at the high concentrations used in these experiments (lane 2). Addition of Zta and hIID produced weak hypersensitive sites at positions +2 and +13 (lane 4). Addition of TFIIA to the hIID-DNA complex partially extended the footprint upstream of the TATA box to position −42 (lane 5). Addition of TFIIA to the hIID-DNA complex strongly enhanced the downstream hypersensitive sites at +2 and +13 and the appearance of an additional site at position +30, which now reveals a downstream footprint between nucleotides +14 to +28 (lane 6) that is clearly distinct from the Zta footprint over this same region (lane 2). These downstream hypersensitive sites and footprint have been shown to be dependent upon the Zta activation domain and the TAFs in the hIID fraction (30). For this reason, the changes downstream of the TATA element are likely to reflect an important aspect of the transcriptional activation mechanism. Interestingly, the addition of TFIIB to the D-A complex had no obvious effect on the DNase I footprint in the absence (lane 7) or the presence (lane 8) of Zta. Similarly, addition of USA also had no obvious effect on the DNase I footprint in the absence (lane 9) or presence (lane 10) of Zta. While TFIIB had no obvious effect in the EMSA (Fig. 6B), USA did interact with the Z-D complex by EMSA (Fig. 3). Furthermore, judging from Sarkosyl-resistant transcription assays, both TFIIB and USA interact functionally with the Z-D-A-DNA complex (Fig. 4B). Nevertheless, DNase I footprinting only reveals an obvious interaction with Z-D-A and the promoter DNA. This interaction does not occur in the absence of TFIIA, even when TFIIB and USA are present (lanes 11 and 12). We also observed that affinity-purified TFIIA produces the extended upstream footprint and the downstream hypersensitive sites in the presence of Zta (lane 14). These results emphasize the significance of the interaction of Zta with the D-A complex, since these appear to be the major preinitiation events that interact directly with the promoter DNA. TFIIB and USA, which are essential for stable preinitiation complex formation, do not appear to directly interact with the promoter DNA in this system.

DISCUSSION

The biochemical difference between activated and basal-level transcription may be reflected in the composition and stability of the preinitiation complex. In an effort to address this possibility, I have identified chromatographically distinct fractions which are required for both high-level transcriptional activation and the formation of a stable preinitiation complex dependent upon the Zta transcriptional activator. A stable intermediate in the Zta-activated preinitiation complex assembly pathway could be identified with the use of either the anionic detergent Sarkosyl or ZRE-specific oligonucleotides. Results presented here indicate that Zta functions at an early step in the formation of a D-B-A complex and that USA significantly stabilizes this intermediate. Gel electrophoresis mobility shift assay showed that Zta stimulated the assembly of both a D-A-promoter complex and a USA-D-promoter DNA complex. DNase I protection experiments indicate that only Zta, hIID, and TFIIA make significant contacts with the Z_7E4T promoter DNA. Taken together, these results support the model that Zta stimulates the interaction of several components of the general transcription factors and recruits the coactivator USA to the preinitiation complex, allowing for high-level transcription.

High-level transcriptional activation with immunopurified hIID requires USA-like activity. While hIID, in contrast to TBP, has the ability to support transcriptional activation by several activators and from TATA-less promoters (55), work presented here demonstrates that high-level transcriptional activation requires additional activity that copurifies with the TFIID fraction. This activity could be separated from TFIID by chromatography over DEAE in the presence of nonionic detergents, similar to the USA cofactor described by Meister-

TABLE 1. Summary of Sarkosyl and ZRE challenge experiments used to identify Zta-dependent preinitiation complex intermediates that were competent for transcription

Activated preinitiation complex	Resistance to:	
	ZRE	Sarkosyl
Z-D	−	−
Z-D-A	NS	−
Z-D-B	NS	−
Z-D-USA	−	−
Z-D-A-USA	−/+	−
Z-D-B-USA	−/+	−
Z-D-A-B	+	−
Z-D-A-B-USA	+	+
Z-D-A-B-USA-E/F-Pol II	++	+

[a] NS, data not shown.

ernst et al. (38). I found that the partially purified TFIID fraction could be separated into immunopurified holo-TFIID (TBP plus TAFs), the stimulatory activity of USA, and TFIIA-like activity. Both USA and TFIIA have been reported to copurify with TFIID (38). USA was originally described as a composite activity consisting of both positive and negative components referred to as PC1 and NC1, respectively. The preparation of USA used in these experiments did not show significant repression of basal-level transcription but was essential for high-level transcriptional activation from both the Zta activation domain and the synthetic acidic activator, GAL4-AH, in reactions reconstituted with immunopurified hIID. A similar conclusion that USA was required for high levels of activation was reached by Chiang et al. (8) using immunopurified flag epitope-tagged TFIID and the GAL4-AH activator. While previous reports suggested that holo-TFIID did not require USA (55), it may be possible that USA was contaminating other general factor preparations and masking a need for its addition in reconstitution experiments.

