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Transcriptional activity of transcription factor IIE is dependent on zinc binding

(RNA polymerase II basal transcription/atomic absorption spectroscopy)

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ABSTRACT **The functions of individual basal transcription factors during the formation of an initiation complex by RNA polymerase II remain largely unknown. Transcription factor IIE (TFIIE) has recently been shown to bind to multiple targets in the initiation complex. To assess the role of zinc binding in basal transcription, we have mutated the predicted zinc-finger domain of human TFIIE. Atomic absorption spectroscopy using purified recombinant proteins revealed that the large subunit, TFIIE-56, is indeed a zinc-binding protein. Mutation of a cysteine residue in the putative zinc-finger domain abolished zinc binding. Moreover, mutant TFIIE-56 failed to support reconstituted basal transcription *in vitro*, suggesting that zinc binding is required for TFIIE function. However, gel-filtration experiments and protein affinity experiments suggest that mutant TFIIE-56 forms a stable heterotetramer with the small subunit, TFIIE-34, that is similar to wild type. Interestingly, gel mobility shift experiments reveal that loss of transcriptional activity by mutant TFIIE is correlated with its inability to stably assemble into the transcription complex. These findings establish that zinc binding by TFIIE may help form a specific structure that is required for stable entry into the transcription complex.**

Transcription of protein-coding genes in eukaryotes is a multienzymatic process and involves RNA polymerase II (Pol II) in addition to a complement of accessory proteins that make up the basal transcription machinery (ref. 1 and references therein). These ancillary components of Pol II transcription exert their effects through core promoter elements, which include the TATA box and initiator motif, and appear to be required for accurate initiation of transcription. The individual contributions of these basal transcription factors (TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH) in the multistep transcription reaction have been of great interest given their potential as targets for regulation by promoter-specific transcription factors. The study of basal transcription factors may also provide mechanistic insight into the events that govern the formation of the first phosphodiester bond during initiation of mRNA transcription. The stages of the transcription process, which include initiation, promoter clearance, elongation, and termination, undoubtedly involve the concerted action of multiple transcription factors. An understanding of how individual basal factors and their subunits help direct the function of a transcription complex is central to decoding the mechanisms that govern mRNA synthesis.

The recent molecular cloning of cDNAs encoding the subunits of basal transcription factors has enabled the biochemical and structural characterization of these proteins. For example, TFIIE is a heterotetramer composed of two 56-kDa and two 34-kDa subunits (2) and has recently been found to contact multiple targets within the basal machinery including TFIID, TFIIF, TFIIH and the nonphosphorylated form of Pol II (3). This finding suggests that TFIIE may play a key role in the assembly and stabilization of the transcription complex. In particular, recent studies suggest that TFIIE may be essential for the recruitment of TFIIH (3, 4). Additionally, recent mechanistic studies have indicated that TFIIE and TFIIH are most likely involved in a step subsequent to initiation, such as promoter clearance, since TFIIE and TFIIH are not required for the formation of an active open promoter complex (5) and are apparently not associated with the elongating Pol II (21). Therefore, a detailed analysis of TFIIE and TFIIH may provide valuable insight into the steps of transcription that follow the initiation event.

Unlike TFIIH, TFIIE activity has been fully reconstituted using recombinant proteins, and computer analysis of the cDNA sequences encoding its two subunits has revealed the presence of several potentially significant structural features (6). Among the predicted structural motifs is a putative zinc-finger domain in the large (56 kDa) subunit of TFIIE (TFIIE-56) that was identified based on its similarity to a zinc-finger domain of the bacterial UvrA protein, a subunit of the UvrABC endonuclease involved in recognition of damaged DNA. Given that zinc-binding domains have been documented to mediate a variety of activities including protein–protein and protein–nucleic acid interactions (7, 8), we were particularly interested in determining whether the predicted zinc-binding domain of TFIIE-56 is involved in imparting functional activity to TFIIE.

