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Analysis of the Role of TFIIE in Basal Transcription and TFIIH-Mediated Carboxy-Terminal Domain Phosphorylation through Structure-Function Studies of TFIIE-α

YOSHIAKI OHKUMA, SHIGERU HASHIMOTO,† C. KATHY WANG, MASAMI HORIKOSHI,‡ and ROBERT G. ROEDER*

Laboratory of Biochemistry and Molecular Biology, The Rockefeller University, New York, New York 10021

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The general transcription factor TFIIE recruits TFIIH at a late stage of transcription initiation complex formation and markedly stimulates TFIIH-dependent phosphorylation of the carboxy-terminal domain (CTD) of RNA polymerase II. To study this function of TFIIE in more detail, systematic deletion mutations were introduced into the large subunit of TFIIE (TFIIE-α) and were analyzed with regard to their effects on TFIIH-dependent CTD phosphorylation, TFIIE-dependent basal and enhancer-dependent transcription, and interactions of TFIIE-α with both TFIIE-β and TFIIH. The amino (N)-terminal half of TFIIE-α, which possesses several putative structural motifs, was sufficient for the phosphorylation and transcription activities and for TFIIE-β interactions, whereas a site effecting both strong interactions with TFIIH and large stimulatory effects on transcription and CTD phosphorylation was localized to an acidic region near the carboxy (C) terminus. The fact that these activities appear to be tightly linked supports the idea that TFIIE interacts physically and functionally with TFIIH and that CTD phosphorylation is essential for transcription under normal conditions. The present results suggest that TFIIE, via its effect on TFIIH, may act as a checkpoint for formation of a preinitiation complex.

Productive transcription initiation events play a key role in the regulation of gene expression. A number of potentially distinct steps can be recognized: (i) formation of a preinitiation complex on the promoter; (ii) transition, accompanied by promoter melting, to an active (competent) initiation complex; (iii) transcription initiation per se (formation of the first phosphodiester bond); and (iv) transition to a productive elongation complex (promoter clearance) (for reviews, see references 42 and 69). In eukaryotes, the formation of a productive (pre)initiation complex, which is defined here as one leading ultimately to a functional elongation complex, may involve at least seven general transcription factors (TFIIA, -IIB, -IID, -IIE, -IIF, -IIG/J, and -IIH) in addition to RNA polymerase II (Pol II) (for reviews, see references 8, 52, and 78). At present, most efforts have been directed toward understanding preinitiation complex formation by characterizing both general transcription factors (for a review, see reference 8) and various sequence-specific transcription regulators (for a review, see reference 43) which affect productive preinitiation complex formation. While early steps, such as TFIIA-stabilized interactions of natural TFIID or the derived TATA box-binding protein (TBP) with the TATA box, and subsequent TFIIB recruitment, are fairly well characterized, analysis of the later steps is not yet complete. This is due, in part, to the increasingly complex interactions as more factors are incorporated into the preinitiation complex.

One of the general transcription factors involved in late steps of preinitiation complex formation is TFIIH (14). This multisubunit factor and its rat and yeast homologs (for a review, see reference 8) have a kinase activity which phosphorylates both the carboxy-terminal domain (CTD) of Pol II and several general transcription factors, as well as ATPase and helicase activities (11, 38, 46, 59, 61). The former kinase activity has recently been identified as the CDK-activating kinase (CAK) (12, 53). That these activities could regulate productive transcription events is suggested by the following. First, early studies on the timing of CTD phosphorylation suggested that it might be involved in the transition from initiation to elongation events (3, 32, 50). Second, transcription from the adenovirus 2 major late promoter was shown to require hydrolysis of the β-γ bond of ATP or dATP (2, 56), even when an initiation-competent proteolyzed form (IIB) of Pol II lacking the CTD was employed (32). Third, formation of a functional preinitiation complex was shown to be more efficient with the nonphosphorylated form of the CTD-containing Pol II (IIA) than with the phosphorylated form (II0) (3, 37). Fourth, earlier studies of open complex formation (promoter melting) have supported a role for ATPase and possibly helicase activities prior to initiation (25–27, 71, 78). More recent studies have suggested conditional requirements for TFIIH in transcription that are dependent on the nature (e.g., superhelical density) of the templates, although the results have not been completely consistent (references 17, 49, 66, and 70 versus reference 14). Thus, there are indications (based on different specificities, for example) that at least two of the three TFIIH activities, the kinase and ATPase activities, may be differentially regulated and may function during different steps of the transcription process. A recent study by Goodrich and Tjian (17) supports a role for TFIIH in promoter clearance.

Of special relevance in the present report is the general transcription factor TFIIE, a heterotetrameric $\alpha_2\beta_2$ structure (23, 47, 48, 51, 63) involved in recruitment of TFIIH to the (pre)initiation complex. TFIIE has been demonstrated to stim-

* Corresponding author. Mailing address: Laboratory of Biochemistry and Molecular Biology, The Rockefeller University, 1230 York Avenue, New York, NY 10021. Phone: (212) 327-7600. Fax: (212) 327-7949.

† Present address: Institute for Molecular and Cellular Biology, Osaka University, Suita, Osaka 565, Japan.

‡ Present address: Institute of Molecular and Cellular Biosciences, University of Tokyo, Bunkyo-ku, Tokyo 113, Japan.

ulate both CTD phosphorylation by TFIIH (38, 46) and TFIIH-dependent ATP hydrolysis (46) even in the absence of transcription initiation and, most recently, has been implicated in promoter clearance in conjunction with TFIIH (16). To further elucidate the mechanisms involved in initiation and early elongation events, we have constructed a series of deletion mutants of TFIIE-α and tested their abilities to mediate CTD phosphorylation as well as both basal and activated transcription. In addition, we have identified regions of TFIIE-α which are essential for physical interactions with TFIIE-β and TFIIH. Our results indicate that TFIIE-stimulated CTD phosphorylation can occur before transcription initiation, in agreement with the results described by Laybourn and Dahmus (32), and that the ability of TFIIE-α mutants to stimulate CTD phosphorylation is correlated with their ability to form a heteromeric complex with TFIIE-β, to interact with TFIIH, and to effect basal transcription. This suggests that the function (processivity) of Pol II in productive initiation complexes might be potentiated by phosphorylation events that release the CTD from associations with DNA and TBP or TFIID (cf. reference 67) even before transcription initiation.

MATERIALS AND METHODS

DNA templates. For basal transcription assays, the adenovirus type 2 major late promoter containing plasmid pML(C_2AT)Δ-50 served as a template (57). To assay transcription activation, the plasmid pG5HM(C_2AT) was used as the test template (4), with the plasmid pML(C_2AT)Δ-53Sh providing the base line control (68). pG5HM(C_2AT) contains five GAL4-binding sites preceding the human immunodeficiency virus TATA box and the AdML promoter initiator element in front of a G-less cassette. Both pML(C_2AT)Δ-50 and pG5HM(C_2AT) give 380-nucleotide (nt) transcripts, and pML(C_2AT)Δ-53Sh gives a 330-nt transcript.

Construction of histidine-tagged expression vectors. The oligonucleotide 5′-ATCTGGGTCTGCCATATGTAGCAACGAACTCCA-3′ was used to create an *Nde*I site at the first methionine of the open reading frame of human TFIIE-α cDNA in p2EYC by site-directed mutagenesis (21, 30). The *Nde*I-*Bam*HI fragment of this clone (p2EA) was subcloned into the 6HisT-pET11d vector as described elsewhere (19).

The plasmid containing cDNA encoding the p62 subunit of TFIIH (pBS62) was kindly provided by J.-M. Egly (13). A duplex oligonucleotide (5′-CCATC CTGCTTCTTTTGACGCACTTTCTTTACAATCAGCAAAACTTCTT CAGATGAGGTTGCCA-3′; 5′-TATGGCAACCTCATCTGAAGAAGTTTT GCTGATTGTAAAGAAAGTGCGTCAAAAGAAGCAGGATGGAGCT-3′) and a *Bam*HI (Klenow fragment digested)-*Sac*I fragment from pBS62 were used to subclone the p62 cDNA into the 6HisT-pET11d vector as described above.

Construction of TFIIE-α mutants. Mutants of the TFIIE-α cDNA (p2EA) were constructed by the described procedure of oligonucleotide-mediated mutagenesis (30). A restriction site was designed in each oligonucleotide to enable selection of mutant plasmids as described elsewhere (21), which was then confirmed by sequencing. The *Nde*I-*Bam*HI fragments of all mutants were subcloned into 6HisT-pET11d. 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.

Expression and purification of recombinant proteins. Recombinant human TFIIE-α and its mutants in 6HisT-pET11d were expressed in *Escherichia coli* BL21(DE3)pLysS by induction with IPTG (isopropyl-β-D-thiogalactopyranoside) (62). Since TFIIE-α and all mutants were highly soluble, lysates were prepared as described elsewhere (47). For large-scale preparations, lysates from 1 to 4 liters of IPTG-induced bacterial culture were fractionated by 33% saturation of $(NH_4)_2SO_4$. Almost all expressed proteins were thus precipitated, resuspended with buffer B (20 mM Tris-HCl [pH 7.9 at 4°C], 10 mM 2-mercaptoethanol, 20% [vol/vol] glycerol, 0.5 mM phenylmethylsulfonyl fluoride [PMSF]) containing 500 mM KCl (BB500), and then purified on an Ni-nitrilotriacetic acid (NTA) column (Qiagen; 1-ml column volume) as described elsewhere (47). After washing with 10 column volumes of the same buffer (BB500) and then washing with 10 column volumes of BB500 containing 20 mM imidazole-HCl (pH 7.9), expressed proteins (>95% pure by Coomassie blue staining of a sodium dodecyl sulfate [SDS]-polyacrylamide gel) were eluted with BB500 containing 100 mM imidazole-HCl (pH 7.9). For miniscale preparations, lysates (1 ml) from 50 to 100 ml of culture were directly suspended in Eppendorf tubes with 100 μl of an Ni-NTA resin and incubated for 4 h at 4°C. The resin samples were washed five times with 1 ml of BB500 and three times with 1 ml of BB500 containing 20 mM imidazole-HCl (pH 7.9), and expressed proteins were eluted twice with 200 μl of BB500 containing 100 mM imidazole-HCl (pH 7.9). Typical preparations were >75% pure. The large-scale preparation was carried out for recombinant human TFIIE-β, resulting in >95% purity. The purification of recombinant human TFIIB and TBP (TFIIDτ) has been described in detail elsewhere (39, 65).

