The Zta trans-activator protein stabilizes TFIID association with promoter DNA by direct protein–protein interaction

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Eukaryotic transcriptional activators are believed to stimulate transcription through direct and/or indirect interactions with one or more of the general transcription factors. We show here that the Zta transcriptional activator protein encoded by the Epstein-Barr virus makes direct physical contact with the basic transcription factor TFIID. Both Zta and TFIID were expressed in and purified from Escherichia coli. Zta stabilized the binding of TFIID to Zta-responsive promoters as assayed by gel electrophoresis mobility-shift and immunoprecipitation of radiolabeled promoter DNA. A deletion mutant of Zta that failed to activate transcription failed to stabilize TFIID binding. DNase I footprinting showed that Zta reduced the dissociation rate of TFIID bound to the TATA element. Protein blotting and immunoprecipitation experiments demonstrated that TFIID and Zta also interact in the absence of promoter DNA. The amino acid residues 25–86 of Zta were essential for the stable association with TFIID and were shown to be required for trans-activation in vivo. We propose that Zta stimulates transcription, in part, by direct physical contact with the conserved domain of TFIID and the formation of a stable Zta–TFIID–promoter complex.

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Transcriptional regulation of eukaryotic protein-coding genes can be accounted for, in large part, by the combinatorial effects of upstream activator proteins targeted to promoter regions by sequence-specific DNA binding (Maniatis et al. 1987; Mitchell and Tjian 1989). These tissue and temporally regulated trans-acting proteins, referred to as upstream factors, are believed to facilitate the assembly of a group of general transcription factors into a stable preinitiation complex at the start site of transcription (Ptashne 1988; Ptashne and Gann 1990). The assembly of these general transcription factors is initiated by the binding of TFIID to the TATA-like element found in most promoters (Van Dyke et al. 1988; Buratowski et al. 1989; for review, see Saltzman and Weinman 1989, Sawadogo and Sentenac 1990; Greenblatt 1991). Addition of TFIIA then forms a complex that is competent for interaction with TFIIIB, followed by RNA polymerase, and then TFIIIE/F (Van Dyke et al. 1988; Buratowski et al. 1989). Presumably, one or more of these steps may be rate limiting for a particular promoter. It is hypothesized that upstream factors accelerate the rate of transcription by facilitating one or more of the rate-limiting steps in the assembly process (Ptashne and Gann 1990).

Because of the fundamental role TFIID plays in assembly of the preinitiation complex, it has been considered a likely target of upstream factor activation. Several upstream activator proteins have been examined for their ability to interact with TFIID. The pseudorabies virus immediate-early protein was shown to enhance the association of TFIID with promoters in in vitro transcription assays (Abmayr et al. 1988). This stimulation of transcription was particularly strong when examined on nucleosome-repressed promoters (Workman et al. 1988). The mammalian activator proteins USF and ATF, and the yeast activator GAL4, increase the ability of partially purified HeLa TFIID to footprint over the region downstream from the TATA box (Sawadogo and Roeder 1985; Horikoshi et al. 1988a, b). In addition, TFIID reduces the off-rate of USF, further demonstrating an interaction between the two factors (Sawadogo and Roeder 1985).

These pioneering experiments were performed with TFIID partially purified from HeLa cells. Recent studies comparing the activity of such partially purified TFIID fractions with TFIID expressed from the cloned gene in Escherichia coli indicate that the partially purified HeLa TFIID fractions contain additional factors—coactivators—required to mediate activation by upstream factors (for review, see Ptashne and Gann 1990; Dynlacht et al. 1991). These findings raised the question of whether the interaction between upstream factors and TFIID is a direct one or whether additional proteins in the HeLa TFIID fraction act as intermediaries, forming a molecular bridge between upstream factors and TFIID (Ptashne and Gann 1990).
A direct test of this question has been performed with the herpes simplex virus VP16 protein. The VP16 acidic activation domain was shown to bind directly to cloned yeast TFIID (Stringer et al. 1990). Furthermore, mutants in a VP16 activation subdomain, which decrease its affinity for TFIID, decrease activation in vivo (Ingles et al. 1991). The adenovirus E1A trans-activator has also been shown recently to bind directly to cloned human TFIID (Hirokoshi et al. 1991; Lee et al. 1991). However, additional recent experiments demonstrate that the VP16 activation domain also binds TFIIB (Lin and Green 1991). Experiments with agarose-bound DNA templates indicate that the VP16 activation domain stabilized TFIIB binding to the promoter but had little effect on the binding of TFIID. TFIID bound stably both in the presence and absence of the VP16 activation domain (Lin and Green 1991). These results brought the significance of the VP16–TFIID interaction into question. It appeared that the VP16 interaction with TFIIB was of much greater functional significance than the interaction with TFIID.

To examine some of these questions, we have been studying the Zta trans-activator protein encoded by the Epstein-Barr virus (EBV). EBV is a predominantly latent herpesvirus in which the majority of viral genes remain transcriptionally silent during the latent stage (for review, see Kieff and Liebowitz 1991). Initiation of lytic cycle transcription can be induced by expression of Zta immediate-early protein (also known as ZEBRA, EB1, BZLF1) (Countryman and Miller 1985; Chevallier-Greco et al. 1986). Zta is a member of the b-Zip family of sequence-specific DNA-binding proteins and binds as a homodimer to multiple sites in promoter regions of target genes (Farrell et al. 1989; Chang et al. 1990; Flemington and Speck 1990; Lieberman and Berk 1990). Structure-function studies show that the basic region is responsible for DNA-binding specificity, that zipper sequences are essential for dimerization, and a region in the amino-terminal domain provides the transcriptional activation function (Chang et al. 1990; Flemington and Speck et al. 1990, Giot et al. 1991). The activation domain of Zta is not rich in acidic amino acid residues; and although it is somewhat proline and glutamine rich, it shows no obvious homology to previously characterized activator sequences (Giot et al. 1991).

In this work we investigate the possibility that Zta alters the association of TFIID with an EBV lytic-cycle promoter. The BHLF1 minimal promoter (MinL) of EBV has been shown to respond to Zta trans-activation in tissue culture transfection experiments (Chavrier et al. 1989; Lieberman and Berk 1989) and in vitro transcription assays with Zta purified from engineered E. coli (Lieberman and Berk 1990). An interesting feature of the MinL promoter is that it has very low constitutive levels of transcription in vivo and in vitro. This low basal activity may be due, in part, to the relatively poor TATA element (GATAA) at the –30 position relative to the transcriptional initiation site. Promoters with weak or missing TATA consensus elements at the –30 position may be more likely to be rate limited by their association with TFIID. For this reason we thought that MinL might be a good candidate for a promoter that requires upstream factors to stabilize the interaction of TFIID with the template. We show here that Zta binds directly to TFIID and that this interaction stabilizes TFIID binding to the MinL GATA box.

