Analysis of the Role of TFIIE in Transcriptional Regulation through Structure-Function Studies of the TFIIEβ Subunit*

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Tomoko Okamoto‡, Seiji Yamamoto‡, Yoshinori Watanabe‡, Tsutomu Ohta¶, Fumio Hanaoka‡, Robert G. Roeder§, and Yoshiaki Ohkuma‡§

From the ‡Institute for Molecular and Cellular Biology, Osaka University, Suta, Osaka 565-0871, Japan and the ¶Laboratory of Biochemistry and Molecular Biology, The Rockefeller University, New York, New York 10021

The general transcription factor TFIIE plays important roles at two distinct but sequential steps in transcription as follows: preinitiation complex formation and activation (open complex formation), and the transition from initiation to elongation. The large subunit of human TFIIE (TFIIEα) binds to and facilitates the enzymatic functions of TFIIBH, but TFIIE also functions independently from TFIIBH. To determine functional roles of the small subunit of human TFIIE (TFIIEβ), deletion mutations were systematically introduced into putative structural motifs and characteristic sequences. Here we show that all of these structures that lie within the central 227-amino acid region of TFIIEβ are necessary and sufficient for both basal and activated transcription. We further demonstrate that two C-terminal basic regions are essential for physical interaction with both TFIIEα and single-stranded DNA, as well as with other transcription factors including the Drosophila transcriptional regulatory gene Krüppel. In addition, we analyzed the effects of the TFIIEβ deletion mutations on TFIIBH-dependent phosphorylation of the C-terminal domain of RNA polymerase II and on wild type TFIIEβ-driven basal transcription. Both responsible regions also mapped within the essential 227-amino acid region. Our results suggest that TFIIE engages in communication with both transcription factors and promoter DNA via the TFIIEβ subunit.

In eukaryotes productive transcription initiation by RNA polymerase II (Pol II)‡ plays a key role in the regulation of gene expression in response to various developmental and environ-
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...cruiting TFIIF into the PIC and for the modulation of the two functions of this factor in transcription initiation and in the transition from initiation to elongation.

Human TFIIEβ, which is a highly basic (pI 9.5) protein of 291 amino acids, also possesses several conserved structural motifs and characteristic sequences (10, 17, 18). Photocross-linking studies revealed that TFIIEβ binds to promoter DNA in the region between positions −14 and −2 from the transcription initiation site (+1), a property that distinguishes it from TFIIEα which cannot be cross-linked with DNA (19). Two-dimensional crystallography of the highly related yeast TFIIE with Pol II has confirmed these observations by showing that TFIIE interacts with the active center of Pol II relative to the transcription initiation site (20). Other recent studies have demonstrated that the introduction of short mismatched heteroduplex DNA regions around the initiation site (minimally from positions −4 to +2) in topologically relaxed templates abolishes the requirement for TFIIE, TFIIF, and β-γ ATP hydrolysis (21–23). Importantly, changes in the short mismatch region create differential requirements for TFIIE and TFIIF with, most notably, a continued requirement for TFIIE but lack of a requirement for the function of TFIIF being observed (23). These results indicate two possibilities as follows: first, that TFIIE and TFIIF play a role around the transcription initiation site during open complex formation, and second, that TFIIE has a unique function, possibly in promoter melting, distinct from its role with TFIIH.

To elucidate further mechanisms of TFIIE function, and especially that of TFIIEβ, we constructed a series of deletion mutants of TFIIEβ. We tested the ability of these mutants to support both basal and activated transcription and to associate with other general transcription factors and transcriptional regulatory factors, and we examined their effects on CTD phosphorylation by TFIIF as well as their dominant negative effects on basal transcription. In so doing, we have succeeded in identifying a central core that is important for the mediation of TFIIE function and which contains the C-terminal basic regions essential for binding both to general transcription factors and to single-stranded (ss) DNA. In addition, we provided a new clue to approach that TFIIE is not only recruiting TFIIF into the PIC and functioning as a bridge between Pol II and TFIIF for its CTD phosphorylation but also playing an unidentified important role during transcription initiation and in the transition from initiation to elongation.

EXPERIMENTAL PROCEDURES

DNA Templates—For basal transcription assays, the plasmid pMLC(ATA−50 containing the adenovirus type 2 major late promoter was used as a template (24). To study transcriptional activation, the plasmid pG5HM(CAT) was used as the test template (25), with the plasmid pMLC(ATA−53S) as the base-line control (26). pG5HM(CAT) contains five GALA-binding sites and the core promoter as described previously (15). The two templates pMLC(ATA−50 and pG5HM(CAT) give 390-nucleotide transcrip...ts, and pMLC(ATA−53S) gives a 290-nucleotide transcript.

Construction of Various Expression Vectors—The isolated plasmid p2EB contains the complete open reading frame of human TFIIEβ (TFIIEβ cDNA) cloned into Bluescript II SK− (−) phagemid (Stratagene) (17). This was first digested with XhoI and CiaI, and the 1.7-kilobase pair cDNA fragment was subcloned into the pGEM-7Zf(−) vector (Promega) to create a BamHI site at the 3′-end of TFIIEβ cDNA. The oligonucleotide 5′-CCCTTCTACCTGACCATGATGACCCACGC- CCTGG3′ was then used to create an NdeI site at the first methionine codon of TFIIEβ cDNA and to disrupt a BamHI site located just after the first methionine by site-directed mutagenesis (27). Finally, the NdeI-BamHI fragment of this cDNA clone (p2EBT) was subcloned into the 6His-T-PE11d vector to construct the histidine (His)-tagged TFIIEβ (6His-TFIIEβ) expression plasmid.