Recombinant human p62 (one of the TFIIH subunits) in 6HisT-pET11d was also expressed in *E. coli* BL21(DE3)pLysS by induction with IPTG (62). The expressed p62 was insoluble and solubilized in 8 M urea with sonication. The recombinant protein was then purified by preparative SDS-10% polyacrylamide electrophoresis (PAGE) and recovered by electroelution. The purity (>99%) and quantity of the gel-purified recombinant protein were determined by SDS-PAGE.

In vitro transcription assay. General transcription factors (TFIIF, TFIIG/J, and TFIIH) were purified either from HeLa nuclear extract or from cytoplasmic S100 fraction as previously described (48, 64), with the following modifications. TFIIF and TFIIH were purified through five columns: phosphocellulose (P11), DEAE-cellulose (DE52), high-performance liquid chromatography (HPLC)-DEAE 5PW, HPLC-SP 5PW, and HPLC-heparin 5PW (46). The DEAE-HPLC TFIIF fraction (64), which contained both TFIIF and TFIIH activities, was dialyzed, loaded onto an HPLC-SP 5PW column, and eluted in a gradient at 280 mM KCl. Peak fractions were diluted to a KCl concentration of 100 mM and loaded onto an HPLC-heparin 5PW column. TFIIF was eluted at 320 mM KCl, and TFIIH was eluted at 370 mM KCl. To prepare TFIIG/J, the dialyzed P11 0.85 M KCl fraction was applied onto a DE52 column twice, and the flowthrough fraction was loaded onto a heparin-Sepharose column and eluted with 0.5 M KCl. Pol II was highly purified from HeLa nuclear pellets through DE52, A25, P11, and HPLC-DEAE 5PW chromatography as described elsewhere (64). In vitro transcription was carried out as described elsewhere (48). For transcriptional activation assays, 20 ng of TFIID containing flag-tagged TBP (4) was used instead of TBP. For activators, 40 ng of either GAL4-VP16 (55) containing the C-terminal acidic activation domain of VP16 (residues 413 to 490) fused to the N terminus of GAL4 (residues 1 to 94) or GAL4-CTF1 (28) containing the C-terminal proline-rich activation domain of CTF1 (residues 399 to 499) fused to GAL4 (residues 1 to 94) was used.

Raising antiserum against human TFIIE-α and the p62 subunit of TFIIH. Two hundred micrograms (100 μl) of purified TFIIE-α (>99% pure) was mixed with the same volume (100 μl) of complete Freund adjuvant (Difco) and injected into each rabbit. Two weeks after the first injection, the second injection was carried out with 100 μg (100 μl) of purified TFIIE-α in 100 μl of incomplete Freund adjuvant (Difco). The third injection was carried out 2 weeks later by the same method used for the second injection. Blood was collected 8 days after the third injection.

Seventy-five micrograms (500 μl) of the purified p62 (>99% pure) was mixed with the same volume (500 μl) of complete Freund adjuvant, and the mixture was injected into each rabbit. Four weeks after the first injection, the rabbits were given booster injections of 50 μg (500 μl) of the purified p62 mixed with the same volume (500 μl) of incomplete Freund adjuvant. Blood was collected 4 weeks after the second injection.

Coimmunoprecipitation of TFIIE-β with TFIIE-α mutants. Histidine-tagged TFIIE-β was expressed in *E. coli* in the presence of 0.1 mCi of [^{35}S]methionine (Amersham) per ml and 200 μg of rifampin per ml. Labeled TFIIE-β was solubilized by sonication and purified by using the Ni-NTA column as described above. Labeled TFIIE-β (50 ng) was incubated with deletion mutants of TFIIE-α (200 ng) in 100 μl of BB100 containing 0.5% Tween 20, 0.005% NaN_3, and 0.2 mg of bovine serum albumin per ml for 1 h at 30°C. Then, the reaction volume was increased to 200 μl with 1× phosphate-buffered saline (136.9 mM NaCl, 5.4 mM KCl, 85.8 mM Na_2HPO_4, 2.9 mM KH_2PO_4) containing 1% Tween 20, 0.01% NaN_3, and 0.25% skim milk, and 2 μl of either rabbit preimmune serum or polyclonal antiserum against TFIIE-α was added. After incubation for 5 h at 4°C with rotation, 5 μl (packed volume) of protein A-Sepharose (Pharmacia) was added. After an additional 1-h incubation at 4°C, the protein A-Sepharose was precipitated, washed with 500 μl of washing buffer I (10 mM Tris-HCl [pH 7.9 at 4°C], 500 mM NaCl, 0.1% Tween 20), and washed twice with 500 μl of washing buffer II (10 mM Tris-HCl [pH 7.9 at 4°C], 150 mM NaCl, 0.1% Tween 20). Finally, the protein A-Sepharose beads were boiled in SDS sample buffer and analyzed by SDS-10% PAGE. Coimmunoprecipitated TFIIE-β was detected by autoradiography after enhancement with Amplify (Amersham).

Coimmunoprecipitation of TFIIE-α mutants with TFIIH. Polyclonal antisera against the p62 subunit of TFIIH (0.02 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 mM EDTA, 10 mM 2-mercaptoethanol, 20% [vol/vol] glycerol, 0.002% [vol/vol] Nonidet P-40, 200 μg of bovine serum albumin per ml, 0.5 mM PMSF) containing 100 mM KCl (BC100) for 2 h at 4°C with rotation. The protein G-agarose beads were precipitated and washed twice with 500 μl of washing buffer I and twice with 500 μl of buffer C containing 1 M KCl (BC1000). The heparin-HPLC TFIIH fraction (200 ng) was then added with 400 μl of BC1000, and the mixture was incubated with anti-p62-protein G beads for 4 h at 4°C with rotation. The beads were washed twice with 500 μl of BC1000, twice with 500 μl of washing buffer I, and three times with 500 μl of BC100 to remove weakly associated contaminants. The beads precipitated TFIIH with both ATPase and CTD kinase activities (46a). Finally, various TFIIE-α mutants (200 ng) were incubated with TFIIH-prebound anti-p62-protein G beads in 500 μl of BC100 for 4 h at 4°C with rotation. The beads were washed three times with 500 μl of BC200, boiled in SDS sample buffer, and analyzed by SDS-11% PAGE. Coimmunoprecipitated TFIIE-α mutants were detected by immunoblotting as de-

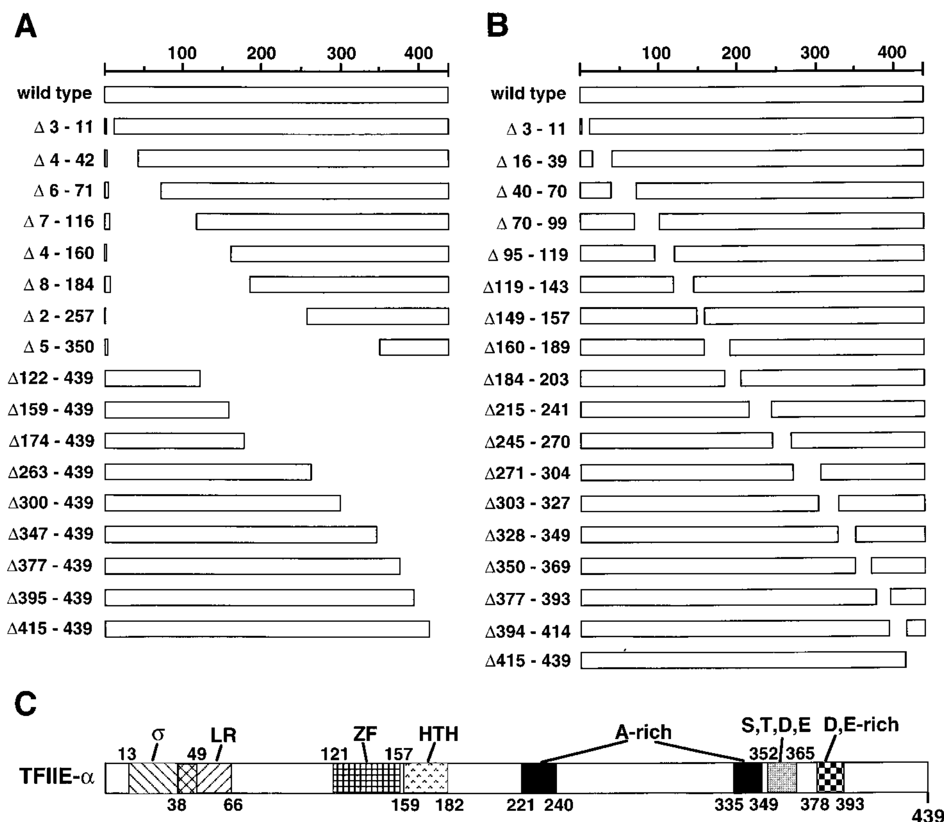

FIG. 1. Structures of deletion mutants of human TFIIE-α. The schematic diagrams of TFIIE-α mutants are presented. The numbers on the left indicate the actual amino acid residues deleted and are used to denote each mutant (the deleted portion of TFIIE-α is indicated by a gap). (A) N-terminal and C-terminal deletion mutants. All mutants were designed according to the putative structural motifs and characteristic sequences as shown in panel C. (B) Internal deletion mutants. These mutants were also constructed according to the proposed structures of TFIIE-α (Fig. 1C). Two mutants (Δ3-11 and Δ415-439) are the same as the mutants presented in panel A. (C) Schematic diagram of representative structures of TFIIE-α. The σ homology region (σ), leucine repeat (LR), zinc finger (ZF), helix-turn-helix (HTH), alanine-rich region (A-rich), a region that consists of serine, threonine, aspartic acid, and glutamic acid residues (S,T,D,E), and acidic region (D,E-rich) are indicated (47).

scribed below. Proteins were transferred to a Millipore Immobilon-P polyvinylidene difluoride membrane. This membrane was blocked in a 5% nonfat milk solution containing 25 mM Tris-HCl (pH 7.5) and 150 mM NaCl and incubated with anti-TFIIE-α polyclonal antiserum (1:3,000 dilution) in rinse solution (10 mM Tris-HCl [pH 7.5], 1 mM EDTA, 150 mM NaCl, 0.1% [vol/vol] Tween 20, 0.4% nonfat milk) and then with horseradish peroxidase-conjugated anti-rabbit second immunoglobulin G (1:10,000 dilution; Amersham). Then, the membrane was developed with an enhanced chemiluminescence detection kit (Amersham) and was detected by PDB-1 film (Kodak).