The Zta transcription factor was shown to facilitate the assembly of USA with the hIID-promoter DNA in Mg^{2+} agarose EMSA, similar to the ability of Zta to enhance the D-A-promoter complex (Fig. 3). We failed to detect a direct interaction of USA with hIID in these experiments, which is in contrast to previous observations that USA directly interacts with TBP in EMSA experiments (37). Several variations in experimental conditions could account for these differences. In this study, EMSA in agarose gels with relatively large promoter DNA fragments and high-molecular-weight hIID was performed, while USA binding activity was previously characterized in polyacrylamide gels with oligonucleotide probes and recombinant TBP (37). Preparations of USA used in this study did not show significant repression of basal level transcription, suggesting that the negative component of USA may have been lost during purification. This negative component was shown to interact directly with TBP (37) and may explain why I failed to observe a direct interaction of USA and TBP. From results presented here, it seems likely that activator proteins like Zta promote the interaction of the positive-acting component of USA with the hIID-promoter DNA complex.

The Sarkosyl-resistant activated preinitiation complex consists of hIID, TFIIB, TFIIA, and USA. Sarkosyl has been used to identify functional steps in the transcriptional initiation process (1, 4, 19, 24, 36, 44). Others have shown that in the absence of activators, a preformed D-A-promoter complex is resistant to 0.015% Sarkosyl, but resistance to 0.03% Sarkosyl requires the preforming of the D-A-B complex (36). In this work, I have used Sarkosyl to distinguish between basal-level preinitiation complexes and preinitiation complexes formed in the presence of the Zta activator protein. Preinitiation complexes formed in the presence of Zta conferred resistance to 0.035% Sarkosyl, while preinitiation complexes formed in the absence of Zta were sensitive to this concentration of Sarkosyl. This experimental design was used to determine which components in the preinitiation complex were required for the preincubation with Zta. Initial experiments utilizing partially purified TFIID fraction (DE-IID), which produced high levels of transcription in vitro, demonstrated the importance of TFIIB and TFIID in the formation of a Zta-dependent Sarkosyl-resistant complex (Fig. 4A). In experiments using immunopurified hIID, promoter DNA required preincubation with Zta, hIID, TFIIB, the TFIIA fraction, and the coactivator USA to establish a Sarkosyl-resistant complex. Thus, the preinitiation complex intermediate which is stable to 0.035% Sarkosyl and dependent upon preincubation with Zta consists of at least TBP-TAFs, TFIIA, USA, and TFIIB. Since USA is a relatively crude preparation, it is possible that contaminating general factors or bulk protein concentration contribute to Sarkosyl resistance. However, TFIIA and TFIIB could not be detected in the USA fraction by Western blotting analysis (Fig. 3C and D). In addition, the amount of USA in transcription reaction was less than 1 μg of protein, compared with the 1.8 μg of TFIIA and 2.5 μg of the Pol II-TFIIE-TFIIF fraction. More interestingly, the Z-D-A complex was resistant to 0.035% Sarkosyl if preformed but sensitive if Sarkosyl was introduced prior to stable complex formation, indicating that Sarkosyl resistance is not simply a function of bulk protein concentration.

In a similar experimental design, Arnosti et al. (1) showed that octamer factor 2 (Oct-2) could form a Sarkosyl-resistant preinitiation complex when preincubated only with TFIID. They concluded that Oct-2 accelerated the rate of TFIID interaction with DNA and that Oct-2 had relatively little effect on TFIIB or TFIIA assembly. In contrast to Oct-2, I found that Zta required both TFIIB and TFIIA to form a Sarkosyl-resistant complex. Arnosti et al. also found that 0.02% Sarkosyl was sufficient to distinguish between preinitiation complexes formed in the presence of Oct-2 and those formed in its absence, while I found that Zta required 0.035% Sarkosyl to distinguish between Zta-preincubated complexes and those incubated with Zta after preinitiation complex formation. Oct-2 and Zta share little obvious sequence similarity in their activation domains and, on the basis of these results, are likely to facilitate the formation of a preinitiation complex by different mechanisms.