To set up the coexpression system of the two TFIIF subunits for the purpose of preparation of transcriptionally active recombinant TFIIF, the human TFIIFβ (RAP30) expression plasmid (pET11d) (29) was digested with XhoI and EcoRI, and both ends of this fragment containing TFIIFβ cDNA were converted to blunt ends, whereas the expression plasmid of human TFIIFα (RAP74) with six His tags at the C terminus (in pET23d) (30) was digested with XhoI, and the XhoI sites were blunted to blunt ends and, after being phosphorylated with calf intestine phosphatase, then the TFIIFβ cDNA fragment was subcloned into the XbaI sites (blunt) of the TFIIFα expression plasmid to place both cDNAs tandemly and the 6His-TFIIF expression plasmid was constructed.

The plasmid pBSK containing cDNA encoding the Drosophila developmentally and tissue-specific gene product (Kr) was kindly provided by S. Belasco and H. Ja¨ckle (31, 32). To create an NdeI site at the first methionine, two oligonucleotides Kt1 (5′-GACCGCTCATAGTGCATATCCAT-GTCTCT-3′) and Kt2 (5′-CATGGACGTAGATGAAGTGC-3′) were used for polymerase chain reaction. This 82-base pair polymerase chain reaction product was digested with NdeI and XbaI, and the NdeI-XbaI fragment was then subcloned with the XbaI-EcoRI fragment of Kr cDNA into the NdeI and EcoRI sites of the HA influenza hemagglutinin epitope sequence-pET11d vector to construct the HA-tagged Kr (full length) expression plasmid (Fig. 7A). The HA-pET11d vector was constructed by inserting the NcoI-NdeI fragment of HA(S)pGEM-7 into the NcoI and NdeI sites of 6His-T-PE11d.

GST fusion constructs were made in pGEX-2TL(−), a pGEX-2TL (Amersham Pharmacia Biotech) derivative in which the polylinker was expanded to contain the multicloning sites NcoI-NdeI-XhoI-HindIII-AflII-BglII-BamHI-Smal-EcoRI (33).

Construction of TFIIEβ Mutants—Deletion mutants of TFIIEβ were constructed using plasmid p2EBT containing the wild type TFIIEβ cDNA and the described procedure of oligonucleotide-mediated mutagenesis (27). A restriction site was designed in each oligonucleotide to select for properly mutated plasmids as described elsewhere (28), and the mutants were then checked by sequencing. The Ndel-BamHI fragments of all mutants were subcloned into 6His-T-pET11d to create a His tag at the N terminus. N-terminal and internal deletions were constructed by deleting the indicated amino acid residues and C-terminal deletions by creating termination codons at the residues shown in Fig. 1. (Because of the large number of oligonucleotides used, we have refrained from describing their exact sequences, but this information will be provided on request.)

Expression and Purification of Recombinant Proteins—Recombinant proteins were expressed in E. coli BL21(DE3)pLyS8 by induction with isopropyl-β-D-thiogalactopyranoside (34). For purification, soluble bacterial lysates were used (17). His-tagged proteins were purified on an Ni2+-nitrilotriacetic acid column (Qiagen) by elution with 100 mM imidazole HCl (pH 7.9). The large scale preparation of TFIIEβ was as described before (15) and resulted in ~95% purity as judged by Coomassie Blue staining of an SDS-polyacrylamide gel. All the deletion mutants of TFIIEβ were miniscule preparations. Lysates (1 ml) representing 50–100 ml of culture were directly reconstituted in Eppendorf tubes with 1 ml of buffer B (20 mM Tris-HCl (pH 7.9 at 4 °C), 0.5 mM EDTA, 10% (v/v) glycerol, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 2 μg/ml aprotinin, 1 μg/ml leupeptin, 0.8 mg/ml pepstatin, 10 mM 2-mercaptoethanol) containing 500 mM NaCl (BB500) and 100 μl of Ni2+-nitrilotriacetic acid resin and incubated for 4 h at 4 °C. The resin samples were washed twice with 1 ml of BB500, twice with 1 ml of buffer D (20 mM Tris-HCl (pH 7.9 at 4 °C), 20% (v/v) glycerol, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 2 μg/ml aprotinin, 1 μg/ml leupeptin, 0.8 μg/ml pepstatin, 10 mM 2-mercaptoethanol) containing 500 mM KCl (BD500), and twice with 500 μl of BB500 containing 20 mM imidazole HCl (pH 7.9). Bound proteins were eluted twice with 300 μl of BD500 containing 100 mM imidazole HCl (pH 7.9). Typical preparations were >75% pure.

Recombinant TFIIF was also expressed in E. coli BL21(DE3)pLyS8. More than 90% of both TFIIFα (RAP74) and TFIIFβ (RAP30) subunits became soluble in bacterial lysate when they were coexpressed, and 6His-TFIIF was purified through Ni2+-nitrilotriacetic acid column just as 6His-TFIIEβ. Purified TFIIF was >95% purity, contained both subunits stoichiometrically, and was fully active in transcription.2 HA-tagged and GST fusion proteins were expressed in E. coli BL21(DE3)pLyS8 by isopropyl-β-D-thiogalactopyranoside induction. Cell extracts were harvested 90 min after induction in 1 ml of BB500, and sonicated. Soluble lysates were separated from insoluble debris by ultracentrifugation and stored at −80 °C until use.