Kinase assay. In addition to transcription factors and DNA added as described in the legends to Fig. 7 and 8, the kinase reaction mixture (25 μl) contained 5 mM HEPES (*N*-2-hydroxylethylpiperazine-*N*′-2-ethanesulfonic acid)-KOH (pH 7.8), 20 mM Tris-HCl (pH 7.9 at 4°C), 7 mM $MgCl_2$, 60 mM KCl, 12% (vol/vol) glycerol, 2% (wt/vol) polyethylene glycol 8000, 2 mM 2-mercaptoethanol, 0.1 mM EDTA, 240 μg of bovine serum albumin per ml, 5 μM ATP, and 3 μCi of [γ-^{32}P]ATP. Phosphorylation reactions were done at 30°C for 1 h and stopped by the addition of 75 μl of phosphorylation stop solution (10 mM EDTA, 0.1% Nonidet P-40, 0.05% SDS). Phosphorylated proteins were precipitated with trichloroacetic acid and analyzed by SDS-PAGE (5.5 or 10% acrylamide).

RESULTS

Putative structural motifs of TFIIE-α are essential and sufficient to support basal transcription. The predicted amino acid sequence of TFIIE-α reveals several putative structural motifs and characteristic sequences: a bacterial σ factor homology region (residues 13 to 49), a leucine repeat motif (residues 38 to 66), a zinc finger motif (residues 121 to 157), a helix-turn-helix motif (residues 159 to 182), alanine-rich sequences (residues 221 to 240 and 335 to 349), a sequence consisting of serine, threonine, aspartate, and glutamate (residues 352 to 365), and an acidic region (D and E rich [residues 378 to 393]), as shown in Fig. 1C (45, 47). On the basis of this information, a systematic series of deletion mutants (N terminal, C terminal, and internal) was constructed (summarized in Fig. 1A and B). For structure-function analyses of TFIIE-α, bacterially expressed mutants were purified and analyzed by SDS-PAGE (Fig. 2A and 3A). The deletion mutant of the acidic domain (Δ377-393) shows the fastest migration in SDS gels among the internal deletion mutants (Fig. 3A). This may be because of the removal of the heavy negative charge. 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 TFIIE. As shown in Fig. 2B, N-terminal deletion mutant Δ3-11 was almost as active as the wild type; however, mutants with further deletions from the N terminus showed no basal transcription activity. In contrast and somewhat surprisingly, all mutants with C-terminal deletions extending to residue 174 supported basal transcription activity, albeit at a reduced level that largely reflects loss of sequences between residues 377 and 395 (see below as well). Additional mutants with C-terminal deletions extending to residues 122 and 159 were expressed efficiently but showed no transcription activity (see Fig. 5A and B). Thus, the minimal functional region of TFIIE-α is localized between residues 12 and 173.

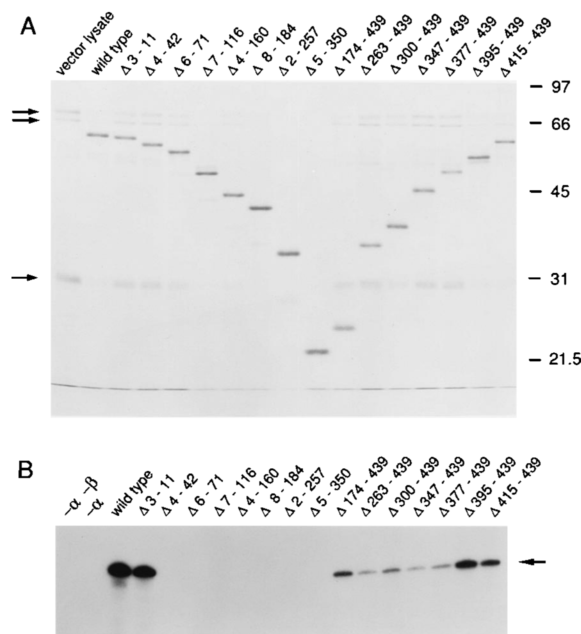

FIG. 2. Basal transcription activities of purified N-terminal and C-terminal deletion mutants of TFIIE-α. (A) SDS-PAGE of TFIIE-α mutants. The N- and C-terminal deletion mutants (diagrammed in Fig. 1A) were purified as described in Materials and Methods, analyzed on a 10% polyacrylamide gel, and stained with Coomassie blue. Vector lysate means the vector lysate purified by the same procedure. The sizes of molecular mass markers are indicated on the right side (in kilodaltons). Arrows indicate the position of contaminating polypeptides. (B) Analysis of basal transcription activities. Reaction mixtures (25 μl) containing 200 ng of the adenovirus type 2 major late promoter template, pML(C_2AT)Δ-50 (55), Pol II, and all general transcription factors except TFIIE (48) were complemented with buffer only (−α−β), 2 ng of TFIIE-β (−α), or 4 ng of various TFIIE-α mutants together with 2 ng of TFIIE-β. The arrow indicates the position of the specific transcript.

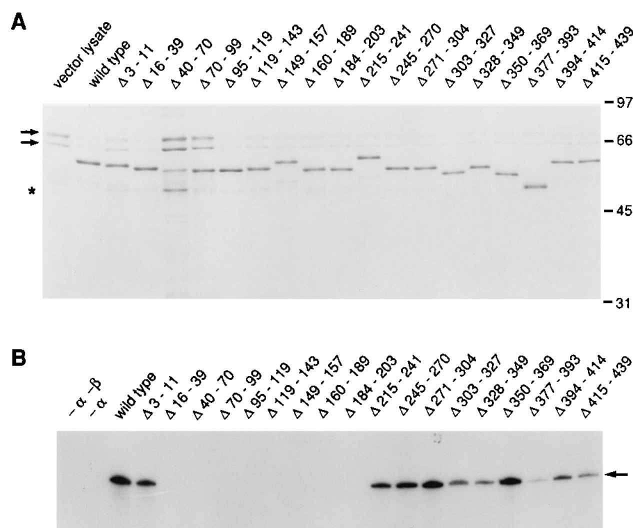

FIG. 3. Basal transcription activities of purified internal deletion mutants of TFIIE-α. (A) SDS-PAGE of TFIIE-α mutants. The internal deletion mutants (diagrammed in Fig. 1B) were purified and analyzed on a 10% polyacrylamide gel stained with Coomassie blue. Arrows indicate the positions of contaminating polypeptides. The asterisk indicates the position of a degradation product in Δ40-70. (B) Analysis of basal transcription activities. A complementation assay of TFIIE-α activity was carried out as described in the legend to Fig. 2B. The arrow indicates the position of the specific transcript.

To further investigate the importance of the individual internal sequences for basal transcription, internal deletion mutants were analyzed (Fig. 3). As shown in Fig. 3B, mutations which selectively delete the σ homology (Δ16-39), leucine repeat (Δ40-70), zinc finger (Δ119-143 and Δ149-157), and helix-turn-helix (Δ160-189) structures, as well as intervening sequences (e.g., Δ70-99 and Δ95-119), all abolished transcription. These results are consistent with those from the C-terminal deletion analyses, with one exception; internal deletion mutant Δ184-203 did not show basal transcription activity, while the C-terminal deletion mutant Δ174-439, which also lacks residues 184 to 203, still supported transcription activity. One explanation for this inconsistency is that the closer apposition of separate peptide fragments in mutant Δ184-203 results in inhibitory conformational changes.

The strong sequence conservation of the N-terminal half (residues 1 to 218) of TFIIE-α between humans and *Xenopus laevis* (45) further supports the functional importance of the N-terminal domain (residues 12 to 173). However, while the C-terminal half of TFIIE-α was not absolutely required for activation, deletion of the acidic domain (residues 378 to 393) decreased transcription activity by 90% (compare also mutant Δ377-439 with Δ395-439 in Fig. 2B and mutant Δ377-393 with Δ394-414 in Fig. 3B). This result suggests that the acidic domain interacts with some component(s) of the general transcription machinery. Deletion of the other characteristic C-terminal sequences (e.g., A-rich and S, T, D, and E regions) had little or no effect on basal transcription. However, it is still possible that these sequences are important for interactions with specific transcriptional regulators.

Regions essential for TFIIE-α–TFIIE-β interactions are located in the N-terminal half of TFIIE-α. To localize the regions of TFIIE-α involved in interactions with TFIIE-β, TFIIE-α mutants were incubated with ^{35}S-labeled TFIIE-β and coimmunoprecipitated by using anti-TFIIE-α polyclonal antibody. The reactivities of this antibody to all mutants were established in advance by both immunoprecipitation and West-

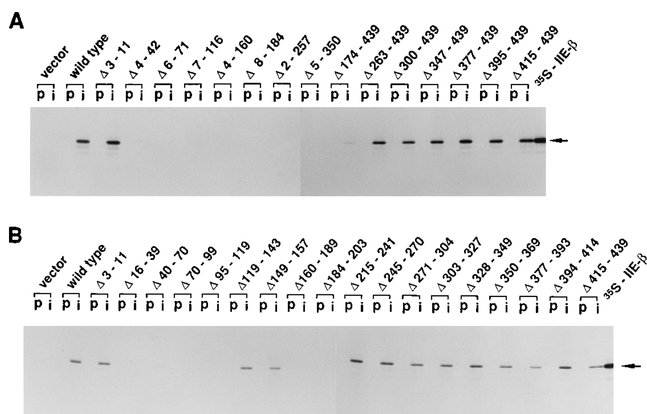

FIG. 4. Characterization of TFIIE-β interaction domains in TFIIE-α. (A) N-terminal and C-terminal deletion mutants. In vivo-labeled ^{35}S-TFIIE-β (50 ng) was incubated with 200 ng of *E. coli* lysate of vector-transformed cells, 200 ng of TFIIE-α (wild type), and 200 ng of various TFIIE-α mutants as indicated at the top of the panel. TFIIE-β was coimmunoprecipitated with TFIIE-α mutants by using either rabbit preimmune sera (p) or immune sera (i) against TFIIE-α as indicated in Materials and Methods. Immunocomplexes were analyzed by SDS-PAGE, and ^{35}S-TFIIE-β was detected by autoradiography. Input ^{35}S-TFIIE-β was run in parallel on the rightmost lane. The arrow indicates the position of TFIIE-β. (B) Internal deletion mutants. The procedure described for panel A was used to detect coimmunoprecipitated ^{35}S-TFIIE-β.

