Factors Involved in Specific Transcription by Mammalian RNA Polymerase II

PURIFICATION AND FUNCTIONAL ANALYSIS OF GENERAL TRANSCRIPTION FACTOR IIE*

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Mammalian RNA polymerase II transcription factor IIE (TFIIE) was purified to apparent homogeneity. The activity copurified with polypeptides of 34 and 56 kDa. The 56-kDa subunit was sufficient for low levels of transcription activity in a transcription system reconstituted in vitro with highly purified general transcription factors and RNA polymerase II. The 34-kDa polypeptide was found to be stimulatory. The native molecular mass of TFIIE, as determined by gel filtration, was estimated to be approximately 200 kDa, suggesting that TFIIE exists in solution as a tetramer composed of two 56-kDa and two 34-kDa polypeptides. Consistent with previous studies demonstrating an interaction of TFIIE with RNA polymerase II, we found that the entry of TFIIE into the transcription cycle was subsequent to the entry of RNA polymerase II.

Transcription of protein-coding genes in higher eukaryotes is carried out by a multienzymatic complex that includes RNA polymerase II and several accessory transcription factors. One class of factors consists of DNA-binding proteins that recognize specific promoter or enhancer elements, through which they regulate gene- or tissue-specific transcription by RNA polymerase II (reviewed in Mitchell and Tjian, 1989; Johnson and McKnight, 1989). Another group termed general transcription factors act through the core promoter elements (TATA box and initiator motif) and are required for basal transcription of all class two promoters (for review, see Saltzman and Weinmann, 1989; Merenstein et al., 1989). The mechanisms by which the general transcription factors determine the position and directionality of transcription initiation and the biochemical events by which they trigger the formation of the first phosphodiester bond by RNA polymerase II are not yet understood.

In order to answer these questions and to learn about the participation of the general transcription factors in the regulation of transcription initiation, our approach has been to isolate each factor in a pure form and to elucidate their separate roles. This work was supported by National Institutes of Health Grant GM 37110 and National Science Foundation Grant DMB 88-19342. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.

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* The abbreviations used are: TF, transcription factor; Hepes, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid; Ad-MLP, adenovirus major late promoter; HPLC, high pressure liquid chromatography; FPLC, fast protein liquid chromatography; SDS, sodium dodecyl sulfate.

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A better understanding of the functional properties of TFIIE has been hampered by the slow progress in its purification. In the work presented here, we report the purification to apparent homogeneity of transcription factor IIE.

In agreement with previous studies indicating an interaction between TFIIE and RNA polymerase II (Reinberg and Roeder, 1987; Flores et al., 1989), we found that the entry of TFIIE into the transcription cycle is dependent on RNA polymerase II and results in the formation of a stable "putative intermediate" during the production of a transcription-competent complex.

**MATERIALS AND METHODS**

**Specific Transcription Assay and Transcription Factors**—Reaction mixtures (40 µl) were incubated at 30 °C for 60 min and contained 20 mM Hepes buffer, pH 7.5, 8 mM MgCl₂, 50 mM KCl, 10 mM ammonium sulfate, 12% (v/v) glycerol, 10 mM β-mercaptoethanol, 2% (w/v) polyethylene glycol 8000, RNase T1 (20 units), 0.6 mM ATP and CTP, 15 µM [α-32P]UTP (10,000 cpm/pmol), and 0.5 µg of pML(C2AT)A-50 DNA (Sawadogo and Roeder, 1985). Transcription factors added to the reactions were TFIIA (Sepharyl S-500 fraction, 0.25 µg (Flores et al., 1987)), TFIIH (DEAE-5PW fraction, 0.1 µg (Flores et al., 1987)), recombinant yeast TFIIID (Superose 12 fraction, 0.04 µg (Maldonado et al., 1990)), TFIIIF/IIH (DEAE-5PW fraction, 3 pg (Flores et al., 1989)), and RNA polymerase II (2.8 units (Reinberg and Roeder, 1987)). For assays measuring TFIIIE activity, the TFII protein fraction was omitted and replaced by the column fractions. Reaction products were separated on a 4% polyacrylamide-urea gel. Gels were dried and exposed to x-ray films.