Results

Amino-terminal sequences of Zta are essential for trans-activation

To investigate the biochemical properties of Zta important for transcriptional activation we wished to construct Zta mutants that were competent for DNA binding but incapable of activating transcription. The DNA-binding domain and dimerization domain have been mapped to the basic region and leucine zipper homology in the carboxy-terminal half of the 240-amino-acid residue protein (Chang et al. 1990; Flemington and Speck 1990). Several Zta mutants were constructed with internal deletions in the amino-terminal half of the protein and tested by transient transfection assay for activation of the MinL promoter. We found that deletion of amino acid residues 25–141 eliminated almost all Zta trans-activation function (Fig. 1). Smaller deletions led to smaller reductions in trans-activation. Deletion of residues 25–86 led to a 90% loss of activity, whereas deletion of sequences 93–141 led to only a 50% loss of activity. We conclude that the bulk of the activation domain resides in sequences 25–86 but that the region spanning sequences up to amino acid residue 141 contributes to the full trans-activating properties of Zta.

Zta has been shown to activate transcription from the MinL promoter in in vitro transcription reactions (Lieberman and Berk 1990). To test whether the amino-terminal sequences of Zta that were found to be essential for in vivo transcriptional activation were similarly required for stimulation of transcription in vitro, we constructed an amino-terminal deletion mutant of Zta (ΔZta) that lacked amino acid sequences 2–141. Full-length Zta and ΔZta were expressed and purified from E. coli in a parallel manner. Both protein preparations were assayed by DNA-binding activity in electrophoretic mobility-shift assay (EMSA) and DNase I footprinting (not shown). When 10 footprinting units of Zta were added to an in vitro transcription reaction containing 50 μg of HeLa cell nuclear extract we observed a substantial stimulation of transcription from the MinL promoter (Fig. 1C, lane 2). When 10 footprinting units of amino-terminal deletion mutant ΔZta were added, far less activation was observed (Fig. 1C, lane 3). This demonstrates that the domain of Zta required to stimulate transcription in vivo is also required to stimulate transcription in vitro.

Transcription reactions reconstituted with partially purified general factors and either recombinant TFIID or a TFIID fraction partially purified from HeLa cells clearly demonstrated that Zta trans-activation required a coactivator that copurified with the TFIID fraction from HeLa cells (P.M. Lieberman and A.J. Berk, unpubl.).
However, the preparation of recombinant TFIID used in the studies reported below could support Zta activation when complemented with HeLa cell nuclear extract that had been depleted of TFIID activity by mild heat treatment at 45°C for 6 min (Fig. 1C, lanes 4–9). This is similar to the observations of Pugh and Tjian (1990) and Peterson et al. (1990) concerning other upstream transcription factors. When the heat treatment was extended to 15 min at 45°C, recombinant TFIID no longer supported Zta trans-activation. Similar results were recently reported by White et al. (1991). Because recombinant TFIID could functionally complement a mild heat-treated HeLa cell extract to support Zta activation, it seemed reasonable to investigate the possibility that Zta interacts directly with recombinant TFIID. As mentioned in the introductory section, several investigators have found evidence for a physical interaction between some upstream activator proteins and TFIID (Sawadogo and Roeder 1985; Horikoshi et al. 1988a, b; Stringer et al. 1990; Horikoshi et al. 1991; Lee et al., 1991). Although an interaction between Zta and cloned TFIID may not be sufficient for reconstitution of transcriptional activation in vitro, it may still be a significant part of the transcriptional activation mechanism.

Zta stabilizes the association of TFIID with responsive promoters in an immunoprecipitation assay

To test the possibility that Zta interacts directly with the cloned TFIID polypeptide we expressed both Zta and TFIID in E. coli and purified them to near homogeneity. The two proteins were then tested for their ability to interact with each other on promoter DNA by an immunoprecipitation assay. The MinL and E1B promoter DNAs were end labeled to similar specific activities and incubated with purified human TFIID (HIID) in the presence or absence of purified Zta. Radiolabeled DNA was then precipitated by antibodies directed against TFIID or Zta (Fig. 2A). Precipitation of promoter fragments by antihuman TFIID antibody (α-HIID) showed that TFIID has a stronger affinity for the E1B promoter, which has a consensus TATA box (TATATAAT) than for the MinL promoter template, which has a nonconsensus TATA box (GATAAAAG) (Fig. 2A, lane 3). However, addition of Zta protein resulted in a significant increase in the amount of MinL promoter template precipitated relative to the E1B template (Fig. 2A, lane 4). Zta alone was not precipitated by the α-HIID antibody (lane 6). These results demonstrate that Zta enhanced the association of recombinant TFIID with the MinL promoter in the absence of any other eukaryotic proteins.

To determine whether the Zta-dependent increase in TFIID association with the MinL promoter correlates with transcriptional activation function, we tested the ability of the amino-terminal deletion mutant ΔZta, lacking amino acid residues 2–141, to affect the ability of TFIID binding. ΔZta had a greatly reduced effect on the in vitro transcription of the MinL promoter relative to full-length Zta (Fig. 1C). When ΔZta was incubated with TFIID and promoter DNA, the amount of MinL DNA
This is the expected consequence for a DNA molecule full-length Zta alone (Fig. 2A, lanes 12, 13; Fig. 2B, lanes 8, 9). These observations indicate that Zta and TFIID stabilize the interactions of one another with MinL DNA. No DNA was precipitated by the α-HIID antibody in the absence of HIID protein (Fig. 2A, B, lanes 6, 7), and pre-immune serum did not precipitate complexes in a nonspecific manner (Fig. 2A, lanes 14-16).

The ability of Zta to enhance the association of TFIID to promoter DNA was not dependent on the unique characteristics of the MinL promoter. A synthetic Zta-responsive promoter, Z₃T, which contains three Zta-binding sites upstream from the E1B TATA element, behaved similarly to the MinL promoter in this analysis (Fig. 2B). The Z₃T promoter can be activated more than fivefold by the addition of full-length Zta to an in vitro transcription reaction, whereas ΔZta did not activate this promoter in vitro (data not shown). Addition of Zta increased the number of Z₃T templates precipitated by HIID and α-HIID antibody relative to control E1B template (Fig. 2B, cf. lanes 3, 4). Again, in the absence of HIID, α-Zta antibody precipitated more of the DNA bound by ΔZta than DNA bound by full-length Zta (lanes 8, 9). Addition of TFIID to the binding reaction with Zta increased the amount of DNA precipitated with the α-Zta antibody (Fig. 2B, cf. lanes 8, 11).

These results demonstrate that transcriptionally active Zta enhances the association of recombinant TFIID with Zta-responsive promoters. Both Zta and TFIID were purified from E. coli and were free of any contaminating eukaryotic proteins. Full-length Zta facilitated the stable binding of TFIID to the MinL promoter in this immunoprecipitation assay, which involves several washing steps. Zta also stabilized the binding of TFIID to the Z₃T promoter. In both cases, this activity required the activation domain of Zta. In addition, TFIID stabilized the binding of full-length Zta to MinL and Z₃T DNA, providing further evidence for an interaction between purified Zta and purified TFIID.