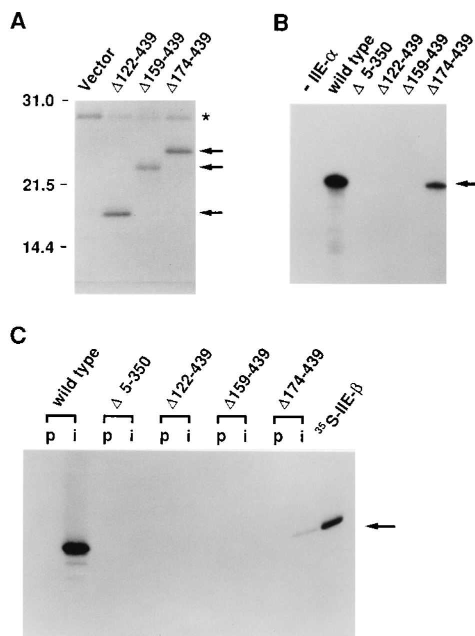

FIG. 5. Identification of the minimal functional domain of TFIIE-α. (A) SDS-PAGE of further C-terminal deletion mutants. Further deletions were constructed from the C terminus. Two mutants (Δ122-439 and Δ159-439), which are diagrammed in Fig. 1A, were purified and analyzed on a 12% polyacrylamide gel stained with Coomassie blue. Sizes of molecular mass markers are indicated on the left side (in kilodaltons). Arrows indicate the positions of Δ122-439, Δ159-439, and Δ174-439. The asterisk shows the position of a contaminant. The mock-purified TFIIE-α fraction was run in the first lane. (B) Analysis of basal transcription activities. Complementation transcription assays of TFIIE-α activity were carried out as described in the legend to Fig. 2B. The N-terminal deletion mutant Δ5-350 was used as a negative control, and the C-terminal deletion mutant Δ174-439 and the wild type were used as positive controls (Fig. 2B). The arrow indicates the position of specific transcripts. (C) TFIIE-β interactions. Coimmunoprecipitation assays were carried out as described in the legend to Fig. 4. In this case, Δ5-350 and Δ174-439 were also used as negative and positive controls (Fig. 4A), respectively. The arrow indicates the position of TFIIE-β. p and i, preimmune and immune sera, respectively.

ern blotting (immunoblotting) (data not shown). As shown in Fig. 4A and B and 5C, and by comparison with the data in Fig. 2B, 3B, and 5B, all mutants which were active in basal transcription also showed an interaction with TFIIE-β. Interestingly, all but two of the mutants which were transcriptionally inactive also failed to interact with TFIIE-β, the exceptions being mutants Δ119-143 and Δ149-157. Although each lacks a part of the putative zinc finger domain, both still interact with TFIIE-β (Fig. 4B). Taken together, these results indicate that two distinct domains (residues 12 to 118 and 158 to 214), which are separated by the zinc finger region, are essential for interaction with TFIIE-β.

All deletion mutants of TFIIE-α which support basal transcription also support transcriptional activation by various activators. Although recent studies have implicated TFIID (both TBP and associated proteins) and TFIIB as targets for transcription activators (6, 16, 18, 20, 22, 29, 35, 36, 75), this does not exclude other general initiation factors from being alternate or simultaneous targets. Since TFIIE-α contains structural motifs of documented importance for other regulatory factors, the possible function of these elements in activator-dependent transcription was tested through the use of TFIIE-α mutants that retained basal transcription activity. The analyses in Fig. 6A and B show effects of the acidic activator GAL4-VP16 on the selected N- and C-terminal and internal deletion mutants, respectively. All mutants tested supported transcriptional activation by 5- to 10-fold, although the basal transcription level varied for each mutant. The results for GAL4-AH were identical to those for GAL4-VP16 (data not shown). Even when the proline-rich activator GAL4-CTF1 was used, all transcriptionally active N- and C-terminal deletion mutants supported activation at levels almost equivalent to those observed with GAL4-VP16 (Fig. 6C). These results demonstrate that the C-terminal half of TFIIE-α modulates basal transcription but apparently is not directly involved in GAL4-based activator function under the conditions tested. It seems likely, therefore, that the N-terminal half is important for both basal and activated transcription. The best way to distinguish the region important for activation from the region for basal transcription will be to identify mutants (possibly point mutants) that possess basal transcription but are refractory to activation.

Effects of TFIIE-α mutations on TFIIH-dependent CTD phosphorylation correlate with effects on basal transcription. TFIIE strongly stimulates phosphorylation of the CTD of Pol II by TFIIH (38, 46) and is required for the maximal level of phosphorylation of the CTD that is observed during formation of the preinitiation complex (46). Thus, given the possible effect of CTD phosphorylation on both preinitiation complex formation (including Pol II entry) and preinitiation complex function (initiation or early elongation events), one or both subunits of TFIIE might help to directly regulate these events. To investigate this possibility for TFIIE-α and to map the regions which are essential for TFIIH-mediated phosphorylation, the abilities of the deletion mutants of TFIIE-α to stimulate CTD phosphorylation during preinitiation complex formation (in the presence of the promoter, all initiation factors, and Pol IIA) were determined.

As shown in Fig. 7A and B, all mutants capable of interacting with TFIIE-β also stimulated CTD phosphorylation under these conditions. These included the zinc finger mutants (Δ119-143 and Δ149-157) that are completely inactive in basal transcription and the C-terminal acidic region mutant (Δ377-393) that shows a 10-fold reduction in basal transcription. Although phosphorylation of the CTD in the presence of promoter DNA is completely dependent on entry (at a late step) of TFIIE into the preinitiation complex (38, 46), phosphorylation (by TFIIH) of the CTD in the absence of promoter DNA is enhanced by TFIIE regardless of the presence of the other general factors (46). Therefore, the effects of TFIIE-α mutations on CTD phosphorylation were also analyzed in the absence of DNA, with only TFIIH, TFIIE-β, and Pol II (Fig. 8). Of the mutants that were analyzed, all of those which were fully or partially active in transcription also stimulated DNA-independent phosphorylation, with the notable exception of the Δ377-393 mutant. This mutant lacks the acidic region and, as shown above (Fig. 3B), showed a significantly reduced (by 90%) basal transcription activity. This suggests that TFIIE-α binds directly to TFIIH and/or Pol II via interactions involving the acidic region (see below). The failure of the Δ377-393 mutation to inhibit CTD phosphorylation in the presence of DNA and the other general transcription factors (Fig. 7B, lane 19) may simply reflect concerted interactions between the factors (e.g., additional contacts of TFIIB and/or TFIIF with TFIIH and/or Pol II) that compensate for loss of the intrinsic ability of the TFIIE-α mutant to interact with TFIIH and/or Pol II (see below).

Interestingly, while deletion mutants lacking the putative zinc finger motif (Δ119-143 and Δ149-157) also supported stimulation of CTD phosphorylation under transcription conditions (Fig. 7B, lanes 9 and 10), they failed to stimulate CTD

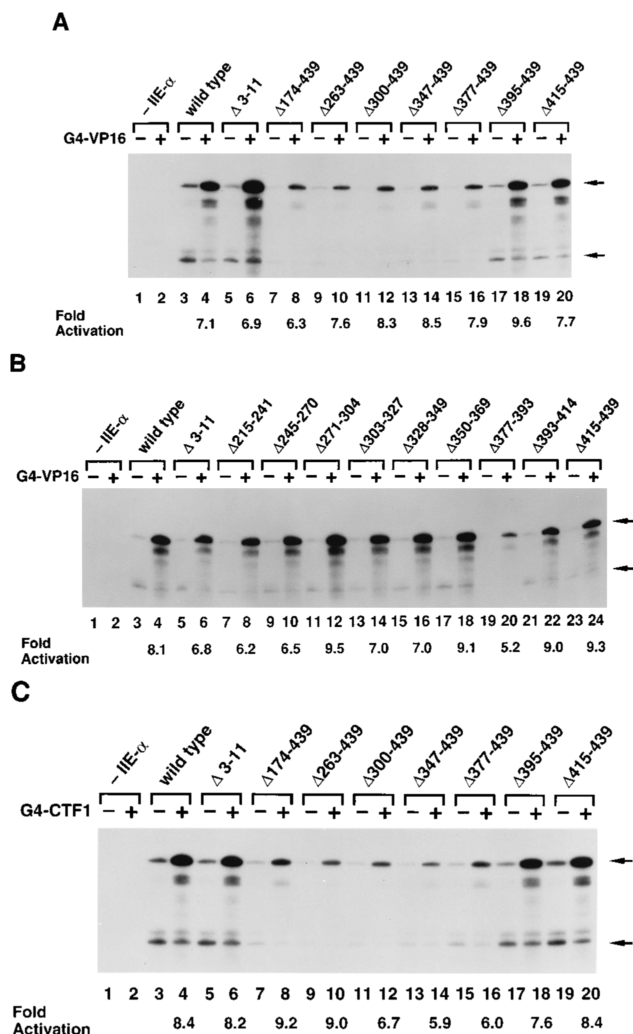

FIG. 6. Effects of deletion mutants of TFIIE-α on transcriptional activation. (A) N-terminal and C-terminal deletion mutants of TFIIE-α with GAL4-VP16. Reaction mixtures (25 μl) contained 100 ng of pG5HM(C_2AT), 50 ng of pML(C_2AT)Δ-53Sh, and all general transcription factors together with Pol II as described elsewhere (48), except that 20 ng of purified flag-tagged TFIID was used as natural TFIID (4). Twenty nanograms of mutant or wild-type TFIIE-α was used for transcription as indicated on the top of the panel. In lanes 1 and 2, TFIIE-α was omitted (−IIE-α). The upper arrow (380 nt) indicates transcripts of pG5HM(C_2AT), and the lower arrow (330 nt) indicates transcripts of pML(C_2AT)Δ-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 with a PhosphorImager (Molecular Dynamics). (B) Internal deletion mutants of TFIIE-α with GAL4-VP16. The procedure used was the same as that described for panel A, except that 20 ng of the internal deletion mutants was employed. (C) N-terminal and C-terminal deletion mutants of TFIIE-α with GAL4-CTF1. Forty nanograms of the proline-rich activator GAL4-CTF1 (G4-CTF1) instead of GAL4-VP16 was used. All conditions were as described for panel A.

phosphorylation by TFIIH in the absence of all other general factors and promoter DNA (Fig. 8, lanes 9 and 10). This most likely reflects a reduced intrinsic ability of these mutants to interact directly with TFIIH and/or Pol II and supports the view that other general factors may help recruit mutant TFIIE and TFIIH to the preinitiation complex and allow phosphorylation activity (cf. above). Additionally, two deletion mutants (Δ16-39 and Δ40-70) which failed either to support basal transcription or to interact with TFIIE-β were still able to stimulate phosphorylation of the CTD (Fig. 8, lanes 5 and 6). These mutants could be missing regions that are normally inhibitory for interactions with TFIIH and/or Pol II or they might contain a newly created domain capable of interacting with TFIIE-β in conjunction with Pol II and TFIIH. Alternatively, their persistent ability to bind independently to TFIIH (Fig. 9, lane 7 for Δ40-70 and data not shown for Δ16-39) may still account for their ability to stimulate CTD phosphorylation in the absence of DNA (Fig. 8, lanes 5 and 6) but not under conditions which necessitate proper entry into an initiation complex (Fig. 7B, lanes 5 and 6). This region (residues 16 to 120) contains several sets of heptad repeats of hydrophobic residues that may suffice for protein-protein interactions (especially with TFIIE-β) even after partial internal deletions within this region.