**Purification of TFIIIE**—TFIIIE was purified from HeLa cell nuclear extracts (Dignam et al., 1983). Twelve different preparations of nuclear extract (from approximately 2.5 × 10⁹ cells, 9.8 g of protein, 1000 ml) were pooled and used as starting material for a representative purification. The first three fractionation steps (chromatography on phosphocellulose, DE-52, and HPLC DEAE-5PW columns) were described previously by Flores et al. (1989). TFIIIE activity derived from the DEAE-5PW chromatographic step (80 ml, 44 mg) was concentrated up to 4 ml of using a Mono-S column (HR10/10, Pharmacia) equilibrated with buffer C with 0.1 M KC1. The hound suspension was incubated at room temperature for 30 min. The reaction mixture (25 µl) contained 20 mM Hepes buffer, pH 7.9, 1 mM MgCl₂, 5 mM (NH₄)₂SO₄, 8% (v/v) glycerol, 2% (w/v) polyethylene glycol 8000, 50–80 mM KCl, 5 mM β-mercaptoethanol, 0.2 mM EDTA, and 25 µg/ml of poly(dG-dC)-(dG-dC).-Activated DNA complexes were separated by electrophoresis through a 4% polyacrylamide gel containing 3% (v/v) glycerol using Tris-borate/EDTA buffer, pH 8.2 (40 mM Tris, 40 mM boric acid, 1 mM EDTA) as the running buffer. Electrophoresis was performed at 100 V until the bromophenol blue dye reached the bottom of the gel.

**Other Methods**—Protein kinase assay conditions were as described by Cisek and Gorden (1989) and contained as substrates either histone H1 or casein. In the peptide containing four copies of the heptapeptide repeated present at the C terminus of the largest subunit of RNA polymerase II (Allison et al., 1988), the phosphorylated (Ilo), or the dephosphorylated (Ila) forms of RNA polymerase II.

DNA helicase and topoisomerase assays were performed essentially as described by Sopata et al. (1989) and Darby and Vosberg (1985), respectively.

**RESULTS**

**Purification of TFIIIE**—TFIIIE activity was purified using a transcription assay reconstituted with the general transcription factors, RNA polymerase II, and Ad-MLP. The first three chromatographic steps in the purification of TFIIIE (phosphocellulose, DEAE-cellulose, HPLC-DEAE-5PW) were essentially as described previously by Flores et al. (1989). The purification of TFIIIE was accomplished by chromatography of the DEAE-5PW protein pool on Superose S200, Mono-S, and phenyl-Superose columns, as described under "Materials and Methods" and summarized in Table I. Purification of TFIIIE to apparent homogeneity was accomplished by chromatography of the phenyl-Superose protein pool on a phenyl-Superose microcolumn as described under "Materials and Methods." This procedure resulted in TFIIIE activity coeluting with polypeptides of 34 and 56 kDa (data not shown, see Fig. 2). The polypeptide compositions of the different chromatographic steps of the purification of TFIIIE are shown in Fig. 1.

This highly purified preparation of TFIIIE was transcriptionally active and devoid of DNA-dependent and -independent ATCase, topoisomerase, and DNA helicase activities (data not shown). The TFIIIE preparation was also devoid of detectable protein kinase activity, as measured using different substrates as described under "Materials and Methods" (data not shown).

The Renatured 56-kDa Subunit of TFIIIE Is Sufficient for...
TABLE I

Purification of TFIIE

| Protein                  | mg | Units* | Specific activity |
|-------------------------|----|--------|------------------|
| Nuclear extract         | 9,800 | ND    | 9,800 units/mg protein |
| Phosphocellulose        | 820  | 700,000 | 853 units/mg protein |
| DEAE-cellulose          | 360  | 450,000 | 1,250 units/mg protein |
| HPLC-DEAE 5PW           | 44   | 210,000 | 4,770 units/mg protein |
| Superdex S-200          | 2.4  | 170,000 | 70,830 units/mg protein |
| Mono-S                  | 0.3  | 165,000 | 550,000 units/mg protein |
| Phenyl-Superose         | 0.05 | 90,000  | 900,000 units/mg protein |
| Micro phenyl-Superose   | 0.04 | 83,000  | 1,075,000 units/mg protein |

*One unit is defined as the amount of protein that catalyzed the incorporation of 1 pmol of nucleotide into a specific transcript in 60 min under the conditions specified under "Materials and Methods." The high recovery of activity on the Mono-S chromatographic step is due to the removal of an inhibitor of transcription. This procedure has been repeated four times with similar results. ND, not determined.