Here we report the analysis of a TFIIE-56 mutant bearing an amino acid substitution in its predicted zinc-finger domain (Zn56). Using atomic absorption spectroscopy, we have demonstrated the ability of the wild-type TFIIE-56 to bind zinc. Additionally, we have tested the ability of this mutant to form a stable heterotetramer with the small (34 kDa) subunit of TFIIE (TFIIE-34) and to associate with a stable Pol II transcription complex. Finally, we have tested the ability of the mutant protein to support reconstituted basal transcription *in vitro*. Our results suggest that the zinc-finger domain and zinc binding by TFIIE-56 contribute an essential structure required for functional transcription complex formation.

MATERIALS AND METHODS

Site-Directed Mutagenesis. Cysteine-154 was changed to alanine using a standard site-directed mutagenesis technique developed by Kunkel *et al.* (9). The sequence of the oligonucleotide used for mutagenesis was 5′-GGCAAAAAG-TAGCGCGGAAAGTTCC-3′, and mutagenesis was per-

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Abbreviations: Pol II, RNA polymerase II; TBP, TATA-binding protein; AdMLP, adenovirus major late promoter; TFIIE-56 and TFIIE-34, large (56 kDa) and small (34 kDa) subunits of TFIIE; Zn56, TFIIE-56 mutant with an amino acid substitution in its predicted zinc-finger domain.

formed on a single-stranded Bluescript construct carrying the coding region of the TFIIE-56 cDNA.

Atomic Absorption Spectroscopy. Approximately 0.25 mg of protein was digested in concentrated nitric acid heated to 100°C for 1 hr. Utilizing the graphite furnace attachment on the Perkin–Elmer model 2380 atomic absorption instrument, the zinc concentrations were determined. A preparation of wild-type TFIIE-56 was found to contain 0.015% zinc while the mutant and control preparations contained 0.0008% and 0.001%, respectively, values at the lower level of detection for the instrument (effectively zero). The instrument was calibrated using a four-point linear calibration curve, and the correlation coefficient for the line was >0.995. The calculated stoichiometry was ≈1 zinc atom per molecule of TFIIE-56, based on a conservative estimate of TFIIE-56 purity in the partially purified preparation.

***In Vitro* Transcription.** *In vitro* transcription assays were performed using a fractionated HeLa transcription system supplemented with recombinant TATA-binding protein (TBP), TFIIB, and TFIIE. Recombinant TBP and TFIIB were gifts from Greg Peterson and had been expressed and purified from bacteria as described (10, 11). TFIIE subunits were expressed in bacteria and isolated as described (6). A fraction containing TFIIF and TFIIH was isolated as described (4) through the DEAE 5PW (Toso Haas, Philadelphia) step. The RNA polymerase preparation used was a mixture of the IIO and IIA forms and was purified as described in ref. 12. Transcription reactions were performed in a final volume of 25 μl in buffer containing 20 mM Tris Cl (pH 7.9), 10% (vol/vol) glycerol, 1 mM dithiothreitol, 4 mM $MgCl_2$, 50 mM KCl, 6.5 units of RNasin, 15 units of RNase T1, and 10 mM ammonium sulfate. Each reaction contained 20 ng of TBP, 10 ng of TFIIB, 600 ng of TFIIF/TFIIH fraction, 30 ng of TFIIE-34, 100 and 200 ng of TFIIE-56 or Zn56, 125 ng of RNA Pol II, and 200 ng of template DNA containing the Adenovirus major late promoter (AdMLP) (−53 to +10) upstream of the G-less cassette (13). Transcription factors were incubated with DNA for 30 min at 30°C. Nucleotides (2 μl of a 13× mixture) were added to give final concentrations of 500 μM ATP, 500 μM CTP, and 25 μM [α-^{32}P]UTP (5 μCi; 1 Ci = 37 GBq). Transcription reactions were incubated for 10 min at 30°C. Reactions were stopped by the addition of 100 μl of a solution containing 3.1 M ammonium chloride, 10 μg of carrier yeast RNA, and 15 μg of proteinase K. The samples were subjected to denaturing PAGE after ethanol precipitation, and the transcripts were subsequently visualized by autoradiography.