The activation domain of Zta is required to stabilize TFIID binding to MinL as determined by EMSA

To corroborate that Zta stabilizes the association of TFIID with the MinL promoter we employed a gel EMSA as an independent measure of the influence of Zta on TFIID binding. Initial studies with low percentage polyacrylamide gels showed that large complexes formed on the MinL promoter (four Zta-binding sites plus a GATA box) did not enter the gel. Consequently, we turned to the use of 1.4% agarose gels, which did resolve these large complexes (Fig. 3). Addition of TFIID to MinL promoter DNA did not result in any detectable retardation of free probe at the concentration of TFIID used in these experiments (Fig. 3A, lanes 1, 2). However, when Zta was added to the binding reaction, addition of TFIID resulted in a further shift over that observed with Zta alone (Fig. 3A, lanes 3, 4). Addition of higher concentrations of Zta resulted in the binding of additional Zta dimers to the probe, as observed by a further decrease in the mobility of the Zta–promoter DNA complex (Fig. 3A, lane 7). At this higher Zta concentration, addition of TFIID led to a
further shift of nearly all of the promoter DNA. Similar results were observed with the ZtgT promoter, which contains the E1B consensus TATA element (data not shown). In contrast, a 30-bp oligonucleotide containing a single ZRE site or a single TATA-binding site did not support the formation of this slower migrating complex [data not shown]. To demonstrate that TFIID was stably associated with the slowest migrating complex. The MinL promoter was incubated with 5 ng of TFIID (lane 2), with 0.5 footprinting units at concentrations of 1 [lanes 3,4] or 5 [lanes 7,8] footprinting units. Free probe mobility is indicated at left. (B) Addition of α-TFIID antibody demonstrates that TFIID is stably associated with the slower mobility complex. The MinL promoter was incubated with 5 ng of TFIID [lane 2], with 0.5 footprinting units of Zta [lane 3], or both [lanes 4–6]. Antibody directed against TFIID was added to lane 5, and control pre-immune serum was added to lane 6. Free probe and antibody–protein–DNA complexes are indicated at left.

These results indicate that binding of Zta to MinL promoter DNA facilitates binding of TFIID in a manner that is stable to the EMSA procedure. To test whether this activity of purified Zta correlates with its transcriptional activation function, we analyzed the influence on TFIID binding of ΔZta. ΔZta, lacking amino acid residues 2–141, formed a similar distribution of complexes on MinL promoter DNA as did full-length Zta, except that the complexes had much faster mobilities [Fig. 3A, lanes 5,9]. In contrast to the result with full-length Zta, addition of TFIID to the binding reaction with ΔZta did not result in a further shifted complex [lanes 6,10]. Thus, the activation domain of Zta is required to facilitate the binding of TFIID in the EMSA-stable complexes.

Zta reduces the off-rate of bound TFIID from TATA elements

The ability of Zta to stabilize TFIID binding was detected by EMSA and immunoprecipitation of radiolabeled promoter DNA. However, in a standard DNase I protection assay or in vitro transcription reaction, we failed to see an apparent change in the concentration of recombinant TFIID protein required to bind to the TATA element nor did we see a significant difference when bound proteins were challenged with competitor oligonucleotide [data not shown]. We reasoned that the difference between the EMSA and immunoprecipitation experiments, on the one hand, and the footprinting and transcription reactions, on the other, was essentially a difference of dilution after binding. In the case of EMSA and immunoprecipitation reactions, bound TFIID is separated from free TFIID. This reduction in the free pool of TFIID would then cause the TFIID off-rate to be a major factor in detection of a TFIID–DNA complex. In principle, there is a significant mechanistic difference between dissociation upon dilution and intersegment transfer, which occurs with increasing competitor concentration [von Hippel and Berg 1990].

To test this hypothesis more directly, we performed DNase I footprinting in the following manner. Protein and DNA were incubated in a volume of 10 μl. After a 1-hr incubation, samples were either treated immediately with DNase I [Fig. 4A, lanes 1–4] or diluted with 200 μl of binding buffer and treated with DNase I at various times after dilution [Fig. 4A, lanes 5–18]. When the MinL promoter was used as a footprinting probe, we found that TFIID bound to the GATAA element under concentrated conditions [Fig. 4A, lane 2]. Interestingly, immediately after a 20-fold dilution of the binding reaction the protection over the GATAA element was completely lost [Fig. 4A, lane 7]. However, in the presence of Zta, TFIID binding over the GATAA element was both stabilized and altered [Fig. 4A, lanes 8,10,12,14,16]. The presence of Zta reduced the size of the TFIID protection and induced strong hypersensitive sites near the start site of transcription and at positions –13 and +16 relative to the transcriptional initiation site [Fig. 4A, cf. lanes 2 and 3, 8,10,12,14,16]. Zta stabilized this altered binding of TFIID to the GATAA and retained the hypersensitive DNase I cleavage sites for up to 4 hr after dilution [Fig. 4A, lanes 6–18]. Consistent with the observations from the EMSA [Fig. 3] and immunoprecipitation of radiolabeled DNA [Fig. 2], the amino-terminal dele-

Figure 3. Zta stabilizes TFIID binding to the MinL promoter in gel EMSA. (A) The MinL promoter probe was incubated in the presence (+) or absence (−) of 5 ng of purified recombinant TFIID. Full-length Zta was included in the reactions at a concentration of 1 [lanes 3,4] or 5 [lanes 7,8] footprinting units. Amino-terminal deletion mutant ΔZta was included in reactions at concentrations of 1 [lanes 5,6] or 5 [lanes 9,10] footprinting units. Free probe mobility is indicated at left. (B) Addition of α-TFIID antibody demonstrates that TFIID is stably associated with the slower mobility complex. The MinL promoter was incubated with 5 ng of TFIID [lane 2], with 0.5 footprinting units of Zta [lane 3], or both [lanes 4–6]. Antibody directed against TFIID was added to lane 5, and control pre-immune serum was added to lane 6. Free probe and antibody–protein–DNA complexes are indicated at left.
Figure 4. Zta reduces the dissociation of TFIID bound to the TATA element as assayed by DNase I footprinting. (A) The MinL promoter was incubated with 5 ng of recombinant TFIID alone (lanes 2,7,9,11, 13,17), with 1 footprinting unit of Zta alone (lanes 4,6,18), or with Zta and TFIID (lanes 3,8,10,12,14,16). Lanes 1 and 5 contain no TFIID or Zta. Proteins were allowed to bind in 10-μl volume for 1 hr at 30°C. Reactions in lanes 1–4 were treated with DNase I immediately. Samples (lanes 5–18) were diluted 20-fold and treated with DNase I at the times indicated above. The MinL GATAA element and ZRE sites are indicated at left. Hypersensitive sites at −13, +16, and near the transcriptional initiation site are indicated by arrows at left. (B) The Z3T promoter was incubated with Zta alone (lanes 2,14), TFIID alone (lanes 3,5,7,9,11), or with TFIID and Zta (lanes 4,6,8,10,12). Proteins were bound to DNA in a 10-μl volume and then diluted 20-fold. Samples were treated with DNase I at 0 min (lanes 1–4), 30 min (lanes 5,6), 60 min (lanes 7,8), 120 min (lanes 9,10), or 240 min (lanes 11–14) after dilution. The Z3T promoter TATA element and ZRE sites are indicated at left.