Carboxy-terminal acidic region of TFIIE-α is involved in binding to TFIIH. In view of the results for basal transcription and CTD phosphorylation presented above, it seemed possible that TFIIE-α might interact directly with TFIIH. To test this possibility, binding of various TFIIE-α mutants to TFIIH was analyzed by coimmunoprecipitation with anti-TFIIH antibody and by detection of precipitated mutants with anti-TFIIE-α antibody after Western blotting of SDS-polyacrylamide gels (Fig. 9). Interestingly, a small fragment (mutant Δ5-350) containing mainly the C-terminal residues of TFIIE-α was sufficient to bind to TFIIH (Fig. 9, lane 3), whereas mutants with C-terminal deletion (Δ377-439, Δ300-439, and Δ174-439) extending to or beyond residue 376 could not (Fig. 9, lanes 4 to 6). Similarly, an analysis of internal deletion mutants demonstrated that only mutant Δ377-393, which lacks the acidic region, could not bind to TFIIH (Fig. 9, lane 17). This clearly indicates that TFIIE-α can directly and specifically bind to TFIIH via the acidic region.

Deletions of the putative zinc finger motif (mutants Δ119-143 and Δ149-157) did not affect binding to TFIIH (Fig. 9, lanes 8 and 9). This is of special significance in view of the fact that zinc finger mutations eliminate both basal transcription (Fig. 3) and the ability of TFIIE to enhance CTD phosphorylation by TFIIH in the absence of other general factors (Fig. 8). These results are consistent with the idea that TFIIE must be involved in multiple interactions, i.e., minimally those through which TFIIE is recruited to the preinitiation complex and those which in turn recruit TFIIH, but possibly including distinct interactions which regulate the enzymatic function of TFIIH (10, 46). While zinc finger motifs could be involved in generalized protein-DNA binding as suggested earlier (for a review, see reference 7), the presence of zinc finger motifs in Pol II (for a review, see reference 77) and the demonstrated contribution of zinc finger motifs to subunit interactions of either RNA polymerase I (76) or RNA polymerase III (74) raise the possibility that the TFIIE-α zinc finger motif is involved in interactions with Pol II. Consistent with this view, others have indicated that TFIIE can interact with Pol II (15) and that a zinc finger mutation can alter TFIIE interactions with the preinitiation complex (41).

A putative zinc finger domain is essential for productive transcription initiation. The deletion mutants (Δ119-143 and Δ149-157) lacking the putative zinc finger domain failed to show basal transcription activity (Fig. 3B) but stimulated CTD phosphorylation during preinitiation complex formation (Fig. 7B, lanes 9 and 10). These results suggest that these mutants have the potential to form a nonfunctional initiation complex. To examine this possibility, the effect of increasing amounts of the mutants on basal transcription by wild-type TFIIE-α was tested. As shown in Fig. 10A, these deletion mutants actively suppressed basal transcription in a dose-dependent manner (lanes 4 to 8 and 9 to 13). On the other hand, TFIIE-α mutants lacking either the N-terminal half (Δ8-184) or the C-terminal

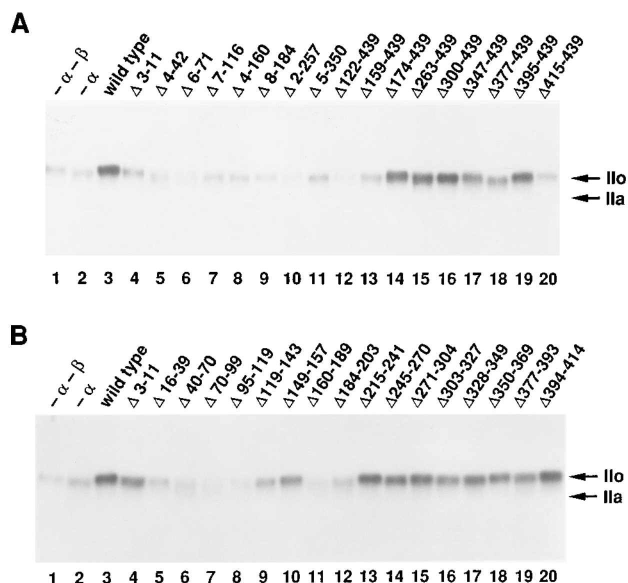

FIG. 7. Effects of deletion mutants of TFIIE-α on CTD phosphorylation by TFIIH during active initiation complex formation. (A) N-terminal and C-terminal deletion mutants. Kinase assays (25-μl mixtures) were carried out as described in Materials and Methods under the conditions of active initiation complex formation. Factors added were as follows: recombinant human TBP (30 ng), recombinant TFIIB (20 ng), heparin-HPLC-purified TFIIF (30 ng), heparin-HPLC-purified TFIIH (30 ng), and DEAE-HPLC-purified Pol II (100 ng). Two nanograms of DNA fragment containing adenovirus type 2 major late promoter sequences from −39 to +29 also was added. Lane 1, reaction without TFIIE (−α−β); lanes 2 to 20, 10 ng of TFIIE-β added; lane 2, reaction in the absence of TFIIE-α (−α). Phosphorylated proteins were analyzed on an 5.5% SDS-acrylamide gel and detected by autoradiography. Arrows indicate the positions of the phosphorylated form of the largest subunit of Pol II (IIo) and the unphosphorylated form (IIa). (B) Internal deletion mutants. All procedures were the same as those described for panel A, except that the internal deletion mutants were used.

half (Δ174-439) had no effect on transcription even when present at a 64-fold excess (Fig. 10A, lanes 14 to 18 and 19 to 23). The same effects were observed in activated transcription when GAL4-AH was used (data not shown). Along with the demonstrated ability of the zinc finger mutants to facilitate CTD phosphorylation in the presence (but not the absence) of template DNA and other general factors, these results clearly indicate formation of a nonfunctional preinitiation complex (which can sequester factors essential for forming active initiation complexes). Moreover, the fact that CTD phosphorylation can occur without active transcription with the zinc finger

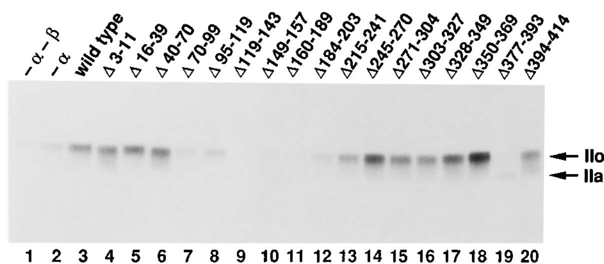

FIG. 8. Effects of internal deletion mutants of TFIIE-α on CTD phosphorylation by TFIIH in the absence of DNA. Kinase assays were carried out as described in Materials and Methods with Pol II, TFIIH, TFIIE-β, and TFIIE-α (or its mutants) in the absence of DNA. Twenty nanograms of the internal deletion mutants was used. Arrows indicate the positions of the two forms of the largest subunit of Pol II.

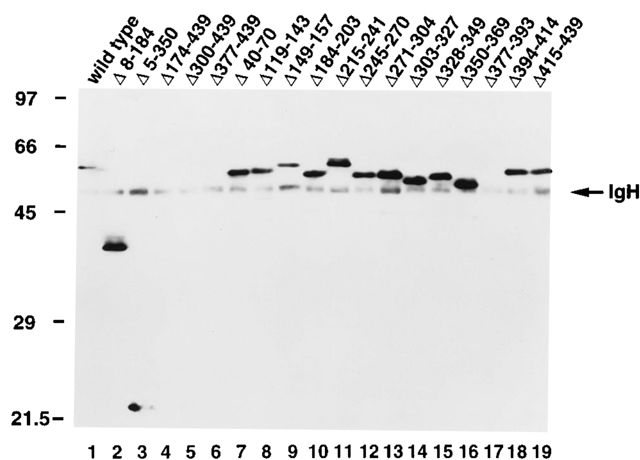

FIG. 9. Direct binding of TFIIE to TFIIH via the C-terminal acidic region. Various deletion mutants of TFIIE-α (200 ng) were incubated with TFIIH prebound to anti-p62-protein G-agarose. Immunocomplexes were washed three times with a buffer containing 200 mM KCl and were analyzed by SDS-PAGE (11% acrylamide) as described in Materials and Methods. Coimmunoprecipitated TFIIE-α mutants were detected by enhanced chemiluminescence (Amersham) after transfer to an Immobilon P membrane (Millipore). The arrow indicates the position of the immunoglobulin heavy chain (IgH). The sizes of molecular mass markers are indicated on the left (in kilodaltons).

mutants is consistent with the experiments described by Laybourn and Dahmus (32) showing CTD phosphorylation under conditions which preclude initiation. Altogether, these observations indicate that a conformational change essential for productive transcription may occur in Pol II, and probably in the general transcription factors, upon interaction of Pol II with TFIIE via the zinc finger domain.

DISCUSSION

This study has investigated the structure and function of TFIIE by analyzing the effects of TFIIE-α mutations on basal transcription, transcriptional activation, interactions with the TFIIE-β subunit and with TFIIH, and stimulation of TFIIH-dependent CTD phosphorylation. Since TFIIE has been found to regulate TFIIH function (10, 38, 46), our present study has focused mainly on characterization of the mechanism of transcriptional enhancement by TFIIE in conjunction with TFIIH activity.