Dimerization Assay. Heterodimerization was assayed by coimmunoprecipitation of TFIIE-56 and Zn56 with TFIIE-34 in 0.1 M KCl/HEMG buffer as described (3). [^{35}S]Methionine-radiolabeled TFIIE-56 and Zn56 were synthesized using a coupled transcription–translation system obtained from Promega and were tested for the ability to bind TFIIE-34 immobilized on protein A-Sepharose beads via affinity-purified polyclonal antibodies against TFIIE-34. Five microliters of affinity-purified antibodies was used per reaction in the presence or absence of ≈600 μg of recombinant TFIIE-34. TFIIE-34 was purified as described (6).

Heterotetramerization. Heterotetramerization was assayed using gel filtration as described (6) with the following modifications. Filtration was performed in a buffer containing 25 mM Tris Cl (pH 7.9), 0.1 mM EDTA, 10 mM 2-mercaptoethanol, 0.2 M KCl, 10% glycerol, 0.01% Nonidet P-40 using a Pharmacia Superose 6 SMART column. A total of 10–15 μg of TFIIE-34–TFIIE-56 (or Zn56) complexes was analyzed after preincubation of a 1:1 mixture of the subunits on ice for 30 min. The column was calibrated using a Bio-Rad protein standard mixture containing thyroglobulin (670 kDa), bovine gamma globulin (158 kDa), chicken ovalbumin (44 kDa), equine myoglobin (17 kDa), and vitamin B-12 (1.35 kDa); the correlation coefficient for the line was >0.997. Graphic analysis of the elution profiles revealed that both wild-type and mutant TFIIE complexes eluted from the column similarly and at a size consistent with a heterotetramer. SDS/PAGE, visualized by both silver staining and Western blotting, was performed to confirm the coelution of wild-type and mutant complexes. Elution values presented represent an average of three independent experiments for each complex.

Gel Mobility Shift Assay. A DNA fragment containing the AdMLP (−50 to +33) was used as a template for gel mobility shift analysis of partially formed transcription complexes. Approximately 500 cps (2–5 fmol) of probe was used per reaction using conditions described elsewhere (6). Yeast TBP (10 ng per reaction) was kindly provided by Cathy Thut (Tjian Laboratory, University of California, Berkeley). RAP74 (12 ng per reaction; RAP30/74 is also known as TFIIF) and RAP30 (5 ng per reaction) were expressed in bacteria and purified as described (14, 15). TFIIB (125 ng per reaction), RNA Pol II (1 μl of Pol IIa, a kind gift from Lalo Flores, Tularik, South San Francisco, CA), and TFIIE (complexes were formed with reactions containing 18 ng of TFIIE-34 and 100 ng of TFIIE-56 or 100 ng of Zn56 were tested at 1× and 2× concentrations) were prepared as described above. Proteins were incubated with the probe for 30 min at 30°C and the resulting DNA–protein complexes were resolved on nondenaturing 4% acrylamide gels containing 0.5× Tris/borate/EDTA buffer and 2% glycerol.

RESULTS AND DISCUSSION

Zinc Binding of TFIIE. To determine the functional relevance, if any, of the predicted zinc-finger domain of TFIIE-56, site-directed mutagenesis was employed to introduce an alternate amino acid in this region of the protein. Fig. 1*A* illustrates the position of the predicted zinc finger domain in the context of the full-length TFIIE-56. Cysteine-154 was changed to alanine (Fig. 1), thereby preventing the potential coordination of a zinc atom by the cysteine residues in the