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Figure 4. Zta reduces the dissociation of TFIID bound to the TATA element as assayed by DNase I footprinting. (A) The MinL promoter was incubated with 5 ng of recombinant TFIID alone (lanes 2,7,9,11, 13,17), with 1 footprinting unit of Zta alone (lanes 4,6,18), or with Zta and TFIID (lanes 3,8,10,12,14,16). Lanes 1 and 5 contain no TFIID or Zta. Proteins were allowed to bind in 10-μl volume for 1 hr at 30°C. Reactions in lanes 1–4 were treated with DNase I immediately. Samples (lanes 5–18) were diluted 20-fold and treated with DNase I at the times indicated above. The MinL GATAA element and ZRE sites are indicated at left. Hypersensitive sites at −13, +16, and near the transcriptional initiation site are indicated by arrows at left. (B) The Z3T promoter was incubated with Zta alone (lanes 2,14), TFIID alone (lanes 3,5,7,9,11), or with TFIID and Zta (lanes 4,6,8,10,12). Proteins were bound to DNA in a 10-μl volume and then diluted 20-fold. Samples were treated with DNase I at 0 min (lanes 1–4), 30 min (lanes 5,6), 60 min (lanes 7,8), 120 min (lanes 9,10), or 240 min (lanes 11–14) after dilution. The Z3T promoter TATA element and ZRE sites are indicated at left.

transcription mutant ΔZta did not stabilize the footprinting of TFIID over the MinL GATAA sequence [data not shown]. The presence of TFIID also influenced the binding of Zta to the ZRE sites. Although Zta remained bound for up to 4 hr in the absence of TFIID [lane 18], the Zta footprints were more complete in the presence of TFIID [lane 16]. These results demonstrate that purified Zta and TFIID affect the DNA-binding properties of one another. The Zta–TFIID–MinL complex showed altered protection over the GATAA element, hypersensitive sites near the start site of transcription and greatly reduced TFIID dissociation rate.

To eliminate the possibility that these observations were a peculiarity of a unique promoter, we performed a similar experiment on the synthetic Zta-responsive promoter, Z3T. Z3T contains three Zta-binding sites upstream of the E1B TATA element, which binds TFIID with higher affinity than does the GATA element of the MinL promoter. Again, binding reactions were carried out in a small volume (10 μl) and then diluted 20-fold. DNase I was added at various times after dilution. With the E1B TATA element, TFIID bound more stably to the TATA box in the absence of Zta than it did to the MinL promoter [Fig. 4B]. At times immediately after dilution, TFIID bound almost identically in the presence or absence of Zta [Fig. 4B, lanes 3,4]. However, when Zta was bound to the Z3T promoter, TFIID dissociated from the TATA box more slowly than when Zta was absent [Fig. 4B, lanes 7–12]. Zta did not protect the TATA element in the absence of TFIID [Fig. 4B, lanes 2,14]. Interestingly, simultaneous binding of Zta and TFIID did not produce the same qualitative changes in the DNase I footprinting pattern over Z3T as observed for the MinL A promoter. The reduction in the size of the TATA element protection and hypersensitive sites near the initiation site was not observed with the Z3T promoter. Again, TFIID did stabilize the binding of Zta to the ZRE1 element [Fig. 4B, cf. lanes 12 and 14], similar to the observed enhancement of Zta binding by TFIID noted with the MinL promoter. Most importantly, the presence of Zta reduced the dissociation rate of TFIID from the consensus E1B TATA element, although to a less dramatic extent than that observed for the stabilization over the MinL GATAA sequence.

Zta stabilizes TFIID binding as assayed by in vitro transcription reactions

To demonstrate that the stable complex formed between Zta and TFIID is functional for transcription in vitro, we performed transcription reactions on DNA templates fixed to agarose beads (Arias and Dynan 1989). This procedure has been used by other investigators to demonstrate that acidic activators increase the affinity of TFIIB for promoter templates (Lin and Green 1991). The MinL promoter fixed to agarose beads was incubated with recombinant TFIID or HeLa cell nuclear extract in the presence or absence of Zta (Fig. 5A). Preinitiation complexes were then washed and resuspended in transcription buffer supplemented with the HeLa cell phosphocellulose C and A fractions (PC-C, PC-A) and ribonucleotides. As expected, Zta preincubated with nuclear
whether Zta stabilized the binding of native TFIID found in HeLa cell phosphocellulose D fraction (PC-D) to the MinL promoter. The MinL promoter DNA fixed to agarose beads was incubated with PC-D in the presence or absence of Zta protein. The resulting DNA–protein complexes were then washed and resuspended in buffer containing the HeLa cell PC-C and PC-A fractions and ribonucleotides [rNTP]. When Zta and PC-D were included in the preincubation step, transcription from MinL was reconstituted by the addition of PC-C, PC-A [Fig. 5B, lanes 1–4]. However, when MinL was preincubated with PC-D in the absence of Zta, transcription could not be restored by Zta alone [Fig. 5B, lane 5]. As expected, transcription was restored by the addition of both Zta and PC-D [Fig. 5B, lane 7], and a low level of transcription was restored by the addition of PC-D alone [Fig. 5B, lane 6]. These results demonstrate that Zta stabilizes the association of the native TFIID activity in the HeLa cell PC-D fraction, as well as the cloned TFIID protein purified from E. coli, with the MinL promoter.

**Transcriptionally active Zta protein makes direct physical contact with TFIID**

The previous set of experiments demonstrated that full-length Zta, but not amino-terminal deletion ΔZta, stabilized the association of TFIID with Zta-responsive DNA templates. The most likely explanation for this finding is that Zta makes direct physical contact with TFIID. To test this possibility, we assayed the ability of Zta to bind directly to TFIID in the absence of promoter DNA. One approach was to perform protein–protein blotting experiments, which we refer to as far Western blotting (Fig. 6; Lee et al. 1991). In this experiment, crude bacterial extracts of Zta, or deletion mutants of Zta, were fractionated on SDS–polyacrylamide gels and transferred to nitrocellulose filters. The filters were renatured and incubated with in vitro-translated [35S]methionine-labeled TFIID protein [Fig. 6A, right]. We found that TFIID specifically bound to full-length Zta protein [Fig. 6A, right], lane 2] but not to amino-terminal deletion mutants Δ25–86 [lane 3], Δ25–141 [lane 4], Δ2–141 [lane 5], or control bacterial extract [lane 1]. All of the amino-terminal deletion mutants were severely impaired in their transcriptional activating functions in vivo [Fig. 1]. The same blots were probed by Zta-specific antisera [Fig. 6A, middle], and polyacrylamide gels were stained with Coomassie brilliant blue [Fig. 6A, left] to demonstrate the relative specificity of the TFIID–Zta interaction. Control 35S-labeled proteins did not react with Zta proteins in this assay [data not shown].