2 A. Hoffmann and R. G. Roeder, unpublished data.

3 Y. Ohkuma, S. Yamamoto, F. Hanaoka, and Y. Ohkuma, unpublished data.
In Vitro Transcription Assay—General transcription factors (TFIIF and TFIIE) were purified either from HeLa nuclear extracts or from cytoplasmic S100 fractions as described previously (15). Pol II was highly purified from HeLa nuclear pellets by DE52, A25, P11, and high performance liquid chromatography-DEAE 5PW chromatography as described (15). In vitro transcription was carried out as described (9, 15). To observe transcriptional activation, 20 ng of TFIIID containing flag-tagged TBP (25) was used instead of 20 ng of TBP. As activators, 40 ng of either GAL4-VP16 (36) containing the C-terminal acidic activation domain of VP16 (residues 413–490) fused to GAL4 (1–94) (residues 1–94) or GAL4-CTF1 (37) containing the C-terminal proline-rich activation domain of CTF1 (residues 399–490) fused to GAL4 (1–94) was used. Autoradiography was performed at −80 °C with Fuji RX-U x-ray film. The incorporation of [α-32P]CTP into transcripts was quantified using the BAS2500 Bio-Imaging analyzer.

Generation of Antibody against TFIIEb—Two hundred micrograms (100 μl) of purified 6His-TFIIEb (>99% pure) was mixed with the same volume (100 μl) of complete Freund’s adjuvant (Difco) and injected into each rabbit. Two weeks after the first injection, a second injection was carried out with 100 μg (100 μl) of purified TFIIEb in 100 μl of incomplete Freund’s adjuvant (Difco). A third injection was carried out 2 weeks later using the same procedures as described for the second injection. Blood was collected 8 days after the third injection. The generated antibody recognized all of the TFIIEb deletion mutants used in this study.

Coimmunoprecipitation of TFIIEβ Mutants with TFIIIEα—Polyclonal antiserum against TFIIIEa (0.01 μl) and 5 μl (packed volume) of protein G-agarose (Pierce) were incubated in buffer C (20 mM Tris-HCl (pH 7.9 at 4 °C), 0.5% EDTA, 20% (v/v) glycerol, 0.5 mM phenylmethylsulfonyl fluoride, 10 mM 2-mercaptoethanol, 0.002% (v/v) Nonidet P-40) containing 100 μM KCl (BC100) and 200 μg/ml bovine serum albumin for 2 h at 4 °C with rotation. The beads were washed twice with 500 μl of washing buffer I (10 mM Tris-HCl (pH 7.9 at 4 °C), 500 mM NaCl, 0.1% Tween 20), twice with 500 μl of buffer C containing 1 mM KCl (BC1000), and twice with 500 μl of BC100. Various TFIIEβ mutant proteins (200 ng) were incubated with TFIIIEa (300 ng) for 1 h at room temperature, and bound proteins were coimmunoprecipitated after incubation with anti-TFIIIEα-protein G beads in a 500-μl reaction volume for 4 h at 4 °C with rotation. The beads were washed twice with 500 μl of washing buffer I, once with 500 μl of BC100, boiled in SDS sample buffer, and analyzed by SDS-PAGE (12% acrylamide).

Coimmunoprecipitated TFIIEβ mutants were detected by Western blotting with anti-TFIIIEβ polyclonal antiserum (1:3000 dilution) after transfer to an Immobilon-P polyvinylidene difluoride membrane (Millipore) as described previously (15). Signals were detected using the enhanced chemiluminescence detection (ECL) system (Amersham Pharmacia Biotech) after incubation of the immunoblots with horseradish peroxidase-linked secondary antibodies. PBD-1 film (Kodak) or RX-U film (Fuji Film) was used to record the chemiluminescence.

GST Pull Down Assays—GST fusion proteins were used for protein interaction assays. Each tester protein (200 ng) was incubated with lysates containing 5 μg of GST protein together with 5 μl (packed volume) of glutathione-Sepharose (Amersham Pharmacia Biotech) in a 500-μl reaction volume of BC100 with 200 μg/ml bovine serum albumin for 4 h at 4 °C with rotation. The beads were washed twice with 500 μl of washing buffer I, once with 500 μl of BC100, boiled in SDS sample buffer, and analyzed by SDS-PAGE. Pulled down tester proteins were detected by Western blotting as described in the coimmunoprecipitation assay.

Single-stranded DNA Binding Assay—Four hundred nanograms of His-tagged TFIIEβ deletion mutants were incubated with 5 μl (packed volume) of ssDNA-agarose (Life Technologies, Inc.) in a 500-μl reaction volume of BC100 with 200 μg/ml bovine serum albumin for 4 h at 4 °C with rotation. The ssDNA-agarose resin was washed twice with 500 μl of buffer C containing 200 mM KCl (BC200), once with 500 μl of BC100, boiled in SDS sample buffer, and analyzed by SDS-PAGE (12% acrylamide). Bound mutants were detected by Western blotting with anti-TFIIIEβ antisera (1:3000 dilution) as described above.

Kinase Assay—Essays were carried out essentially as described (14, 15) with all general transcription factors in the presence of Pol II and a DNA fragment containing the adenovirus type 2 major late promoter sequence (residues 51–278). In addition, except that 10% of bacterially expressed recombinant TFIIF was used instead of 30 ng of high performance liquid chromatography heparin-purified TFIIIF from HeLa nuclear extract. Phosphorylation reactions were done at 30 °C for 1 h and stopped by addition of 75 μl of phosphorylation stop solution (10 mM EDTA, 0.1% Nonidet P-40, and 0.05% SDS). Phosphorylated proteins were trichloroacetic acid-precipitated, analyzed by SDS-PAGE (5.5% acrylamide), and detected by autoradiography performed at −80 °C with Fuji RX-U x-ray film.