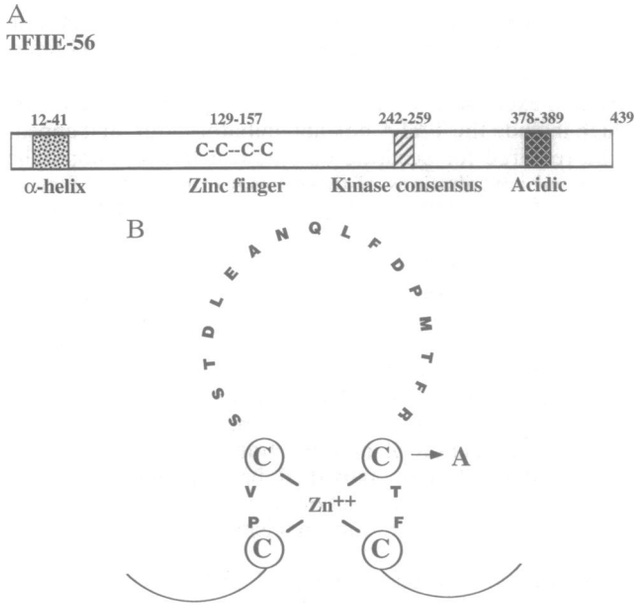

FIG. 1. (*A*) Schematic diagram of TFIIE-56. The residues spanned are shown above each feature: α-helix, region predicted to form an amphipathic α-helix; zinc finger, sequence predicted to form a zinc-finger domain; kinase consensus, region with similarity to protein kinase C-β and -δ; acidic, a region rich in acidic amino acids [after Peterson *et al.* (6)]. (*B*) Schematic diagram of the zinc-finger domain mutation. Using site-directed mutagenesis, cysteine-154 of TFIIE-56 was replaced by an alanine residue.

putative zinc-finger domain. Both wild-type TFIIE-56 and Zn56 proteins were expressed in bacteria and purified in parallel as described (6). Atomic absorption spectroscopy was used to ascertain whether zinc was actually present in the protein preparations. The wild-type TFIIE-56 preparation was found to contain approximately one atom of zinc per molecule of TFIIE-56 (Fig. 2, bar 2). By contrast, the Zn56 preparation and the empty vector control extract were found to have little or no detectable zinc (Fig. 2, bars 1 and 3). These results show that TFIIE-56 is indeed able to coordinate a zinc atom and that altering cysteine-154 to alanine abolishes the ability of the protein to bind zinc.

Zinc Binding Is Required for Transcriptional Activity. To determine whether zinc binding by TFIIE is functionally significant, the Zn56 subunit was tested for its ability to support transcription in an assay using human TBP, TFIIB, TFIIF, TFIIE-34, TFIIH, and Pol II. When transcription was reconstituted in the absence of TFIIE-56, a low level of transcription was observed (Fig. 3, lane 1). This level of transcription may be due to an incomplete dependence on TFIIE for transcription from the supercoiled AdMLP promoter (5, 16) or contamination of the Pol II and/or TFIIH preparations with TFIIE-56. When purified recombinant TFIIE-56 was added, transcription was greatly stimulated (Fig. 3, lanes 2 and 3). By contrast, addition of the recombinant zinc-binding mutant, Zn56, resulted in little or no stimulation of transcription above background (Fig. 3, lanes 4 and 5). Interestingly, at the higher concentration of Zn56, we observed an inhibition of transcription, perhaps resulting from a trans-dominant negative effect. Given that TFIIE has recently been reported to be essential for transcription from all linear templates analyzed (5, 17), we tested the ability of Zn56 to support basal transcription from a linear AdMLP template. As expected, Zn56 was also unable to support transcription from linear AdMLP DNA (data not shown). Thus, in contrast to the wild-type TFIIE-56, Zn56 failed to support transcription in a reconstituted system, indicating that zinc coordination and/or cysteine-154 is required for transcriptional activity from either supercoiled or linear AdMLP templates.