The far Western blotting approach was used to map the region of TFIID responsible for the interaction with the amino terminus of Zta [Fig. 6B]. HIID, yeast TFIID (YIID), the conserved carboxy terminus of HIID (C-term), the unique amino-terminal domain of HIID (N-term), and a carboxy-terminal deletion of HIID (Δ270–339) were all radiolabeled by in vitro translation, normalized by autoradiography of SDS–polyacrylamide gels of the translation products, and used as probes for far Western analysis. We found that the conserved carboxy-terminal domains of HIID and YIID were capable of binding to Zta [Fig. 6B]. The amino terminus of human HIID extract formed a preinitiation complex stable to wash conditions [lane 2]. In the absence of any TFIID, no transcription could be reconstituted with HeLa cell fractions PC-C and PC-A [lanes 3,4]. Preincubation of cloned TFIID alone failed to reconstitute transcription from MinL when the template was washed before the addition of PC-C, PC-A, and NTPs [lane 5]. Addition of Zta with PC-C, PC-A, and NTPs after washing MinL template preincubated with cloned TFIID also failed to induce detectable transcription [data not shown]. Significantly, in the presence of Zta, recombinant TFIID remained stably associated with the MinL promoter throughout the wash procedure and was sufficient to reconstitute transcription with fractions PC-C and PC-A [lane 6]. It is important to mention that recombinant TFIID did bind to the MinL promoter in the absence of washing procedures and was in sufficient concentration to produce readily detectable levels of transcription from the MinL promoter in the absence of Zta [data not shown]. However, when the TFIID–MinL promoter complexes were washed in the absence of Zta, TFIID activity dissociated during the wash procedure. These results show that Zta stabilized the association of recombinant TFIID with the MinL promoter in the absence of additional eukaryotic proteins and that this stable complex is transcriptionally competent.

This transcription system was used to determine whether Zta stabilized the binding of native TFIID found in HeLa cell phosphocellulose D fraction (PC-D) to the MinL promoter. The MinL promoter DNA fixed to agarose beads was incubated with 5 footprinting units of Zta (lane 2, 3 ng of HeLa cell phosphocellulose C and A fractions [PC-C, PC-A] and ribonucleotides [rNTP]. RNA was analyzed by primer extension. (B) The MinL promoter was fixed to agarose beads by biotin–avidin linkage. MinL promoter-containing beads were preincubated with cloned TFIID also failed to induce detectable transcription [data not shown]. Significantly, in the presence of Zta, recombinant TFIID remained stably associated with the MinL promoter [Fig. 6A, right], lane 2] but not to amino-terminal deletion mutants Δ25–86 [lane 3], Δ25–141 [lane 4], Δ2–141 [lane 5], or control bacterial extract [lane 1]. All of the amino-terminal deletion mutants were severely impaired in their transcriptional activating functions in vivo [Fig. 1]. The same blots were probed by Zta-specific antisera [Fig. 6A, middle], and polyacrylamide gels were stained with Coomassie brilliant blue [Fig. 6A, left] to demonstrate the relative specificity of the TFIID–Zta interaction. Control 35S-labeled proteins did not react with Zta proteins in this assay [data not shown].
for that protein (lane 4). As positive and negative controls for this analysis, we observed that the adenovirus E1A protein coprecipitates with TFIID, but the adenovirus E1B 55K protein does not (Fig. 7A, lanes 6,8; Lee et al. 1991).

To confirm the specificity of the interaction of TFIID with Zta and to correlate the association with Zta trans-activating function, we tested three amino-terminal deletion mutants of Zta for their ability to coprecipitate with TFIID (Fig. 7B). We found that Δ25–86 and Δ25–141, which are defective in transcriptional activity, did not coprecipitate with TFIID (Fig. 7B, lanes 6,12). In contrast, transcriptionally active wild-type Zta and Δ93–141 were coprecipitated with TFIID (Fig. 7B, lanes 3,9). The coimmunoprecipitation experiments confirm that TFIID and Zta interact directly in the absence of any promoter DNA. This interaction was dependent on an intact activation domain in the amino terminus of Zta, correlating TFIID binding with transcriptional activation function.

**Discussion**

Zta directly influences TFIID binding to TATA sequences

We have shown by several different biochemical assays that TFIID and Zta form a stable complex in vitro. Although several other transcriptional activator proteins have been reported to interact with TFIID, our investigation of the Zta transcriptional activator is unique among these in several respects. In contrast to the early studies with USF (Sawadogo and Roeder 1985), ATF (Horikoshi et al. 1988b), and GAL4 (Horikoshi et al. 1988a) upstream activators, in this study Zta and TFIID were cloned proteins expressed and purified from E. coli. Consequently, we can rule out the possibility that other eukaryotic proteins mediate the interaction. More recently, the activator proteins VP16 and E1A also have been shown to bind directly to the conserved carboxyl terminus of TFIID (right) or with [35S]methionine-labeled HIID (left) generated by in vitro translation in rabbit reticulocyte lysates. Control bacterial extracts (lane 1), full-length Zta (lane 2), Δ25–86 Zta (lane 3), Δ25–141 Zta (lane 4) and Δ2–141 Zta (lane 5) were loaded at 5–15 μg of total protein normalized for levels of mutant Zta expression. Full-length Zta is indicated by arrow at left. (B) Crude bacterial extracts containing full-length Zta (lanes Z) or control vector (lanes C) were blotted and probed with [35S]methionine-labeled HIID, YIID, the conserved carboxyl terminus of HIID, ΔΣ–147 TFIID (C-Term), the unique amino-terminal domain of HIID, Δ150–339 TFIID (N-Term), or the carboxy-terminal truncation of HIID, Δ270–339 TFIID (Δ270–339). Full-length Zta is indicated by the arrow at left; a strong nonspecific signal is noted by the arrow at right (ns).

and carboxy-terminal truncation Δ270–339 did not bind to Zta.

Direct association of Zta with TFIID was confirmed by coimmunoprecipitation experiments with radiolabeled Zta proteins (Fig. 7). [35S]Methionine-labeled in vitro-translated proteins were incubated with purified E. coli-expressed human TFIID and precipitated with antisera raised against human TFIID. We found that Zta was specifically precipitated by TFIID antisera in a TFIID-dependent manner (Fig. 7A, lane 3). Zta was not precipitated in the absence of TFIID protein (lane 2) and was not precipitated with control immune complexes formed by the addition of β-galactosidase protein and antibody specific for that protein (lane 4). As positive and negative controls for this analysis, we observed that the adenovirus E1A protein coprecipitates with TFIID, but the adenovirus E1B 55K protein does not (Fig. 7A, lanes 6,8; Lee et al. 1991).