RESULTS

The Central Region of TFIIEβ Is Important for Both Basal and Activator-dependent Transcription—Human TFIIEβ is the small subunit of TFIIE and consists of 291 amino acid residues (10, 17). As judged from the predicted open reading frame of TFIIEβ cDNA, it possesses several putative structural motifs and characteristic sequences: a serine-rich sequence (residues 26–71), a region similar to the Pol II binding region of RAP30 (TFIIEβ) (residues 79–111), a leucine repeat motif (residues 145–193), a region similar to the bacterial sigma factor subdomain 3 (residues 163–193), a basic region-helix-loop-helix motif (residues 197–238), and a region with both basic-helix-loop sequence (residues 258–291). Except for the leucine repeat, these regions are different from the putative motifs and sequences observed in TFIIEx (15). Based on these sequences, a systematic series of N-terminal, C-terminal, and internal deletion mutants were constructed (shown in Fig. 1, A and B). Vectors encoding hexahistidine-tagged mutant and wild type TFIIEβ proteins were expressed in bacteria, purified through nickel affinity chromatography, and analyzed by SDS-PAGE (Fig. 2A and 3A). All mutants were highly soluble in the bacterial cell extracts and were easily purified, although some (Δ224–291, Δ96–119, and Δ117–153) were poorly expressed (Fig. 2A, lane 11; Fig. 3A, lanes 7 and 8, respectively).

The basal transcription activity of each deletion mutant was measured by complementation of a reconstituted transcription system containing the adenovirus type 2 major late promoter and all general transcription factors except for TFIIEβ (15). As shown in Fig. 2B, the N-terminal deletion mutants Δ8–25 and Δ8–50 were almost as active as the wild type (lanes 4 and 5 versus lane 3). However, further deletions from the N terminus, including that extending to residue 75, abolished basal transcription activity (lanes 6–9). In contrast, none of the C-terminal mutants (lanes 10–14) other than mutant Δ278–291 supported basal transcription activity, and the activity of Δ278–291 was less than 20% of that of the wild type TFIIEβ (lane 14 versus lane 3). In a further investigation, the internal deletion mutants were analyzed (Fig. 3B). The internal mutant Δ26–47 was as active as the wild type (lane 5 versus lane 3), but the other internal mutants (lanes 6–14) failed to identify another region dispensable for basal transcription. Therefore, the minimal functional region of TFIIEβ was deduced to lie between residues 51 and 277. This means that the N-terminal half of the serine-rich sequence (residues 26–50) and the C-terminal third of the helix region and all of the loop region of the basic region-helix-loop region (residues 278–291) are non-essential, although loss of the latter C-terminal region (residues 278–291) did reduce the basal transcription level at least 5-fold (Fig. 2B, lane 3 versus lane 14). Apart from these regions, all other putative motifs and characteristic sequences were found to be indispensable for TFIIEβ function.

Recent studies have revealed the existence of several targets for transcriptional regulatory factors among the general transcription factors (for a review, see Ref. 38), and these include TFIIE (for a review, see Ref. 5). This suggests that TFIIE functions not only as a signal transducer for transcriptional regulation as a result of its binding to the other general transcription factors such as TFID and TFIIB but also that such signals are also generated through the direct binding of regulators. In an attempt to identify the region essential for transcriptional activity in TFIIEβ, the deletion mutants that supported basal transcription were tested with various activators. Fig. 4A shows the effects of the acidic activator GAL4-VP16 on these N-terminal, C-terminal, and internal deletion mutants. All four
mutants that exhibited basal transcription activity (Δ8–25, Δ8–50, Δ278–291, and Δ26–47), as well as wild type TFIIEβ, supported a 5–7-fold level of transcriptional activation. Similar results (7–8-fold activation) were observed even when the proline-rich activator GAL4-CTF1 was used (Fig. 4B). These results indicate that the 227-amino acid region (residues 51–277) supports both basal and activated transcription. Of course, it is still possible that this region might be actually important for activation, but this issue can only be definitively resolved by the isolation and testing of point mutants.

The C-terminal Basic Regions of Human TFIIEβ Bind to Several General Transcription Factors—TFIIE functions in two distinct but sequential steps, one during open complex formation and the other during the transition from transcription initiation to elongation. During both steps, the binding of TFIIE to various general transcription factors and to Pol II is expected to be quite important. In the present study, pull down assays (39) using GST fusion proteins for each subunit of the general transcription factors and recombinant TFIIEβ showed that TFIIEβ binds strongly to TFIIEα, TFIIB, and TFIIFβ (RAP30) and weakly to TFIIFα (RAP74) and TBP (Fig. 5A). Human TFIIEβ also bound to Pol II and strongly to itself. These results suggest that TFIIEβ plays essential roles in both steps of transcription by binding to various general transcription factors. To better understand these roles, we first identified the binding regions of TFIIEβ for three strongly associating factors TFIIEα, TFIIB, and TFIIFβ.

Since TFIIE forms a heterotetramer with two TFIIEα and two TFIIEβ subunits (9, 10), we identified the α-β heterodimerization domain in TFIIEβ using a coimmunoprecipitation assay containing various TFIIEβ deletion mutants, the TFIIEα subunit, and antibody against TFIIEα (Fig. 5B). The N- and C-terminal deletion mutants (Fig. 5B, lanes 2–12) showed that the TFIIEβ mutants failed to bind to TFIIEα when they contained deletions that stretched further into the C terminus of mutant Δ224–291 (mutants Δ155–291 and Δ176–291; Fig. 5B, lanes 8 and 9, respectively). Regarding the internal deletion mutants (Fig. 5B, lanes 13–21), mutant Δ197–232 failed to bind to TFIIEα (Fig. 5B, lane 20). In summary, residues 197–223, which contain the basic region and adjoining helix and loop regions of the basic region-helix-loop-helix motif, are essential for heterodimerization.