Recent purification and functional characterization of human TFIIH and its homologs from other species demonstrate that all possess kinase, ATPase, and helicase activities, possibly indicating a conserved role in transcription initiation (10–13, 38, 46, 53, 59, 61). All of these activities require hydrolyzable ATP (or dATP), except for the kinase activity, which also shows some activity with GTP (61). Correspondingly, previous studies have suggested that hydrolyzable ATP (or dATP) is required for basal transcription (2, 56) at the following three key steps: (i) modification of the preinitiation complex to form a productive initiation complex, via phosphorylation of the CTD of Pol II (31, 32) and possibly a subset of the general transcription factors (46); (ii) unwinding of template duplex DNA (promoter melting) at the transcription start site (71, 72); and (iii) dissociation of the general transcription factors from the initiation complex to form a competent elongation complex via promoter clearance (17, 24). Other studies have suggested conditional requirements for ATP (or dATP) and TFIIH that may be dependent on the nature and state of the template (promoter strength and DNA topology, etc.), al-

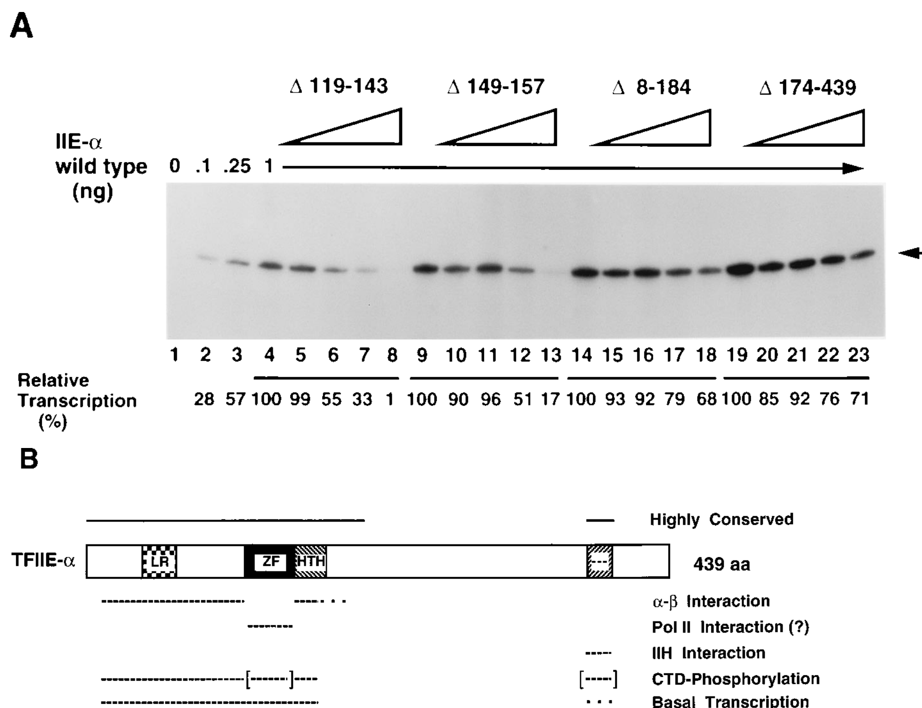

FIG. 10. Dominant negative effect on basal transcription by putative zinc finger deletion mutants of TFIIE-α. (A) Basal transcription assays were performed as described in Materials and Methods. Reaction mixtures contained 100 ng of the DNA template pML(C_2AT)Δ-50. Reactions were carried out without TFIIE-α (lane 1) and with increasing amounts of TFIIE-α (0.1 ng for lane 2, 0.25 ng for lane 3, and 1 ng for lanes 4 to 23). Two deletion mutants of the putative zinc finger domain (Δ119-143 and Δ149-157) and the N-terminal deletion (Δ8-184) and C-terminal deletion (Δ174-439) mutants were used to characterize the effect on basal transcription with 1 ng of wild-type TFIIE-α. The mutant Δ8-184 itself is negative in basal transcription, and the mutant Δ174-439 itself is positive; both were used as controls. Increasing amounts of mutants were added as follows: no mutant (lanes 4, 9, 14, and 19), 1 ng (lanes 5, 10, 15, and 20), 4 ng (lanes 6, 11, 16, and 21), 16 ng (lanes 7, 12, 17, and 22), and 64 ng (lanes 8, 13, 17, and 23). Relative transcription activities (in percentages) were calculated on the basis of the activity without each mutant being 100%. (B) Functional mapping of TFIIE-α. Functional features are summarized and mapped. Dashed lines, functional (essential) regions; dotted line, the stimulatory region; brackets, the conditional regions; LR, leucine repeat; ZF, zinc finger; HTH, helix-turn-helix; – – –, acidic region. The N-terminal half is essential for function, whereas the acidic region in the C terminal half is stimulatory. aa, amino acid.

though there is some inconsistency among these reports (references 17, 49, 66, and 70 versus reference 14).

Structure-function relationships in TFIIE-α. Our mutational analysis of TFIIE-α has shown a link between basal transcription, TFIIE complex formation, and stimulation of CTD phosphorylation by TFIIH (Table 1). As summarized in Fig. 10B, the N-terminal half of TFIIE-α contains the σ homology, leucine repeat, zinc finger, and helix-turn-helix structures. Two separated regions (residues 12 to 118 and 158 to 215) containing the leucine repeat and helix-turn-helix sequences are important for interaction with TFIIE-β. Remarkably, a high concentration of hydrophobic residues is located on the same surface of putative α-helices (heptad repeats) in both regions, including the leucine repeat domain (residues 38 to 66). The zinc finger domain may be important for interaction with Pol II, as evidenced by CTD phosphorylation and basal transcription assays with corresponding mutants (Fig. 7B, 8, and 10A). Other results indicate that the N-terminal half of TFIIE-α is an essential domain for transcription and phosphorylation, while the C-terminal half (notably an acidic domain) may serve as a regulatory domain. TFIIE has been demonstrated to mediate TFIIH recruitment during transcription initiation complex formation (14) and to interact functionally with TFIIH (17, 38, 46). As an extension of these observations, we have shown that the C-terminal acidic domain (residues 378 to 393) of TFIIE-α is a direct binding site for TFIIH (Fig. 9) and that mutation of this site also reduces basal transcription by 90%. These data provide direct evidence for both physical and functional associations of TFIIE and TFIIH and, in addition, support the hypothesis that CTD phosphorylation is functionally important for basal transcription under physiological conditions (see below) requiring TFIIE and TFIIH. These results are consistent with the evolutionary conservation of these regions within the sequence of the *Xenopus* TFIIE-α homolog (45).

Functional role of TFIIE and TFIIH in transcription. Another question addressed was the step(s) at which TFIIE and TFIIH may function. The CTD kinase and ATPase-helicase activities of TFIIH appear to be differentially regulated on the basis of the following observations. First, the IIB form of Pol II (Pol IIB), which lacks the CTD, still requires ATP (or dATP) for transcription (32). Second, the CTD kinase and ATPase activities have distinct DNA requirements (46, 54). Third, Sarkosyl inhibits both ATPase and helicase activities but does not inhibit the kinase activity, whereas the kinase inhibitor staurosporine inhibits only the kinase activity (54). Further, Goodrich and Tjian (17) recently have reported an involvement of TFIIE and TFIIH after the initial phosphodiester bond formation (during promoter clearance), which could be related to coupled ATPase and helicase activities of TFIIH.

Here, we have focused on TFIIH-driven CTD phosphorylation and its stimulation by TFIIE. This modification of Pol II may induce drastic conformational changes in both crude (31) and purified (46) in vitro reconstitution systems. This could be a specific regulatory event for class II (Pol II transcribed) promoters, because the CTD is a unique structure of hep-

TABLE 1. Summary of structure-function studies of TFIIE-α mutants

Type of deletion mutant	Activity result[a]		
	Basal transcription	α-β interaction	Phosphorylation
N- or C-terminal deletion mutant			
Wild type	++	++	++
Δ3-11	++	++	++
Δ4-42	−	−	−
Δ6-71	−	−	−
Δ7-116	−	−	−
Δ4-160	−	−	−
Δ8-184	−	−	−
Δ2-257	−	−	−
Δ5-350	−	−	−
Δ122-439	−	−	−
Δ159-439	−	−	−
Δ174-439	+	+	++
Δ263-439	+	++	++
Δ300-439	+	++	++
Δ347-439	+	++	++
Δ377-439	+	++	+
Δ395-439	++	++	++
Δ415-439	++	++	+
Internal deletion mutant			
Wild type	++	++	++
Δ3-11	++	++	++
Δ16-39	−	−	(−)
Δ40-70	−	−	(−)
Δ70-99	−	−	−
Δ95-119	−	−	−
Δ119-143	−	++	(++)
Δ149-157	−	++	(++)
Δ160-189	−	−	−
Δ184-203	−	−	−
Δ215-241	++	++	++
Δ245-270	++	++	++
Δ271-304	++	++	++
Δ303-327	++	++	++
Δ328-349	++	++	++
Δ350-369	++	++	++
Δ377-393	+	++	(++)
Δ394-414	++	++	++

[a] ++, activities similar to those of the wild type; +, weak-positive activities; −, negative activities. Phosphorylation results were equivalent under transcription conditions (all factors plus DNA) and in the presence of only TFIIE, TFIIH, and Pol II, except for the mutants under the last condition for the results indicated in parentheses (see also text).

tapeptide repeats found only in eukaryotic Pol II and not other RNA polymerases (for a review, see reference 9). In vivo studies indicate that almost all stalled Pol II molecules are in the nonphosphorylated (IIA) form and, conversely, that almost all elongating Pol II molecules are in the phosphorylated (II0) form (44, 73). In addition, in vitro experiments have shown that CTD phosphorylation can occur subsequent to formation of a functional preinitiation complex but before transcription initiation (3, 32, 38, 46). Significantly, and with relation to the above observations, we observe a tight functional link between basal transcription activity and stimulation of CTD phosphorylation. Thus, TFIIE might have dual regulatory roles for TFIIH enzymatic activities: one (after TFIIH recruitment) in the stimulation of phosphorylation of the CTD (and possibly some general transcription factors) either before, coincident with, or just after transcription initiation and a second (after initiation) in promoter clearance involving the ATPase and helicase activities of TFIIH.