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Unlike VP16 and E1A, Zta contains a natural DNA-binding domain. In this respect Zta is the first DNA-binding protein shown to interact directly with cloned TFIID when bound to promoter DNA. Most signifi-
It significantly, we show that Zta bound to promoter DNA stabilized the binding of TFIID to the low-affinity MinL promoter GATA box (Fig. 4). This is the first time a direct interaction with an upstream factor has been shown to stabilize TFIID binding to a TATA element. We also observed that Zta induced several DNase I hypersensitive sites in the TFIID footprint over the MinL promoter, several of which were near the transcriptional initiation site. Also, TFIID enhanced the binding activity of Zta for the ZRE sites nearest the TATA element. These results demonstrate that Zta and TFIID influence the DNA-binding properties of one another, and this effect requires an intact Zta activation domain. Finally, the functional significance of the Zta–TFIID–MinL promoter complex was demonstrated by the in vitro transcriptional activity of DNA affinity-purified complexes (Fig. 5A).

Three distinct activation domains bind directly to the conserved domain of TFIID

The results reported here firmly establish that the Zta transcription factor polypeptide binds directly to the TFIID polypeptide. No intermediary eukaryotic polypeptides are required for the interaction, as it was observed using Zta and TFIID expressed in and purified from E. coli. Zta–TFIID binding was observed by immunoprecipitation of promoter DNA (Fig. 2), EMSA (Fig. 3), DNase I footprinting (Fig. 4), and direct coimmunoprecipitation (Fig. 7) and protein–protein interaction detected by far Western blotting (Fig. 6). Furthermore, TFIID bound by Zta is associated with the GATA box of the MinL promoter in a manner that is functional for transcription (Fig. 5). Most importantly, all of these TFIID interactions were dependent on an intact Zta activation domain. Amino acid residues 25–86 in the Zta amino terminus were required for the interaction with TFIID. These same amino acid residues were essential for transcriptional activation in vivo (Fig. 1; Giot et al. 1991).

Three different types of activation domains have now been shown to bind directly to the evolutionarily conserved ~180-residue carboxyl terminus of TFIID. The first demonstration of this interaction was with the acidic activation domain of VP16 (Stringer et al. 1990). The affinity of the interaction between TFIID and mutants of a subdomain of the VP16 activation domain was shown later to correlate with in vivo activation (Ingles et al. 1991). The adenovirus E1A activation region known as conserved region 3 also interacts specifically and stably with the conserved domain of HIID (Lee et al. 1991). The E1A activation domain is distinct from acidic activation domains in that multiple different single-amino-acid substitutions in E1A severely inhibit activation (Martin et al. 1990). The Zta protein does not contain a high fraction of acidic amino acid residues, nor does it obviously fall into the glutamine- or proline-rich class of activation domains (Courey and Tjian 1988; Mermod et al. 1989). Furthermore, the Zta activation domain shows no obvious homology to the E1A activation region. Thus, these three structurally distinct activation domains may fall into a more general class of upstream transcription factors that bind directly to TFIID. However, not all upstream factors need fall into this class. Whereas there is good evidence that the viral-encoded
E1A, VP16, and Zta proteins make direct contact with TFIID, there was no indication that Spl interacts directly with TFIID [Schmidt et al. 1989; Peterson et al. 1990; Pugh and Tjian 1990]. However, experiments that successfully demonstrated an interaction between TFIID and Zta have not yet been attempted with the Spl trans-activator.

The fact that three different types of activation domains bind TFIID argues that the interactions are not merely coincidental, nonspecific interactions. The specificity of the Zta–TFIID interaction is dramatically demonstrated by the far Western blotting experiments [Fig. 6]. TFIID bound preferentially to Zta even though Zta was not a major protein on the blot. Zta on the blot bound only to forms of TFIID with an intact conserved carboxy-terminal domain. Similar specificity in protein–protein blotting experiments was observed for E1A [Horikoshi et al. 1991; Lee et al. 1991]. The correlation between the affinity of VP16 activation subdomain mutants with in vivo activity [Ingles et al. 1991] also strongly supports the conclusion that the interaction between TFIID and these activation domains is an important part of the activation process.

Function of coactivators

The Zta–TFIID interaction stabilizes TFIID binding to the weak TATA box (GATAAAAG) of the MinL promoter. Under conditions where the DNA protein complexes fixed to agarose beads were washed, this stabilization alone resulted in Zta activation of transcription in a reaction using purified bacterially expressed TFIID [Fig. 5A]. However, in standard in vitro transcription reactions with plasmid templates in solution, Zta did not activate transcription significantly with purified bacterially expressed TFIID [P.M. Lieberman and A.J. Berk, unpubl.]. Zta did activate in standard transcription reactions using TFIID fractions partially purified from HeLa cells [P.M. Lieberman and A.J. Berk, unpubl.]. Clearly, additional components—coactivators [Dynlacht et al. 1991]—in the HeLa cell TFIID fraction were required for activation. One could argue that HeLa cell TFIID associated with coactivators may not interact with Zta in the same way as did recombinant TFIID. Therefore, we tested whether Zta could stabilize the association of TFIID with the MinL template when TFIID was only partially purified from HeLa cells and still complexed to coactivators. Using the agarose-bound template wash protocol, stabilization of TFIID binding was observed for HeLa TFIID as well as it was with recombinant TFIID [Fig. 5B]. Thus, the interaction of Zta with TFIID can occur when TFIID is associated with coactivator molecules.

Other investigators have shown that recombinant TFIID cannot substitute for the HeLa cell fraction of TFIID for the in vitro reconstitution of upstream factor activation [Hoffman et al. 1990; Kambadur et al. 1990; Meisterernst et al. 1990, 1991; Pugh and Tjian 1990; White et al. 1991]. Several lines of evidence point to the requirement for additional components that have been referred to as coactivator, mediator, or adaptor molecules [Berger et al. 1990; Kelleher et al. 1990; Meisterernst 1990, 1991; Peterson et al. 1990; Pugh and Tjian 1990; Dynlacht et al. 1991]. Our unpublished results show that Zta activation in a standard in vitro transcription reaction also depends on the presence of a HeLa cell TFIID-associated coactivator molecule[s] [P.M. Lieberman and A.J. Berk, unpubl.]. Coactivators have been suggested to mediate the physical interaction of upstream activators with the cloned TFIID polypeptide [Pugh and Tjian 1990]. However, we have observed that Zta interacts with TFIID in the absence of any other eukaryotic proteins. Thus, it appears that the coactivator is not essential for the physical bridging of the Zta activation domain with the TFIID polypeptide. Also, Zta stabilizes the binding of TFIID to the TATA box. Consequently, the coactivator is not likely to be required for enhancing the binding of TFIID to the TATA element. This would suggest that stabilization of TFIID binding to promoter DNA by Zta is only a part of the transcriptional activation mechanism. On the basis of these results and recent reports from other investigators [Lin and Green 1991], we speculate that Zta must enhance the ability of the coactivator–TFIID complex to interact with other components of the preinitiation complex.