The interactions of TFIIEβ with TFIIB and TFIIFβ (RAP30) were analyzed next (Fig. 5A). The TFIIEβ mutants were mixed with GST-fused TFIIB (GST-TFIIB), pulled down with GST-TFIIB, and detected by anti-TFIIEβ antibody in an immunoblot analysis (Fig. 6). Mutants lacking the basic and helix regions (residues 257–277) of the basic region-helix-loop sequence near the C terminus failed to bind to TFIIB (Fig. 6A, lanes 8–11; Fig. 6B, lane 12). Almost equivalent results were obtained with GST-TFIIFβ (RAP30). Taken together, these results indicate that the two basic and adjacent C-terminal regions of TFIIEβ may be targets of various general transcription factors.

Two C-terminal Basic Regions of TFIIEβ Are Involved in Binding to the Drosophila Transcriptional Repressor Krüppel—Recently, it has become apparent that vast numbers of transcriptional regulators target various transcription factors, as well as histones, during the multiple steps of gene transcription (for reviews, see Refs. 1 and 38). Since TFIIE plays key roles in two of those steps, transcription initiation and promoter clearance, it would be surprising if it were not a target for transcriptional regulatory factors (for a review, see Ref. 5). As analyzed both in tissue culture and in vitro, the Drosophila segmentation gene product Krüppel (Kr), which is a zinc finger protein, functions as a transcriptional regulatory factor (31, 32). It has been demonstrated that monomeric Kr acts as a transcriptional activator by binding to TFIIB and that the Kr dimer, on the other hand, acts as a transcriptional repressor by binding to TFIIEβ (36, 40). Since this result was the first report of the existence of transcriptional regulatory factor targeting general transcription factor TFIIE, we analyzed Kr binding to TFIIEβ.
As shown in Fig. 7A, Kr binding to general transcription factors was studied using a GST pull down assay. Various GST general factor fusion proteins were tested for their ability to interact with HA-tagged Kr (HA-Kr). Kr binds tightly to TFIIB and TFIIEβ, as demonstrated by Sauer et al. (40), and weakly to TBP and TFIIEβ (RAP30) (25–30% of TFIIB and TFIIEβ) (Fig. 7A, lanes 2 and 4 versus lanes 6 and 7). Kr also bound to TFIIB only weakly (less than 5% of TFIIB and TFIIEβ) (Fig. 7A, lane 10). These results are in good agreement with the observations described above. Therefore, the Kr binding regions within TFIIEβ were identified by using a GST-Kr fusion protein in conjunction with TFIIEβ deletion mutants (Fig. 7, B and C). Kr bound to all the N-terminal deletion mutants, but binding was considerably reduced by deletion of the C-terminal basic region (mutant Δ257–291) and completely abolished by further deletion of the other basic region (mutant Δ176–291) (Fig. 7B, lanes 3–8 versus lanes 10-12). Internal deletion mutants confirmed that deletion of either basic region had a severe effect upon Kr binding (Fig. 7C, lanes 11 and 13).

The C-terminal Basic Region of the C-terminal Basic Region-Helix-Loop-Loop Domain in TFIIEβ—As we reported previously (17), the C-terminal basic region of the basic region-helix-loop (BR-HL) sequence in TFIIEβ is similar to the basic regions of the basic region-helix-loop-helix (BR-HLH) domains of the Myc-related family of proteins (such as Myo-D1 and E12) (41–43). Thus, it was predicted that this basic region may bind directly to single-stranded (ss) and/or double-stranded (ds)DNA. Recent studies have lent further support to this idea as follows: (i) photocross-linking studies revealed that TFIIEβ binds to a core promoter region (between 214 and 222) where dsDNA is melted by transcription initiation (19); (ii) two-dimensional crystallography of yeast TFIIE with Pol II revealed that yTFIIE actually interacts with the active center of Pol II, which is located near the transcription initiation site on DNA (20); (iii) short mismatched heteroduplex DNA around the initiation site in topologically relaxed templates abolishes the requirement for TFIIE, TFIIH, and ATP (22, 23). Therefore, we tested whether TFIIEβ can bind to DNA by using both the gel retardation assay and the pull down binding assay with ssDNA and dsDNA. While TFIIEβ preferentially bound to ssDNA, TFIIEβ alone bound only weakly to ssDNA (less than 5% of the level observed for wild type TFIIEβ), and neither subunit alone was able to bind to dsDNA. Surprisingly, how-
Analysis of the internal deletion mutants showed that all ex-
cept (Fig. 8) did not abolish ssDNA bind-

FIG. 5. Binding of TFIIEB to general transcription factors. A, binding to the GST fusion derivatives of the various general transcription factors. Various general transcription factors were fused to GST and expressed in bacteria. A GST pull-down assay was carried out as described under “Experimental Procedures.” Two hundred nanograms of 6His-TFIIEB was used for each assay. After SDS-PAGE on a 10% polyacrylamide gel, bound TFIIEB was detected by anti-TFIIEB antibody after Western blotting. The sizes of the molecular mass markers are indicated on the left side (in kilodaltons). Lane 1, control bacterial lysate (no GST proteins) (−); lane 2, GST alone (no fusion proteins) (GST); lane 3, GST-TFIIB (B); lane 4, GST-TFIIEα (Eα); lane 5, GST-