**FIG. 1.** Analysis of the polypeptides copurifying with TFIIE activity. Equivalent transcription units from different steps of the TFIIE purification, as indicated at the top of the figure, were loaded onto a 12% SDS-polyacrylamide gel, and the proteins were visualized by silver staining. Protein loaded on lane 4 is derived from the pool of transcriptional activity of the phenyl microcolumn. Migration of molecular weight standards is indicated at the left of the figure. Solid Arrowheads indicate the migration of the 34- and 56-kDa subunits of TFIIE. Lane numbers are shown at the bottom.

**Basal Levels of Activity**—The results presented above suggested that TFIIE activity resided in a heterodimer composed of 34- and 56-kDa subunits. In order to assess the native molecular mass of TFIIE, the highly purified preparation was subjected to gel filtration chromatography on an FPLC-Superdex S200 column. TFIIE activity eluted from the column with an apparent molecular mass of approximately 200 kDa (Fig. 2C). Both the 34- and 56-kDa polypeptides coeluted with TFIIE activity (Fig. 2, compare panels A and B) and appeared to be present in stoichiometric amounts, as determined by densitometric scanning of a protein gel stained with Coomassie Blue (data not shown). Together these results strongly suggest that in solution TFIIE is a tetramer composed of two 56- and two 34-kDa polypeptides.

In order to analyze further whether the 34- and 56-kDa polypeptides copurifying with TFIIE activity were both necessary for transcriptional activity, the polypeptides were separated by chromatography on a reverse phase column (Fig. 3A). The 34- and/or 56-kDa polypeptides were denatured, followed by renaturation as described under "Materials and Methods," and then assayed independently or in combination for their ability to support transcription from the Ad-MLP in

**Fig. 2.** TFIIE activity copurifies with polypeptides of 34 and 56 kDa and has a native molecular mass of approximately 200 kDa. Panel A, TFIIE, micro phenyl-Superose step, was subjected to gel filtration chromatography on an FPLC-Superdex 200 column. The elution volume of TFIIE was determined by assaying 2-μl aliquots of the column fractions in a specific transcription complementation assay for TFIIE activity. Panel B, aliquots (40 μl) of the column fractions were loaded onto an SDS-polyacrylamide gel, and proteins were visualized by silver staining. M, molecular weight standards. Solid arrowheads on the right side indicate the positions of the 34- and 56-kDa polypeptides that coelute with TFIIE activity. Panel C, TFIIE native molecular mass was determined by interpolation from a curve generated from molecular mass standards as described under "Materials and Methods." The arrows on panel A denoted with letters from a to e indicate the elution of molecular weight markers. a, apoferritin, 440 kDa; b, β-amylase, 200 kDa; c, alcohol dehydrogenase, 150 kDa; d, bovine serum albumin, 68 kDa; e, cytochrome c, 14 kDa. Ve, elution volume; Vo, void volume.
a system reconstituted with the other general transcription factors and RNA polymerase II. The 34-kDa polypeptide was unable to support transcription (Fig. 3B, lane 3); however, the 56-kDa polypeptide was capable of low levels of activity (lane 4). The level of activity observed with the 56-kDa polypeptide was approximately 11% of that observed with native TFIIIE (compare lane 4 with 9, respectively). The addition of renatured 34-kDa polypeptide to reaction containing the 56-kDa renatured protein resulted in an approximately 2-fold increase of transcriptional activity (compare lane 4 with lanes 5–7). A different situation was observed when the isolated 34- and 56-kDa polypeptides were renatured together. Under this condition, the level of TFIIIE activity recovered was higher than that observed with the independently renatured polypeptides (Fig. 3B, compare lanes 1 and 2 with lanes 5–7). The recovered TFIIIE activity approached an approximately 40% of the native TFIIIE (compare lane 2 with 9, data not shown). These results may indicate that the association of the two subunits is optimal or that the renaturation of the 34- and/or 56-kDa subunit is better when both polypeptides are renatured together. Similar results were observed when the transcription assay was reconstituted using a partially purified human TFIIID protein or with recombinant human or yeast TFIIID proteins isolated from overproducing Escherichia coli cells. The results presented above indicate that the 56-kDa subunit of TFIIIE is sufficient for basal levels of activity and that the 34-kDa subunit is stimulatory.