Zinc Binding Is Not Required for Heterodimerization or Heterotetramerization. One possible explanation for the deficiency of Zn56 in reconstituted transcription could be an

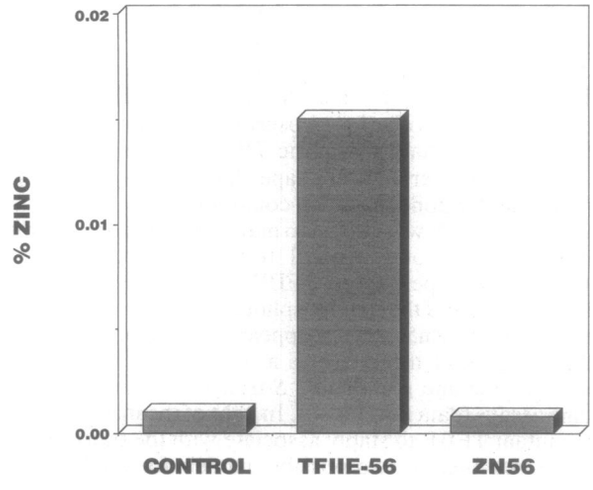

FIG. 2. Atomic absorption spectroscopy analysis reveals the ability of TFIIE-56 to bind zinc. Partially purified wild-type TFIIE-56, zinc-binding domain mutant (Zn56), and an empty vector control extract were subjected to atomic absorption spectroscopy to determine the percentage of zinc present in the samples. A preparation of wild-type TFIIE-56 was found to contain zinc while the mutant and control preparations contained little or no detectable zinc.

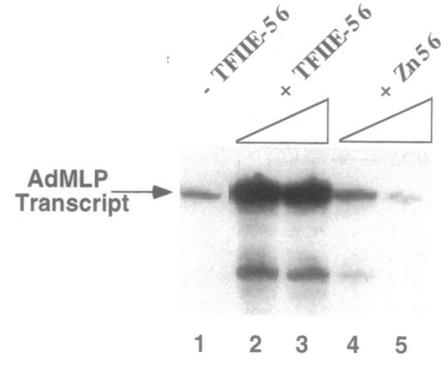

FIG. 3. Mutant TFIIE-56 (Zn56) does not support basal transcription *in vitro*. Recombinant wild-type and mutant TFIIE-56 proteins were purified in parallel and tested for transcriptional activity in a reconstituted HeLa transcription system using supercoiled AdMLP as a template. A low level of basal transcription was observed in the absence of TFIIE-56 (lane 1). The addition of purified TFIIE-56 greatly stimulated transcription (lanes 2 and 3). The addition of Zn56 did not result in the stimulation of transcription (lanes 4 and 5).

inability of the mutant protein to bind the smaller subunit, TFIIE-34. To analyze the potential contribution of zinc binding (or cysteine-154) to interactions between TFIIE-34 and TFIIE-56, coimmunoprecipitation experiments were carried out to detect direct protein–protein interactions. Radiolabeled TFIIE-56 and Zn56 proteins were synthesized using a coupled transcription–translation system and tested for their ability to coimmunoprecipitate with purified recombinant TFIIE-34 using affinity-purified polyclonal antibodies against TFIIE-34. In the presence of TFIIE-34, both the wild-type TFIIE-56 and mutant Zn56 subunits were coimmunoprecipitated (Fig. 4*A*, lanes 5 and 6, respectively). Neither TFIIE-56 nor Zn56 was precipitated by the anti-TFIIE-34 antibodies in the absence of TFIIE-34 (Fig. 4*A*, lanes 3 and 4), indicating that no cross-reactivity of TFIIE-56 exists with anti-TFIIE-34 antibodies. These data suggest that a direct protein–protein interaction between TFIIE-34 and TFIIE-56 occurs independent of the ability of TFIIE-56 to bind zinc.