FIG. 4. Effects of deletion mutants of TFIIEB on transcrip-
tional activation. A, deletion mutants of TFIIEB with GAL4-VP16. Reaction mixtures (25 μl) contained 100 ng of pG5HM(C2AT), 50 ng of pML(C2AT)-53Sh, and all general transcription factors together with Pol II as described (8), except that 20 ng of purified flag-tagged TFIIID was used as a natural TFIIID (TFIID) was used as natural TFIIID (22). Two ng of mutant or wild type TFIIEB were used for transcription as indicated on the top of the panel. In lanes 1 and 2, TFIIEB was omitted (−IEB). The upper arrow (390 nucleotides) indicates transcripts of pG5HM(C2AT), and the lower arrow (290 nucleotides) indicates transcripts of pML(C2AT)-53Sh. The extent of activation was calculated by comparing transcription levels in the absence (−) and presence (+) of 40 ng of GAL4-VP16 (G4-VP16) measured by a Fuji BAS2500 Bio-Imaging analyzer. B, deletion mu-
tants of TFIIEB with GAL4-CTF1. Forty ng of the proline-rich activator GAL4-CTF1 (G4-CTF1) was used instead of GAL4-VP16. All conditions are the same as described in A.

However, as described above, there exists the possibility that TFIIIE may have unique function(s) to stabilize promoter melting by binding to the ssDNA region of the promoter DNA (Fig. 8, Ref. 23). To check this novel function of TFIIIE, the effects of TFIIEB deletion mutations on CTD phosphorylation were analyzed (Fig. 9).

All four mutants of TFIIEB with basal transcription activity (Δ8–25, Δ8–50, Δ278–291, and Δ26–47) stimulated CTD phospho-

Effects of TFIIEB Mutations on TFIIE-dependent CTD Phos-
phorylation Correlate with Effects on Basal Transcription—

Previous studies demonstrated that TFIIIE strongly stimulates CTD phosphorylation by TFIIH by itself and during formation of the active initiation complex (12, 14) and that TFIIIEα is essential for this stimulation which correlates well with the increase in basal transcription activity (15). It was considered that TFIIIE might simply provide a bridge between TFIIH and Pol II to assist TFIIH-mediated phosphorylation of Pol II, as TFIIH by itself had been found not to interact well with Pol II. However, binding to ssDNA was observed when both subunits were mixed together to form active TFIIIE.3

Since TFIIEB binds strongly to ssDNA, ssDNA binding re-

region in TFIIEB was identified by using ssDNA-agarose with the TFIIEB deletion mutants (Fig. 8). Deletions from the N terminus up to amino acid residue 152 did not abolish ssDNA bind-

(290 nucleotides) indicates transcripts of pG5HM(C2AT), and the lower arrow (290 nucleotides) indicates transcripts of pML(C2AT)-53Sh. The extent of activation was calculated by comparing transcription levels in the absence (−) and presence (+) of 40 ng of GAL4-VP16 (G4-VP16) measured by a Fuji BAS2500 Bio-Imaging analyzer. B, deletion mu-
tants of TFIIEB with GAL4-CTF1. Forty ng of the proline-rich activator GAL4-CTF1 (G4-CTF1) was used instead of GAL4-VP16. All conditions are the same as described in A.
stimulation of CTD phosphorylation, but Pol II shift was incomplete (stopped in between IIa and IIo) (Fig. 9A, lane 26 versus lane 3). Two other N-terminal deletion mutants of TFIIE showed a modest negative effect on basal transcription (56% of wild type) (Fig. 10A, lane 7–11). On the other hand, the internal deletion mutant (Δ197–232) lacking the TFIIEα binding region had no effect on transcription even when added at a 64-fold excess over the amount of wild type TFIIEβ (Fig. 10A, lane 26 versus 22). The Δ230–255 mutant described above showed a modest negative effect on basal transcription (56% of wild type transcription at a 64-fold excess) although this mutant has so far failed to display an interaction with other transcription factor or with DNA (Fig. 10A, lane 31 versus 27).

**DISCUSSION**

Elucidation of the precise mechanisms involved in transcription initiation by Pol II has been a long-standing issue in molecular biology. During stepwise assembly of the preinitiation complex (PIC), TFIIE is essential for TFIIH recruitment...
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and completion of PIC formation, as well as for stabilization and activation of the PIC. Recent studies have indicated that TFIIE, through interactions with other factors and possibly with DNA, is localized near the transcription initiation site (between positions –14 and –2) within the PIC and near the active center of Pol II (19–21). It also appears that TFIIE has a novel but unclear function during promoter melting (23). Here, we have investigated the structure and function of the small subunit of human TFIIE (TFIIEb). This included an examination of the effects of TFIIEb deletion mutations on both basal and activator-mediated transcription and identification and characterization of TFIIEb interactions with the general transcription factors TFII Ea, TFII B, and TFII F (RAP30), the Drosophila transcriptional repressor Kr, and ssDNA.

Structure-Function Relationships in TFIIEb—Analysis of TFIIEb deletion mutants showed that a central 227-amino acid region (residues 51–277) was sufficient to mediate both basal and activated transcription. As summarized in Fig. 10B, this region contains all of the previously noted structural motifs and characteristic sequences with the exception of the N-terminal half of the serine-rich sequence and the loop region of the basic region-helix-loop sequence. All of the internal subdomains of the central 227 amino acid region were essential for transcription activity (Figs. 2B and 3B). Protein interaction studies indicated that two basic regions and associated sequences located near the C terminus (residues 197–210 and 258–270) are important for direct TFIIEb interactions with various general transcription factors and transcriptional regulatory factors as well as with ssDNA (Figs. 5–8).3. These basic regions have similar primary structures but may have different (context-dependent) binding preferences, as summarized in Fig. 10B. Thus the N-terminal half of the basic region-helix-loop-helix (BR-HLH) motif is involved in TFII Ea interactions, whereas the second basic region and the following N-terminal half of the helix region may be part of a basic region-helix-loop (BR-HL) domain involved in interactions with TFII B and TFII F (RAP30) as well as with ssDNA. In contrast, interaction of the Drosophila transcriptional repressor Kr with TFIIEb appears to involve both regions.