One limitation of many current studies of TFIIE and TFIIH is that in vitro systems reconstituted with purified factors generally show no requirement for either the CTD or CTD kinase activity for productive transcription (33, 60). In contrast, transcription appears to be selectively blocked at the level of elongation in crude systems in the absence of the CTD or CTD kinase activity (5, 33). Coupled with studies documenting the in vivo function of the CTD (1, 34, 58), these results may indicate the presence (in the context of the normal nuclear environment) of constraints to elongation (mediated by unknown factors) that are reversed by phosphorylation of the CTD. The present studies are consistent with a model in which TFIIE- and TFIIH-mediated CTD phosphorylation events, under conditions in which they are essential for productive transcription (5, 33, 58, 60), are linked to other initiation or promoter clearance events mediated by TFIIE and TFIIH.

Alternative pathways for productive transcription. Taking all the data into consideration, models for alternative pathways for productive transcription events can be proposed. Recently, Goodrich and Tjian (17) have proposed a novel pathway on the basis of dinucleotide priming and abortive initiation assays. In this pathway, abortive transcription occurs following formation of a minimal preinitiation complex (TBP, TFIIB, TFIIF, and Pol II), after which TFIIE and TFIIH are recruited and serve to convert the stalled complex to a competent elongation complex through a promoter clearance step involving ATP hydrolysis and TFIIH helicase functions. The alternative pathway suggested here, and implicit in previous studies (32, 38, 40, 52), invokes formation of a complete preinitiation complex (with TFIIE and TFIIH) prior to transcription initiation. On natural templates dependent on TFIIE and TFIIH for efficient transcription, and under physiological conditions, TFIIH-mediated phosphorylation could play an important role earlier than the point of promoter clearance suggested in the model described by Goodrich and Tjian (17), or it might simply occur prior to initiation and be of functional significance only later. In the presence of ATP (or dATP), TFIIH phosphorylates the CTD of Pol II (38, 52) and possibly other general factors as well (52), releasing it from promoter-bound TBP and forming an elongation-competent initiation complex. Nucleoside triphosphates are then utilized for actual transcription initiation (initiated complex), which is followed directly by ATP-dependent DNA unwinding (mediated by another TFIIH activity) and ultimately promoter clearance and productive elongation. These alternative pathways may be variably utilized, depending on template topology, the strength of individual promoters, and the relative concentrations of various general factors and interacting activators. Although further studies are required to validate these models, the recent demonstration that addition of TFIIE and TFIIH to a naturally initiated (with four nucleoside triphosphates) and stalled minimal complex does not result in productive elongation (51a) favors (for the promoter studied) the latter of the two models. The initiation-elongation mechanisms are currently being characterized by further mutations of the TFIIE-β subunit as well as of the TFIIE-α subunit.

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REFERENCES

1. **Bartolomei, M. S., N. F. Halden, C. R. Cullen, and J. L. Corden.** 1988. Genetic analysis of the repetitive carboxy-terminal domain of the largest subunit of mouse RNA polymerase II. Mol. Cell. Biol. **8:**330–339.
2. **Bunick, D., R. Zandomeni, S. Ackerman, and R. Weinmann.** 1982. Mechanism of RNA polymerase II specific initiation of transcription: ATP requirement and uncapped run-off transcripts. Cell **29:**877–886.
3. **Chesnut, J. D., J. H. Stephens, and M. E. Dahmus.** 1992. The interaction of RNA polymerase II with the adenovirus-2 major late promoter is precluded by phosphorylation of the C-terminal of subunit IIa. J. Biol. Chem. **267:** 10500–10506.
4. **Chiang, C.-M., H. Ge, Z. Wang, A. Hoffmann, and R. G. Roeder.** 1993. Unique TATA-binding protein-containing complexes and cofactors involved in transcription by RNA polymerase II and III. EMBO J. **12:**2749–2762.
5. **Chodosh, L. A., A. Fire, M. Samuels, and P. A. Sharp.** 1989. 5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole inhibits transcription elongation by RNA polymerase II in vitro. J. Biol. Chem. **264:**2250–2257.
6. **Choy, B., and M. R. Green.** 1993. Eukaryotic activators function during multiple steps of preinitiation complex assembly. Nature (London) **366:**531–536.
7. **Coleman, J. E.** 1992. Zinc proteins: enzymes, storage proteins, transcription factors, and replication proteins. Annu. Rev. Biochem. **61:**897–946.
8. **Conaway, R. C., and J. W. Conaway.** 1993. General initiation factors for RNA polymerase II. Annu. Rev. Biochem. **62:**161–190.
9. **Corden, J. L.** 1990. Tails of RNA polymerase II. Trends Biochem. Sci. **15:**383–387.
10. **Drapkin, R., J. T. Reardon, A. Ansali, J.-C. Huang, L. Zawel, K. Ahn, A. Sancar, and D. Reinberg.** 1994. Dual role of TFIIH in DNA excision repair and in transcription by RNA polymerase II. Nature (London) **368:**769–772.
11. **Feaver, W. J., J. Q. Svejstrup, L. Bardwell, A. J. Bardwell, S. Buratowski, K. D. Gulyas, T. F. Donahue, E. C. Friedberg, and R. D. Kornberg.** 1993. Dual roles of a multiprotein complex from S. cerevisiae in transcription and DNA repair. Cell **75:**1379–1387.
12. **Feaver, W. J., J. Q. Svejstrup, N. L. Henry, and R. D. Kornberg.** 1994. Relationship of CDK-activating kinase and RNA polymerase II CTD kinase TFIIH/TFIIK. Cell **79:**1103–1109.
13. **Fischer, L., M. Gerard, C. Chalut, Y. Lutz, S. Humbert, M. Kanno, P. Chambon, and J.-M. Egly.** 1992. Cloning of the 62-kilodalton component of basic transcription factor BTF2. Science **257:**1392–1395.
14. **Flores, O., H. Lu, and D. Reinberg.** 1992. Factors involved in specific transcription by mammalian RNA polymerase II. Identification and characterization of factor IIH. J. Biol. Chem. **267:**2786–2793.
15. **Flores, O., E. Maldonado, and D. Reinberg.** 1989. Factors involved in specific transcription by mammalian RNA polymerase II. Factors IIE and IIF independently interact with RNA polymerase II. J. Biol. Chem. **264:**8913–8921.
16. **Goodrich, J. A., T. Hoey, C. J. Thut, A. Admon, and R. Tjian.** 1993. Drosophila TAFII40 interacts with both a VP16 activation domain and the basal transcription factor TFIIB. Cell **75:**519–530.
17. **Goodrich, J. A., and R. Tjian.** 1994. Transcription factors IIE and IIH and ATP hydrolysis direct promoter clearance by RNA polymerase II. Cell **77:** 145–156.
18. **Hoey, T., R. O. Weinzierl, G. Gill, J. L. Chen, B. D. Dynlacht, and R. Tjian.** 1993. Molecular cloning and functional analysis of Drosophila TAF110 reveal properties expected of coactivators. Cell **72:**247–260.
19. **Hoffmann, A., and R. G. Roeder.** 1991. Purification of his-tagged proteins in non-denaturing conditions suggests a convenient method for protein interaction studies. Nucleic Acids Res. **19:**6337.
20. **Horikoshi, M., T. Hai, Y.-S. Lin, M. R. Green, and R. G. Roeder.** 1988. Transcription factor ATF interacts with the TATA factor to facilitate establishment of a preinitiation complex. Cell **54:**1033–1042.
21. **Horikoshi, M., T. Yamamoto, Y. Ohkuma, P. A. Weil, and R. G. Roeder.** 1990. Analysis of structure-function relationship of yeast TATA box bining factor TFIID. Cell **61:**1171–1178.
22. **Ingles, C. J., M. Shales, W. D. Cress, S. Triezenberg, and J. Greenblatt.** 1991. Reduced binding of TFIID to transcriptionally compromised mutants of VP16. Nature (London) **351:**588–590.
23. **Inostroza, J., O. Flores, and D. Reinberg.** 1991. Factors involved in specific transcription by mammalian RNA polymerase II: purification and functional analysis of general transcription factor IIE. J. Biol. Chem. **266:**9304–9308.
24. **Jacob, G. A., J. A. Kitzmiller, and D. S. Luse.** 1994. RNA polymerase II promoter strength in vitro may be reduced by defects at initiation or promoter clearance. J. Biol. Chem. **269:**3655–3663.
25. **Jiang, Y., and J. D. Gralla.** 1993. Uncoupling of initiation and reinitiation rates during HeLa RNA polymerase II transcription in vitro. Mol. Cell. Biol. **13:**4572–4577.
26. **Jiang, Y., S. T. Smale, and J. D. Gralla.** 1993. A common ATP requirement for open complex formation and transcription at promoters containing initiator or TATA elements. J. Biol. Chem. **268:**6535–6540.
27. **Jiang, Y., S. J. Triezenberg, and J. D. Gralla.** 1994. Defective transcriptional activation by diverse VP16 mutants associated with a common inability to form open promoter complexes. J. Biol. Chem. **269:**5505–5508.
28. **Kim, T. K., and R. G. Roeder.** 1993. Transcriptional activation in yeast by the proline-rich activation domain of human CTF1. J. Biol. Chem. **268:**20866–20868.
29. **Kim, T. K., and R. G. Roeder.** 1994. The proline-rich activator CTF1 targets TFIIB for transcriptional activation. Proc. Natl. Acad. Sci. USA **91:**4170–4174.
30. **Kunkel, T. A., J. D. Roberts, and R. A. Zakour.** 1987. Rapid and efficient site-specific mutagenesis without phenotypic selections. Methods. Enzymol. **154:**367–382.
31. **Laybourn, P. J., and M. E. Dahmus.** 1989. Transcription-dependent structural changes in the C-terminal domain of mammalian RNA polymerase subunit IIa/o. J. Biol. Chem. **264:**6693–6698.
32. **Laybourn, P. J., and M. E. Dahmus.** 1990. Phosphorylation of RNA polymerase IIA occurs subsequent to interaction with the promoter and before the initiation of transcription. J. Biol. Chem. **265:**13165–13173.
33. **Li, Y., and R. D. Kornberg.** 1994. Interplay of positive and negative effectors in function of the C-terminal repeat domain of RNA polymerase II. Proc. Natl. Acad. Sci. USA **91:**2362–2366.
34. **Liao, S.-M., I. C. A. Taylor, R. E. Kingston, and R. A. Young.** 1991. RNA polymerase II carboxy-terminal domain contributes to the response to multiple acidic activators in vitro. Genes Dev. **5:**2431–2440.
35. **Lin, Y.-S., and M. R. Green.** 1991. Mechanism of activation of an acidic transcriptional activator in vitro. Cell **64:**971–981.
36. **Lin, Y.-S., I. Ha, E. Maldonado, D. Reinberg, and M. R. Green.** 1991. Binding of general transcription factor TFIIB to an acidic activator region. Nature (London) **353:**569–571.
37. **Lu, H., O. Flores, R. Weinmann, and D. Reinberg.** 1991. The nonphosphorylated form of RNA polymerase II preferentially associates with the preinitiation complex. Proc. Natl. Acad. Sci. USA **88:**10004–10008.
38. **Lu, H., L. Zawel, L. Fisher, J.-M. Egly, and D. Reinberg.** 1992. Human general transcription factor IIH phosphorylates the C-terminal domain of RNA polymerase II. Nature (London) **358:**641–645.
39. **Malik, S., K. Hisatake, H. Sumimoto, M. Horikoshi, and R. G. Roeder.** 1991. Sequence of general transcription factor TFIIB and relationships to other initiation factors. Proc. Natl. Acad. Sci. USA **88:**9553–9557.
40. **Marshall, N. F., and D. H. Price.** 1992. Control of formation of two distinct classes of RNA polymerase II elongation complexes. Mol. Cell. Biol. **12:** 2078–2090.
41. **Maxon, M. E., and R. Tjian.** 1994. Transcriptional activity of transcription factor IIE is dependent on zinc binding. Proc. Natl. Acad. Sci. USA **91:**9529–9533.
42. **McClure, W. R.** 1985. Mechanism and control of transcription initiation in prokaryotes. Annu. Rev. Biochem. **54:**171–204.
43. **Mitchell, P. J., and R. Tjian.** 1989. Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. Science **245:**371–378.
44. **O'Brien, T., S. Hardin, A. Greenleaf, and J. Lis.** 1994. Phosphorylation of RNA polymerase II C-terminal domain and transcriptional elongation. Nature (London) **370:**75–77.
45. **Ohkuma, Y., S. Hashimoto, R. G. Roeder, and M. Horikoshi.** 1992. Identification of two large subdomains in TFIIE-α on the basis of homology between Xenopus and human sequences. Nucleic Acids Res. **20:**5838.
46. **Ohkuma, Y., and R. G. Roeder.** 1994. Regulatory effect of TFIIE on both ATPase and kinase activities of TFIIH during active initiation complex formation. Nature (London) **368:**160–163.