We next tested the possibility that the zinc-binding domain participates in heterotetramerization. Previous studies have shown that TFIIE-34 migrates as a stable dimer (68 kDa) over a gel-filtration column while TFIIE-56 travels heterogeneously at 110 kDa and larger. The addition of TFIIE-34 to TFIIE-56 has been shown to result in the formation of a TFIIE heterotetramer, which is stable to gel filtration (6). Similar experiments were performed with a TFIIE complex containing the Zn56 mutant. TFIIE-34 and Zn56 were mixed in a 1:1 ratio and incubated on ice for 30 min prior to application to a Pharmacia Superose 6 gel-filtration column. The elution volume (V_e) divided by the void volume (V_o) was determined after SDS/PAGE and was used to graph the elution profiles of both mutant and wild-type complexes. As shown in Fig. 4*B*, the elution of the TFIIE-34–Zn56 complex over a gel-filtration column is quite similar to that of the wild-type complex (V_e/V_o = 1.63 vs. 1.65). Therefore, zinc binding (or cysteine-154) does not appear to be involved in the formation of a stable TFIIE heterotetrameric complex. These data support the notion that, given its ability to form a characteristic TFIIE higher order structure, the Zn56 mutant is clearly not so grossly altered in its secondary structure that heterotetramerization is disrupted.

Zinc Binding Is Required for Entry into the Transcription Complex. It is possible that the failure of Zn56 to reconstitute transcription lies in its inability to interact with other components of the basal transcription factor complex. Earlier

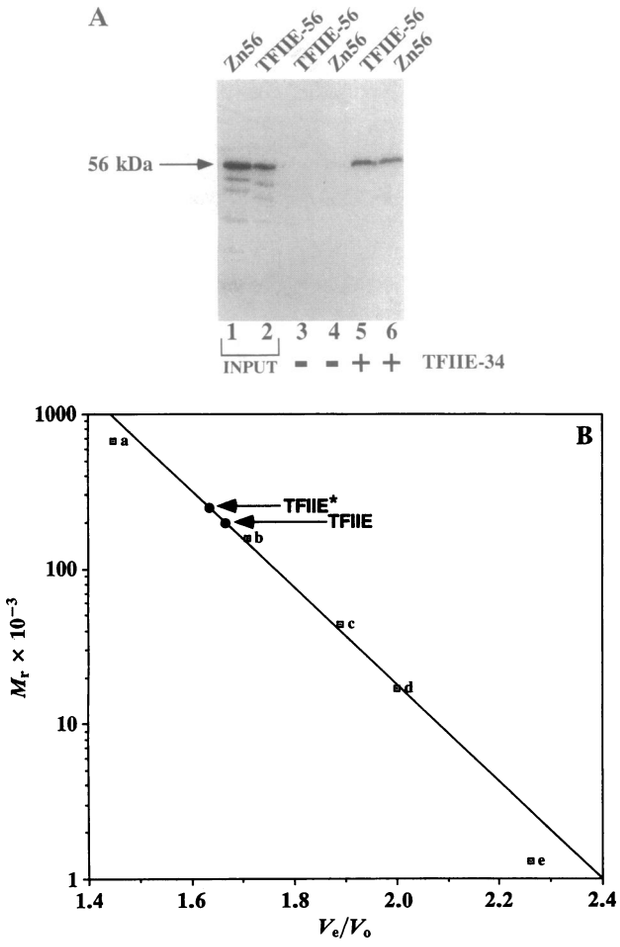

FIG. 4. Zn56 interacts with TFIIE-34. (*A*) Zn56 dimerizes with TFIIE-34. Radiolabeled TFIIE-56 and Zn56 were tested for the ability to coimmunoprecipitate with TFIIE-34. Lanes 1 and 2 show 10% input Zn56 and 20% input TFIIE-56, respectively. Proteins were tested for nonspecific binding on control resins consisting of protein A-Sepharose and α-TFIIE-34 affinity-purified antibodies in the absence of added TFIIE-34 (lanes 3 and 4). The addition of TFIIE-34 resulted in the coimmunoprecipitation of TFIIE-56 (lane 5) and Zn56 (lane 6). (*B*) Zn56 mutant TFIIE complex (TFIIE*) forms a heterotetramer. TFIIE-34 was mixed 1:1 with either TFIIE-56 or Zn56 to form higher order TFIIE complexes. The elution volume (V_e) divided by the void volume (V_o) of wild-type (TFIIE) and mutant (TFIIE*) complexes from a Superose 6 gel-filtration column is shown. The curve was constructed using thyroglobulin (670 kDa; point a), bovine gamma globulin (158 kDa; point b), chicken ovalbumin (44 kDa; point c), equine myoglobin (17 kDa; point d), and vitamin B-12 (1.35 kDa; point e) as standards.