A ZRE-resistant complex consists of hIID, TFIIA, and TFIIB. Sarkosyl is likely to act as a relatively nonspecific detergent that interferes with weak hydrophobic interactions. Multiple protein-protein interactions presumably are required to stabilize a complex in the presence of Sarkosyl. In contrast to Sarkosyl, the ZRE oligonucleotide is a specific competitor of the interaction of Zta with the promoter DNA. A simple interpretation of ZRE oligonucleotide challenge is that events occurring after addition of excess oligonucleotide no longer require direct binding of Zta to the promoter template. However, direct proof of this assumption is difficult by using the transcription assay in which a small fraction of the assembled templates are actively transcribed. Furthermore, loss of direct binding of Zta activator to the promoter ZREs does not exclude Zta from being stably retained in the preinitiation complex. Nevertheless, ZRE oligonucleotide challenge did affect some preinitiation complexes more than others. The

Z-D complex was highly sensitive to addition of ZRE oligonucleotide, suggesting that Zta must persistently interact with hIID before other components can assemble into an activated complex. Addition of either TFIIB or TFIIA stabilized this complex, suggesting that TFIIB and TFIIA can assemble independently of each other. Addition of both TFIIA and TFIIB to the preincubation with hIID strongly enhanced ZRE-resistant transcription, indicating that Zta functions during the formation of the D-A-B complex. It is interesting that while Zta may interact directly with hIID, its function is likely to alter the ability of hIID to interact with TFIIB or TFIIA.

Activator-specific oligonucleotide challenge has been used previously to determine the role of the ATF transcriptional activator in preinitiation complex assembly (17, 23). Using a DNase I footprinting assay, Horikoshi et al. (23) observed that a stable complex consisting of TFIID, TFIIB, TFIIE, and Pol II could be formed in the presence of ATF and was refractory to ATF oligonucleotide competition. Using an in vitro transcription assay, Hai et al. (17) observed that an oligonucleotide refractory complex could be formed by the preincubation of ATF with TFIID, TFIIB, and RNA Pol II. Although it is difficult to directly compare the specific results of these early works with those presented here, a similar general conclusion may be reached concerning the role of the activator in transiently facilitating the formation of a stable preinitiation complex. The precedent for transient assembly factors has been clearly established in RNA Pol III transcription whereby TFIIIA and TFIIIC mediate the formation of a highly stable TFIIIB-promoter complex (25). It seems likely that RNA Pol II transcriptional activators have a role similar to that of transient assembly factors.

The effects of Sarkosyl and ZRE challenge on complex assembly are summarized in Table 1. Formation of the preinitiation complex is probably not a simple serial assembly pathway, since EMSA experiments suggest that USA can partly bind in the absence of TFIIA or TFIIB (Fig. 3A). USA had little effect on the resistance to ZRE challenge but a strong effect on resistance to Sarkosyl. From these experiments, it is not clear when USA assembles into the preinitiation complex, although results of the ZRE challenge experiments suggest that USA may be able to interact with the activated D-B-A complex after Zta has dissociated. These results further suggest that Zta locks TFIIA and TFIIB into a stable activated conformation with hIID, which is now capable of producing high-level transcription with the assistance of USA.

Target molecules of transcriptional activation domains have been difficult to determine precisely. Zta has been shown to directly interact with TBP (29) and to facilitate the formation of a D-A-promoter complex (30). The Sarkosyl and ZRE challenge experiments described here clearly implicate TFIIB, in addition to TFIIA and hIID, as an essential target of Zta action. TFIIB was unambiguously required for the formation of a Zta-activated preinitiation complex that was stable to both ZRE challenge and 0.035% Sarkosyl. The requirement of TFIIB in the preincubation with Zta strongly suggests that Zta alters the association of TFIIB with the preinitiation complex. The Sarkosyl resistance and the EMSA experiments in this work also indicate that Zta effects the ability of USA to interact with the preinitiation complex. At present, only TBP has been shown to interact directly with Zta (29). While the effect of Zta on the formation of the D-A-B-USA complex may be a result of Zta's singular interaction with TBP, it seems more likely that Zta interacts with several additional components of this complex. A similar conclusion has been reached by Choy and Green (9), who demonstrate that activators function during multiple steps in the formation of a preinitiation complex. The characterization of stable intermediates of the preinitiation complex, as attempted in this work, may reflect some of the multiple steps in which activators function.

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