46a. **Ohkuma, Y., and R. G. Roeder.** Unpublished results.

47. **Ohkuma, Y., H. Sumimoto, A. Hoffmann, S. Shimasaki, M. Horikoshi, and R. G. Roeder.** 1991. Structural motifs and potential σ homologies in the large subunit of human general transcription factor TFIIE. Nature (London) **354:**398–401.
48. **Ohkuma, Y., H. Sumimoto, M. Horikoshi, and R. G. Roeder.** 1990. Factors involved in specific transcription by mammalian RNA polymerase II: purification and characterization of general transcription factor TFIIE. Proc. Natl. Acad. Sci. USA **87:**9163–9167.
49. **Parvin, J. D., and P. Sharp.** 1993. DNA topology and a minimal set of basal factors for transcription by RNA polymerase II. Cell **73:**533–540.
50. **Payne, J. M., P. J. Laybourn, and M. E. Dahmus.** 1989. The transition of RNA polymerase II from initiation to elongation is associated with phosphorylation of the carboxyl-terminal domain of subunit IIa. J. Biol. Chem. **264:**19621–19629.
51. **Peterson, M. G., J. Inostroza, M. E. Maxon, O. Flores, A. Admon, D. Reinberg, and R. Tjian.** 1991. Structure and function of the recombinant subunits of human TFIIE. Nature (London) **354:**369–373.

51a. **Reinberg, D.** Personal communication.
52. **Roeder, R. G.** 1991. The complexities of eukaryotic transcription initiation: regulation of preinitiation complex assembly. Trends Biochem. Sci. **16:**402–408.
53. **Roy, R., J. P. Adamczewski, T. Seroz, W. Vermeulen, J.-P. Tassan, L. Schaeffer, E. A. Nigg, J. H. J. Hoeijmakers, and J.-M. Egly.** 1994. The MO15 cell cycle kinase is associated with the TFIIH transcription-DNA repair factor. Cell **79:**1093–1101.
54. **Roy, R., L. Schaeffer, S. Humbert, W. Vermeulen, G. Weeda, and J.-M. Egly.** 1994. The DNA-dependent ATPase activity associated with the class II basic transcription factor BTF2/TFIIH. J. Biol. Chem. **269:**9826–9832.
55. **Sadowski, I., J. Ma, S. Triezenberg, and M. Ptashne.** 1988. GAL4-VP16 is an unusually potent transcriptional activator. Nature (London) **335:**563–564.
56. **Sawadogo, M., and R. G. Roeder.** 1984. Energy requirement for specific transcription initiation by the human RNA polymerase II system. J. Biol. Chem. **259:**5321–5326.
57. **Sawadogo, M., and R. G. Roeder.** 1985. Factors involved in specific transcription by human RNA polymerase II: analysis by rapid and quantitative in vitro assay. Proc. Natl. Acad. Sci. USA **82:**4394–4398.
58. **Scafe, C., D. Chao, J. Lopes, J. P. Hirsch, S. Henry, and R. A. Young.** 1990. RNA polymerase II C-terminal repeat influences response to transcriptional enhancer signals. Nature (London) **347:**491–494.
59. **Schaeffer, L., R. Roy, S. Humbert, V. Moncollin, W. Vermeulen, J. H. J. Hoejimakers, P. Chambon, and J.-M. Egly.** 1993. DNA repair helicase: a component of BTF2 (TFIIH) basic transcription factor. Science **260:**58–63.
60. **Serizawa, H., J. W. Conaway, and R. C. Conaway.** 1993. Phosphorylation of C-terminal domain of RNA polymerase II is not required in basal transcription. Nature (London) **363:**371–374.
61. **Serizawa, H., R. C. Conaway, and J. W. Conaway.** 1993. Multifunctional RNA polymerase II initiation factor δ from rat liver: relationship between carboxyl-terminal domain kinase, ATPase, and DNA helicase activities. J. Biol. Chem. **268:**17300–17308.
62. **Studier, W., A. H. Rosenberg, J. J. Dunn, and J. W. Dubendorff.** 1990. Use of T7 RNA polymerase to direct expression of cloned genes. Methods Enzymol. **185:**60–89.
63. **Sumimoto, H., Y. Ohkuma, E. Sinn, H. Kato, S. Shimasaki, M. Horikoshi, and R. G. Roeder.** 1991. Conserved sequence motifs in the small subunit of human general transcription factor TFIIE. Nature (London) **354:**401–404.
64. **Sumimoto, H., Y. Ohkuma, T. Yamamoto, M. Horikoshi, and R. G. Roeder.** 1990. Factors involved in specific transcription by mammalian RNA polymerase II: identification of general transcription factor TFIIG. Proc. Natl. Acad. Sci. USA **87:**9158–9162.
65. **Takada, R., Y. Nakatani, A. Hoffmann, T. Kokubo, S. Hasegawa, R. G. Roeder, and M. Horikoshi.** 1992. Identification of human TFIID components and direct interaction between a 250-kDa polypeptide and the TATA box-binding protein (TFIIDτ). Proc. Natl. Acad. Sci. USA **89:**11809–11813.
66. **Timmers, H. T. M.** 1994. Transcription initiation by RNA polymerase II does not require hydrolysis of the β-γ phosphoanhydride bond of ATP. EMBO J. **13:**391–399.
67. **Usheva, A., E. Maldonado, A. Golding, H. Lu, D. Houbavi, D. Reinberg, and Y. Aloni.** 1992. Specific interaction between the nonphosphorylated form of RNA polymerase II and the TATA-binding protein. Cell **69:**871–881.
68. **Van Dyke, M. W., M. Sawadogo, and R. G. Roeder.** 1989. Stability of transcription complex on class II genes. Mol. Cell. Biol. **9:**342–344.
69. **von Hippel, P. H., D. G. Bear, W. D. Morgan, and J. A. McSwigger.** 1984. Protein-nucleic acid interactions in transcription: a molecular analysis. Annu. Rev. Biochem. **53:**389–446.
70. **Wampler, S. L., and J. T. Kadonaga.** 1992. Functional analysis of Drosophila transcription factor IIB. Genes Dev. **6:**1542–1552.
71. **Wang, W., M. Carey, and J. D. Gralla.** 1992. Polymerase II promoter activation: closed complex formation and ATP-driven start site opening. Science **255:**450–453.
72. **Wang, W., J. D. Gralla, and M. Carey.** 1992. The acidic activator GAL4-AH can stimulate polymerase II transcription by promoting assembly of a closed complex requiring TFIID and TFIIA. Genes Dev. **6:**1716–1727.
73. **Weeks, J. R., S. E. Hardin, J. Shen, J. M. Lee, and A. L. Greenleaf.** 1993. Locus-specific variation in phosphorylation state of RNA polymerase II in vivo: correlation with gene activity and transcript processing. Genes Dev. **7:**2329–2344.
74. **Werner, M., S. Hermann-Le Denmat, I. Treich, A. Sentenac, and P. Thuriaux.** 1992. Effect of mutations in a zinc-binding domain of yeast RNA polymerase C (III) on enzyme function and subunit association. Mol. Cell. Biol. **12:**1087–1095.
75. **Workman, J. L., S. M. Abmayr, W. A. Cromlish, and R. G. Roeder.** 1988. Transcriptional regulation by the immediate early protein of pseudorabies virus during in vitro nucleosome assembly. Cell **55:**211–219.
76. **Yano, R., and M. Nomura.** 1991. Suppressor analysis of temperature-sensitive mutations of the largest subunit of RNA polymerase I in *Saccharomyces cerevisiae*: a suppressor gene encodes the second-largest subunit of RNA polymerase I. Mol. Cell. Biol. **11:**754–764.
77. **Young, R. A.** 1991. RNA polymerase II. Annu. Rev. Biochem. **60:**689–715.
78. **Zawel, L., and D. Reinberg.** 1993. Initiation of transcription by RNA polymerase II: a multi-step process. Prog. Nucleic Acid Res. Mol. Biol. **44:**67–108.