work established that TFIIE enters the transcription complex subsequent to the assembly of a complex of TBP, TFIIB, TFIIF, and Pol II (2, 6). Both TFIIE subunits have been reported to be required for entry of the factor into the transcription complex as measured by DNA mobility shift assays (6). Using this assay, we tested the ability of the Zn56 mutant heterotetramer to bind a partial transcription complex (Fig. 5). Recombinant TBP and TFIIB interact with a DNA template bearing the AdMLP TATA box and initiator element to generate a specific TBP–B complex (Fig. 5, lane 2). Addition of TFIIF (F) and Pol II results in a protein–DNA complex with slower mobility (TBP–B–F–Pol II). As shown in lanes 3 and 4, the addition of the wild-type TFIIE heterotetramer (E) supershifts the TBP–B–F–Pol II complex to form the TBP–B–F–Pol II–E complex, which migrates more slowly through the gel. By contrast, the addition of the Zn56 mutant heterotetramer failed to induce the characteristic

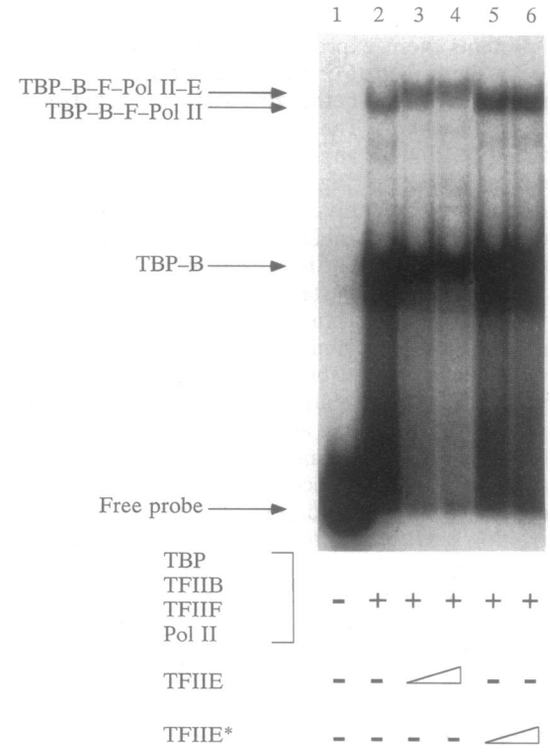

FIG. 5. Zn56 mutant TFIIE complex (TFIIE*) does not stably enter the transcription complex. Lane 1 shows radiolabeled AdMLP fragment in the absence of added transcription complex components. The addition of TBP, TFIIB (B), TFIIF (F), and Pol II results in the formation of the TBP–B–F–Pol II complex (lane 2). Addition of wild-type TFIIE complex (E), a 1:1 mixture of TFIIE-56 and TFIIE-34, results in a supershifted species, TBP–B–F–Pol II–E (lanes 3 and 4). Zn56 mutant TFIIE complex (TFIIE*) does not contribute to the characteristic TBP–B–F–Pol II–E supershift of the TBP–B–F–Pol II complex (lanes 5 and 6).

TBP–B–F–Pol II–E supershift (lanes 5 and 6), indicating that the cysteine to alanine change in Zn56 prevents the mutant heterotetramer from stably entering the preformed partial transcription complex. Thus, it is possible that zinc binding by the TFIIE-56 subunit of TFIIE is required for interactions that result in its stable association with other components of the transcription complex. The failure to form a stable complex in transcription may be responsible, at least in part, for the transcriptional defect of the Zn56 mutant. The possibility exists, however, that the mutant TFIIE complex associates stably with the transcription assembly but migrates indistinguishably from the TBP–B–F–Pol II complex due to a minor alteration in shape that severely affects gel migration under nondenaturing conditions.