Implications for the mechanism of upstream factor activation

The activation domains of upstream activating proteins have been shown to be highly heterogeneous and poorly defined in their primary amino acid sequence. Several instances of synergistic transcriptional activity have been observed when multiple copies of the same activating protein are placed upstream of a TATA box element [Carey et al. 1990]. One interpretation of these results is that some activation domains have more than one target. There is considerable evidence that the acidic trans-activator VP16 may interact with at least three different cellular target proteins. VP16 has been shown to bind recombinant TFIID [Stringer et al. 1990; Ingles et al. 1991] and partially purified fractions of HeLa cell TFIIF [Lin and Green 1991]. In addition, VP16 has been shown to sequester a component of the transcriptional machinery that cannot be replaced by recombinant TFIID or the cellular TFIIB fraction [Kelleher et al. 1990, White et al. 1991], indicating a role for a third component, referred to as a mediator, adaptor, or coactivator molecule [Berger et al. 1990; Kelleher et al. 1990; Pugh and Tjian 1990]. All of these upstream factor interactions [direct binding to TFIID and TFIIB, interactions with coactivators, as well as additional interactions not yet discovered] may be required for an activation domain to stimulate transcription. Multiple target interactions might enhance the precision of transcriptional regulation and provide a rationale for why the polymerase II preinitiation complex consists of so many proteins.

Lin and Green [1991] have observed that partially purified HeLa cell TFIID bound stably to the E4 TATA box in the absence of an acidic activator protein. They concluded that acidic activator proteins act at a step subsequent to TFIID binding to the TATA box, specifically by...
recruiting TFIIB. We found that Zta stabilized both recombinant TFIID [Figs. 2, 3, 4, and 5] and a partially purified HeLa cell TFIID fraction [Fig. 5B] binding to the MinL GATAA sequence. This stabilization of TFIID binding to the MinL promoter was partly dependent on the weak TATA box (GATAAA) of the MinL promoter, as a more subtle stabilization was observed for the E1B consensus TATA box element [TATAAT] in the Z3T promoter [Figs. 2 and 4]. Stimulation of transcription owing to the stabilization of recombinant TFIID template binding by Zta was only observed when bound template was washed extensively (30 min total in the experiment of Fig. 5). In a standard transcription reaction Zta failed to stimulate transcription from the recombinant TFIID, presumably because TFIID binding is not rate limiting under these conditions. We suggest that the process of stabilizing TFIID bound to promoter DNA is only a part of the mechanism by which Zta activates transcription and that Zta must also effect a step in initiation subsequent to TFIID binding. In this respect, our results are not inconsistent with those of Lin and Green (1991).

Although it seems likely that activator proteins interact with more than one target protein, the exact functional significance of each of these contacts has yet to be determined. In promoters with TATA boxes having low affinity for the DNA-binding domain of TFIID, such as the MinL promoter, stabilization of TFIID binding by direct interaction with upstream factors may be an important component of the overall activation process. In promoters with a high-affinity TATA box, such as the adenovirus E4 TATA box analyzed in the experiments of Lin and Green (1991), stabilization of TFIID binding may not be an important component of activation. Even in these situations, however, the direct interaction of upstream factors with TFIID might result in TFIID conformational changes that stimulate transcription initiation. The rate-limiting step in transcriptional initiation facilitated by a particular upstream factor may depend on several constraints, including the particular promoter sequence, nucleosome formation, and the presence of other upstream factors.

**Materials and methods**

**Plasmids**

Promoter templates pPL204 and pAD1B4 have been described previously [Schmidt et al. 1989; Lieberman and Berk 1990]. pZ2, which was derived from inserting three copies of 30-bp ZRE5 (Lieberman and Berk 1990) oligonucleotide into the XhoI site of pAd1B4LS ~48/-40 (Wu et al. 1987). The ZRE5 oligonucleotide and the XhoI site of pAd1B4LS ~48/-40 were filled in with Klenow polymerase and blunt-end-ligated. The target promoter pPL98 used in transfections consists of the BHFL1 sequences from the Kpn1 site at ~250 to the BglI site at position 10 cloned into the Bam site of pCATB, as described previously [Lieberman et al. 1989]. Effector genes were cloned into the expression vector pCDL-SRao296, which contains an SV40-HTLVIII hybrid promoter (Takebe et al. 1988). Zta mutants were constructed in pPL102 (Lieberman et al. 1990) and cloned into the EcoRI site of pCDL-SRao296. The Zta mutant Δ25–86 resulted from a HindIII deletion. Mutant Δ25–144 was constructed by blunt-end ligation between the HindIII site at amino acid position 25 and the Nhel site, which were both filled in with Klenow DNA polymerase. Mutant Δ25–136 was constructed by the blunt ligation between the HindIII site at position 25 and the Pvull site. E. coli expression vectors were derived from pPL122 as described previously [Lieberman and Berk 1990]. Deletion mutants were substituted for wild-type sequences by cloning into the EcoRI site of pPL122. The amnon-terminal deletion mutant Δ2–143 was derived from the Zta Nhel-BamHI fragment cloned into the Ndel–BamHI sites of the pET3b expression vector [Rosenberg et al. 1987]. Ndel and Nhel ends were filled in with the Klenow fragment of DNA polymerase.

**Protein purification**

TFIID was purified from E. coli following induction of the pET3b vector described in Kao et al. (1990a). The soluble extract was passed over DEAE-Sepharose in 0.15 M KCl in HEMGN buffer [25 mM HEPES [pH 7.9], 12.5 mM MgCl₂, 0.1 mM EDTA, 10% glycerol, 0.1% NP-40, 1 mM DTT, 0.1 mM PMSF]. The flows through protein was diluted to 0.1 M KCl and bound to heparin–Sepharose. The heparin–Sepharose column was eluted with 0.4, 0.6, and 1.0 M KCl, all in HEMGN buffer. The 0.6–1.0 M KCl fraction was used as the source of E. coli-expressed recombinant TFIID and was estimated to be ~30% pure. Zta was expressed from pPL122 as described previously [Lieberman and Berk 1990]. ΔZta was expressed from the Δ2–143 expression vector as described previously [Rosenberg et al. 1987]. For purification of Zta and ΔZta, soluble E. coli extract was bound to heparin–Sepharose in 0.1 M KCl in HEG buffer [20 mM HEPES [pH 7.9], 0.2 mM EDTA, 20% glycerol, 2 mM DTT, and 0.1 mM PMSF]. The heparin–Sepharose column was washed with 0.4 M KCl and eluted with 0.6 M KCl in HEG buffer. The 0.4–0.6 M KCl fraction was diluted to 0.1 M KCl with HEG buffer and bound to phosphocellulose. The column was washed with 0.4 M KCl and eluted with 0.6 M KCl in HEG buffer. The 0.4–0.6 M KCl fraction was used as the source of Zta or ΔZta. Zta was estimated to be >90% pure, and ΔZta was estimated to be ~50% pure. One footprinting unit of Zta was ~50 ng of protein estimated by Coomassie brilliant blue staining of SDS–polyacrylamide gels with BSA as a standard. HeLa cell nuclear extracts and phosphocellulose fractions were derived as described [Dignam et al. 1983; Reinberg and Roeder 1987].