We also demonstrated that the TFIIEb subunit is essential for the stimulation of TFIIH-dependent CTD phosphorylation of Pol II by TFIIE as is TFII Ea (Fig. 9 and Ref. 15). The complete shift of the largest subunit of Pol II from IIa to II o form upon CTD phosphorylation was dependent entirely on the basal transcription activity of TFIIEb. An important difference with respect to TFII Ea was that some TFIIEb mutants supported neither basal transcription nor CTD phosphorylation even though they could bind to TFII Ea subunit, Pol II and ssDNA. This is in contrast to TFII Ea whose stimulation of CTD phosphorylation was dependent on its binding to TFIIEb. Another difference was that the transcription-defective TFIIEb mutants gave various extents of Pol II shift upon phosphorylation, whereas all TFII Ea mutants showed a unique profile of Pol II shift (II o form) except for mutants lacking the TFIIH binding region (Fig. 9 and Ref. 15). It is also interesting that
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TFIIENa together with Pol II and TFIIH stimulated CTD phosphorylation more than 10-fold in the absence of DNA (14) but that almost no stimulation was observed when Pol II and all the general transcription factors including TFIIENa were incubated with promoter DNA in the absence of TFIIENb (Fig. 9, A and B, lane 2 versus lane 1). Under these latter conditions, stimulation could only be restored by adding TFIIENb along with the other general transcription factors (Fig. 9, A and B, lane 3 versus lane 2). These results strongly suggest that TFIIENb plays important but different roles from TFIIEna for both transcription and CTD phosphorylation in the active initiation complex.

**Novel Functional Role(s) of TFIEA Mediated by Binding to Single-stranded DNA**—Intriguingly, as described above, TFIIENa was found to bind to ssDNA through its second basic region (Fig. 8). During the preparation for initiation, TFIIEN joins the PIC after recruitment of TFIIF and Pol II and becomes located near the active center of Pol II through interactions with both Pol II and, most likely, with promoter DNA in the region between positions −14 and −2, resulting in the recruitment of TFIIH (for a review, see Refs. 1, 2, and 5). In the step where Pol II is phosphorylated by TFIIH, TFIIEN activates TFIIH by stimulating its kinase and ATPase activities (12, 14). However, the recent finding of differential requirements for TFIIEN and TFIIH when short mismatched heteroduplex DNAs are created around the initiation site has raised the possibility that TFIIEN has a TFIIH-independent function during promoter melting (23). Our results showing ssDNA binding activity by TFIIEN suggest that TFIIENb may play an additional role by binding to the single-stranded region present within melted promoter DNA. Thus, although it is generally held that TFIIEN and TFIIH work together during the promoter clearance step necessary for the transition from initiation to elongation (44), it also is possible that TFIIEN works independently of TFIIH to remove certain general transcription factors from the initiation complex on the promoter.

**Functionally Important Regions of TFIIENb in Transcription**—In addition to identifying the regions important for basal transcription, the effects of adding excess amounts of TFIIENb mutant proteins on basal transcription assays containing wild type TFIIENb were studied (Fig. 10A). This approach is especially effective for both the identification of dominant negative mutants that lack the active center of TFIIENb but maintain functional interactions with other PIC components and, conversely, for the identification of dominant negative mutants that have lost their capacity to interact with other PIC components but still possess an active center. The deletion mutant Δ257–291 of the C-terminal basic region of the basic region-helix-loop sequence was the strongest dominant negative mutant (13% of wild type transcription level when added at a 14-fold excess) (Fig. 10A, lanes 12–16). Since this basic region was identified as a target for the general transcription factors
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TFIIand TFIIbf (RAP30), as well as the Drosophila transcriptional repressor Kr and ssDNA, it appears to be quite important for TFIIβ function. Mutant Δ257–291 may still possess Pol II and TFIIα binding regions, resulting in the depletion of factors necessary for functional PIC formation. Two other deletion mutants (Δ4–125 and Δ75–96) have similar dominant negative effects (about 30% of wild type level when added at a 64-fold excess) (Fig. 10, lanes 7–11 and lanes 17–21). The former mutant lacks the serine-rich sequence and the region similar to the Pol II binding region of RAP30 (TFIIβ), whereas the latter lacks only the region similar to the Pol II binding region of RAP30 (TFIIβ). However, because the first 50 amino acids of TFIIβ, which contain the N-terminal half of the serine-rich sequence, are dispensable for transcription, the former mutant, Δ4–125, may be almost equivalent to the latter mutant, Δ75–96. Importantly, we confirmed here that the region similar to the Pol II binding region of RAP30 (TFIIβ) might also be essential for TFIIβ function. These mutants may not be recruited to their proper position in the PIC complexes because of a failure to bind to Pol II.