Recently, TFIIE was shown to make specific contacts with multiple subunits of the basal transcription complex. In protein binding experiments, TFIIE-56 interacted with TBP, TFIIF, TFIIH, and the nonphosphorylated form of Pol II (3). Surprisingly, the Zn56 mutant appears to be unimpeded in its binding to each of these targets as determined by coimmunoprecipitation and glutathione *S*-transferase fusion protein binding assays (data not shown). In light of the inability of the Zn56 mutant TFIIE to stably associate with the transcription complex, we suggest two possible models for the failure of Zn56 to support reconstituted transcription. First, TFIIE-56 may contact the DNA template directly, and disruption of the zinc-binding domain may abolish DNA binding, resulting in an inability to stably bind the transcription complex. Second, it is possible that the Zn56 mutant interacts with some of its target proteins with an attenuated affinity. While a weakened binding to various protein targets could greatly affect the

stable entry of the Zn56 mutant complex into the transcription assembly as measured by gel mobility shift analysis, slightly weaker binding may not be readily observed in the protein–protein binding assays we have employed.

NMR analysis of the putative nucleic acid-binding domain of transcription factor TFIIS, a highly conserved transcription elongation factor, has revealed a three-stranded antiparallel β-sheet (designated the "Zn ribbon") in the C-terminal nucleic acid binding domain (18). This Cys_4 motif coordinates a single Zn^{2+} with tetrahedral geometry. An analogous Cys_4 motif was proposed by Qian *et al.* (18) for TFIIE-56 based on a two-part computer search employing a degenerate template Cys-Xaa_2-Cys-Xaa_{6-9}-Haa-Xaa_{14}-Cys-Xaa_2-Cys-Xaa_3-Haa (where Haa designates a hydrophobic amino acid). Given the predicted similarity in the zinc ribbon of TFIIS and TFIIE-56, it is tempting to favor the nucleic acid-binding model for TFIIE function. Further support for a potential role of TFIIE in DNA binding stems from the predicted similarity of TFIIE-56 and UvrA, a protein reported to be involved in the recognition of damaged DNA (19, 20). However, all attempts to demonstrate direct double-stranded or single-stranded DNA binding by TFIIE have thus far proven unsuccessful. The possibility exists that stable DNA binding by TFIIE may require assembly of the complete transcription complex and/or formation of an open promoter complex.

The generation of recombinant TFIIE-56 carrying a mutation in the predicted zinc-binding domain has enabled us to demonstrate that TFIIE is a zinc-binding factor and that zinc binding is likely to be required for transcriptional activity. Interestingly, zinc binding is not necessary for the assembly of TFIIE into heterodimeric and heterotetrameric conformations but is implicated in the stable association of TFIIE with the transcription complex. These results support the notion that the zinc-binding domain of TFIIE plays an essential role in the assembly of a transcriptionally competent RNA Pol II nucleoprotein complex and that disruption of this structure confers local instability to the transcription complex, resulting in a loss of function. That a single amino acid change in TFIIE-56 (cysteine-154 to alanine) renders it defective for transcription is significant in that it demonstrates the importance of the zinc-binding domain for a critical step in transcription. Zinc binding domains have been shown to participate in protein–protein interactions as well as interactions with both double-stranded and single-stranded nucleic acids (ref. 8 and references therein). Given that TFIIE has been reported to function in transcription at a step subsequent to the formation of an open complex (5), it is possible that the zinc domain of TFIIE-56 may act at the open transcription "bubble" in some way to facilitate promoter clearance. It is expected that the Zn56 mutant will serve as a molecular tool to provide useful insight into the mechanism of transcription.

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