**Transfections and chloramphenicol acetyltransferase assay**

HeLa cell monolayers were plated at 3.0 × 10⁶ cells per 25-cm² well and transfected by the calcium phosphate method with 1 µg of target DNA and 1–2 µg of effector plasmid DNA [Graham and van der Eb 1973]. Cells were harvested 48 hr later and processed as described previously [O’Hare and Hayward 1984].

**DNA-binding assays**

Binding reactions with Zta and TFIID proteins were performed in binding buffer [12.5 mM HEPES [pH 7.9], 12.5% glycerol, 5 mM MgCl₂, 70 mM KCl, 0.2 mM EDTA, 1 mM DTT] containing 40 µg/ml of poly[dG-C]·poly[dG-C]), and 1 mg/ml of BSA with 2–6 molar of DNA probe. Binding was performed at 30°C for 60 min. DNase I footprinting has been described previously [Lieberman and Berk 1990]. Footprinting reactions were set up in 10 µl and then either treated directly with DNase I, or diluted with 200 µl of binding buffer. Reactions were then made 10 mM MgCl₂, 3.5 mM CaCl₂, and 360 ng/ml DNase I and processed as described previously. Alternatively, complexes were analyzed.
by native gel electrophoresis on 1.4% agarose gels cast in ½ × TBE and run in 1 × TBE at 100 V for 2–3 hr [1 × TBE: 0.045 M Tris, 0.045 M boric acid (pH 8.3), 1 mM EDTA]. Anti-TFIID antibody and control preimmune serum used for EMSA were purified by chromatography over DEAE-Affi-Gel Blue column [Bio-Rad].

DNA-immunoprecipitation assay

Protein–DNA complexes were incubated as described above for binding reactions. Reactions of 50 μl were precleared by incubation for 15 min at 25°C with 20 μl of 1:3 dilution (solid to liquid) of fixed protein A-positive *Staphylococcus aureus* beads that had been washed three times in binding buffer. Samples were then incubated with 3 μl of TFIID or Zta-specific rabbit antisera for 1 hr at 25°C. Protein A–Sepharose beads (300 μl) that were washed three times in binding buffer and diluted to 1:16 (solid to liquid) were added and rocked at 25°C for 1 hr. Protein A–Sepharose beads were then washed three times in 1 ml of binding buffer. Radiolabeled DNA was eluted with 200 μl of stop buffer [1% SDS, 10 mM EDTA, 100 mM NaCl], ethanol-precipitated, and loaded onto 6% polyacrylamide gels.

In vitro transcription

In vitro transcription reactions with the MinL template were described previously [Lieberman and Berk 1990]. HeLa cell nucleolar extracts were heat treated to inactivate endogenous TFIID activity essentially by the method described previously [Nakajima et al. 1988] except that the temperature was reduced to 45°C and the time was only 6 min (White et al. 1991). Transcription reactions performed on templates fixed to agarose beads have been described previously [Arias and Dynan 1989; Lin and Green 1991]. Briefly, 100 μg of MinL DNA linearized at the *HindIII* site (−155) was filled in with biotinylated dATP (GIBCO-BRL) and digested with EcoRI. The 200-bp promoter fragment was then gel purified and incubated with 2.5 ml of streptavidin–agarose (GIBCO-BRL) overnight at 4°C. After washing the beads in 1 M KCl, followed by three washes in TE [10 mM Tris-Cl (pH 8.0); 1 mM EDTA], 20 μl of beads was incubated with transcriptional components for 50 min at 30°C. After the first incubation, the beads were pelleted, washed once in 1 ml of binding buffer containing 2 mM DTT, and rocked for 15 min in 1 ml of the fresh binding buffer two times, pelleting between washes. The beads were then resuspended in transcription reaction buffers containing the protein components indicated for the second incubation. RNA was isolated and analyzed by primer extension as described previously [Schmidt et al. 1989].

Far Western blotting

Crude soluble protein extracts (5–15 μg) from *E. coli* were boiled for 5 min in Laemmli loading buffer, layered on 12.5% SDS–polyacrylamide gels, and transferred to nitrocellulose filters [Schleicher & Schuell, BA83]. The protein blots were then denatured in 6 M guanidine-HCl in renaturation buffer [20 mM HEPES (pH 7.9), 10% glycerol, 60 mM KCl, 6 mM MgCl₂, 0.6 mM EDTA, 2 mM DTT] for 30 min and renatured in 100 mM guanidine-HCl in renaturation buffer supplemented with 0.02% polyvinylpyrrolidone twice for 2 hr at room temperature. The blots were then blocked with 50 μl/ml of BSA in renaturation buffer for 1 hr at room temperature. Protein probes were generated by in vitro transcription–translation in rabbit reticulocyte lysates (Promega) supplemented with [35S]methionine. Probes were normalized for incorporation by autoradiography of SDS–polyacrylamide gels. Typically, 10–50 μl of reticulocyte lysate was then incubated with blots in a 10-ml volume of renaturation buffer for 16 hr at room temperature with gentle shaking. The blots were washed twice in 20 mM HEPES (pH 7.9), 100 mM KCl, 6 mM MgCl₂, 0.2 mM EDTA, and 2 mM DTT for 10 min at room temperature. The blots were dried and exposed to Kodak X-AR film for autoradiography [Lee et al. 1991].

Commmunoprecipitation assay

35S-Labeled proteins were generated by in vitro transcription–translation procedures [Promega]. Briefly, Zta derivatives in pBluescript [SKII+] (Stratagene) were linearized with *XbaI* and transcribed with T3 RNA polymerase. E1B 55K protein was transcribed with SP6 RNA polymerase as described previously [Kao et al. 1990b]. E1A 13S was transcribed with T7 RNA polymerase as described previously [Lee et al. 1991]. cRNA was incubated with rabbit reticulocyte lysate in the presence of [35S]methionine trans label [ICN]. Approximately 200 ng of *E. coli*-expressed TFIID [heparin–Sepharose fraction] was mixed with 1–3 μl of in vitro-translated proteins in a 50-μl volume of binding buffer (see above). Proteins were incubated at 30°C for 1 hr and subjected to a preclearing with *Staphylococcus aureus* Antisera against TFIID or β-galactosidase was then added and allowed to incubate at 25°C for 1 hr. The immune complex was then precipitated with protein A–Sepharose after 1 hr incubation at 25°C with rocking. The protein A–Sepharose beads were then washed three times with 1 ml of 10S buffer [50 mM HEPES (pH 7.2), 250 mM NaCl, 0.3% NP-40, 0.1% Tritton X-100, 0.005% SDS, 10 mM NaPO₄ (pH 7.0), 1 mM Na₂HPO₄ (pH 7.0), 1 mM NaF, 0.1 mM PMSF, and 1 mM DTT], followed by one wash with PBS. The protein A–Sepharose beads were then resuspended in 2 x Laemmli sample buffer, and proteins were loaded onto 12.5% SDS–polyacrylamide gels. Radiolabeled proteins were visualized by autoradiography after enhancement with 1 μm sodium salicylate.

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