Functional Implications of TFIIIE (and TFIIH) in Productive Transcription—In this study we have presented an initial characterization of TFIIβ structure and function, and we have proposed novel function(s) for TFIIIE during both transcription initiation and in the transition from initiation to elongation. As described above, three recent studies dealing with the role of TFIIIE in transcription initiation have suggested that TFIIIE, as part of the PIC, binds both to the core promoter just upstream from the transcription initiation site (between positions –14 and –2) and to Pol II near its active center (for a review, see Refs. 1, 2, and 5). In this way, in conjunction with TFIIH, it would play an important role in promoter opening, a step which can be circumvented by premelting the promoter between positions –4 to +2 (19–23). Our results demonstrate that the C-terminal basic region of TFIIβ binds to both TFII and TFIIβ (RAP30). These observations agree with photocross-linking results showing that TFII and TFIIβ (RAP30) bind to the promoter DNA just upstream of TFIIβ and stabilize the Pol II-TBP interaction at around position –19 (19). Since TFII and TFIIβ (RAP30) may bind to different surfaces on the promoter DNA (19) and since TFIIβ exists as a dimer in the PIC (9, 10), we can envisage a model in which TFII and TFIIβ (RAP30) bind to different TFIIβ subunits that are docked to the DNA in parallel with their C termini facing upstream. As mentioned above, TFIIIE was able to bind to dsDNA, in contrast to the TFIIα or TFIIβ subunit which could not bind to dsDNA when added individually.3 On the other hand, TFIIβ was able to bind to ssDNA (Fig. 8).3 One possibility is that TFIIIE is recruited into the PIC and at that time TFIIα assists TFIIβ to bind to the promoter region (between –14 and –2). This result fits well with the observation made by Coulombe and colleagues (19) that TFIIα could bind to dsDNA but that TFIIβ could bind to dsDNA with the assistance of TFIIα.

The promoter premelting study using short mismatched heteroduplex DNAs and the KMnO4 sensitivity assays described by Timmers and co-workers (23) have demonstrated that TFIIIE and TFIIH open DNA from positions –9 to +1 in the presence of hydrolyzable ATP. Importantly, our finding of ssDNA binding by TFIIβ (Fig. 8) supports the possibility that this factor may contribute to the stabilization of single-stranded regions with the promoter DNA, thereby assisting the DNA unwinding activity of TFIIH which depends on energy supplied by ATP hydrolysis. At the same time, TFIIIE may stimulate the CTD kinase activity of TFIIH and, ultimately, open the DNA between positions –9 to +1. The stimulation of CTD phosphorylation by TFIIβ in the PIC complex (Fig. 9) also supports this model. The “open” region further extends downstream to +8 after formation of the first phosphodiester bond (23). During the transition from initiation to elongation, “promoter clearance” occurs before the transcript attains the size of 10 nucleotides. It is noteworthy that TFIIIE is released from the transcription complex before Pol II reaches position +10 and that TFIIH, on the other hand, is released later between positions +30 and +68 (45). Within the extended DNA bubble found during promoter clearance, TFIIIE might stabilize the single-stranded region and might work together with TFIIH to alter the conformation of the PIC, resulting in the disassembly and removal of the general transcription initiation factors (TFIID and TFIIIB) from the initiation complex. In addition, TFIIIE could help recruit transcription elongation factors. It is intriguing to consider the possibility that the general cofactor PC4, which is also an ssDNA-binding protein, may be involved in promoter clearance by displacing TFIIIB from the initiation complex, as suggested by studies on the yeast PC4 homolog SUB1 (46–49). Nonetheless, elucidation of the mechanisms of promoter clearance still awaits further structural and functional studies of TFIIIE, TFIIH, and Pol II in conjunction with studies focused upon the elongation step that follows initiation.

An increasing number of transcriptional regulatory factors have been reported to target TFIIIE and/or TFIIH in order to express their functions (for a review, see Ref. 5). Thus, transcription activation may, in part, affect promoter opening either by stabilizing single-stranded regions or by regulating the functions of TFIIIE and/or TFIIH. The Drosophila developmental gene product and transcriptional regulatory factor, Kr, is one such factor that may target TFIIIE (40). Our study clearly demonstrated that Kr predominantly binds to TFIIIB and TFIIIE (Fig. 7A). By using TFIIβ deletion mutants, the C-terminal basic regions of both the basic region-helix-loop-helix motif and the basic region-helix-loop sequence were identified as Kr binding regions in TFIIβ (Fig. 7, B and C). Since these basic regions are targets for at least three general transcription factors and for ssDNA, Kr binding might inhibit their binding through competition and, thus, may cause transcriptional repression. Furthermore, it was recently found that the human TFIIID subunit TAF60 (TBP Associated Factor for RNA polymerase I) binds to TFIIα (50). Therefore, the possibility exists that TAF60 transduces signals not only from activators to the general transcription factors (like TFIID and TFIIH) involved in early PIC formation but also to the general transcription factors that affect open complex formation and promoter clearance (like TFIIIE and TFIIH). Another recent finding of the histone-like structures in human TAF60, TAF60, and TAF920 and modification of these structures by the enzymes, histone acetyltransferases and histone deacetylases, has evoked renewed interest in the mechanisms of transcriptional regulation (3, 8, 33, 51). This modification might be important for chromatin remodeling by modifying histones but also for transcriptional regulation. In addition, the recent finding that several histone acetyltransferases also acetylate TFIIβ (52) suggests TFIIβ involvement in such acetylation-mediated transcriptional regulation. In eukaryotic cells, DNAs are wrapped in histones, resulting in the formation of chromatin in the nucleus. Therefore, it is important to consider at the same time both the contributions of histone regulation and general transcription factors in transcription initiation. We are currently pursuing our investigation of the mechanisms of promoter opening and promoter clearance in an attempt to understand better how transcriptional regulatory factors contribute to transcription initiation and elongation through studies on TFIIIE within this context.
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