The Entry of TFIIIE into the Transcription Cycle Is Dependent on RNA Polymerase II—Previous studies analyzing a partially purified preparation of TFIIIE indicated that TFIIIE interacted with RNA polymerase II (Flores et al., 1989) and was required prior to the initiation of transcription (Reinberg and Roeder, 1987; Flores et al., 1989). We have analyzed, using a gel mobility shift assay and highly purified TFIIIE, whether a specific DNA-protein complex could be isolated that resulted from the entry of TFIIIE into the transcription cycle.

We have demonstrated previously that the entry of RNA polymerase II into the transcription cycle was dependent on a DNA-protein complex formed at the TATA motif and surrounding sequences of the Ad-MLP and included transcription factors IIA, IID, and IIB (DAB complex). In addition, we also showed that the binding of RNA polymerase II to the DAB complex was dependent on TFIIIE. We have observed that a highly purified preparation of TFIIIE (renatured polypeptides) could bind the DABPolF complex (Fig. 4B, lane 1). The association of TFIIIE with the transcription complex resulted in the production of a DNA-protein complex (DABPolFE) migrating more slowly than the DABPolF complex on a native polyacrylamide gel (Fig. 4A, compare lanes 9–12; Fig. 4B, compare lanes 1 and 2). In agreement with previous results demonstrating an interaction of TFIIIE with RNA polymerase II, the entry of TFIIIE into the transcription cycle was dependent on RNA polymerase II. The omission of RNA polymerase II from a DNA-binding assay containing the Ad-MLP and factors IIA, IID, IIF, and IIE resulted in the formation of the DAB complex (Fig. 4B, lane 3). The migration of the DAB complex was also not affected by the presence of TFIIIE (Fig. 4A, lanes 5 and 6). Also, the omission of TFIIIF from the DNA-binding assay resulted in the formation of the DA complex (Fig. 4B, lane 4); the migration of this complex was also not affected by the presence of TFIIIE (Fig. 4A, compare lanes 3 and 4). The formation of the DABPolFE complex was, as expected, also dependent on TFIIID and TFIIIE (Fig. 4B, lanes 5 and 6, respectively).

Interestingly, while we were able to demonstrate that the 56-kDa component of TFIIIE was sufficient for basal levels of transcriptional activity, we found that both the 56- and 34-kDa polypeptides copurifying with TFIIIE activity were required to form the DABPolFE complex (Fig. 4B, lane 1). Independently, neither the 34- nor the 56-kDa polypeptides altered the mobility of the DABPolF complex in a mobility shift assay (data not shown). It is possible that the 56-kDa

**FIG. 3. Renaturation of TFIIIE.** Panel A, TFIIIE micro phenyl-Superose fraction was chromatographed on a reverse phase column as described under Materials and Methods. Aliquots (2 μl) of the column fractions were loaded onto an SDS-polyacrylamide gel, and the proteins were visualized by silver staining. The 34- and 56-kDa polypeptides that copurified with TFIIIE activity are indicated by solid arrowheads. Lane numbers are indicated at the bottom. Panel B, fractions containing the 34- and 56-kDa polypeptides (panel A, lanes 3–5 and 7–9, respectively) were pooled, then denatured and renatured as described under Materials and Methods. Approximately 25% of the material obtained from the pools containing the 34- and 56-kDa polypeptides was combined prior to renaturation (34/56). Different amounts of the 34, 56, and 34/56 renatured material, as indicated at the top of the panel, were added to a specific transcription complementation assay for TFIIIE activity. 2X and 4X denote microliters of protein added. The symbol represents a reaction in which TFIIIE was omitted form the transcription reaction.
subunit binds weakly to the complex and requires the 34-kDa subunit for stable isolation using the mobility shift assay. On the other hand, it is also possible that the 34-kDa component of TFIIIE is necessary for transcription and binding of TFIIIE to the DABPolF complex and is present as a contaminant in the TFIIH protein preparation, the only factor not present in the DNA-binding assay but present in the transcription assay (see "Discussion"). Another less likely possibility is that the 56-kDa polypeptide, in the absence of the 34-kDa component of TFIIIE, can bind stably to the complex; however, any change (in conformation, charge, or mass) induced to the DABPolF complex is such that it cannot be resolved using the mobility shift assay.

The results presented above demonstrate that TFIIIE enters the transcription cycle after RNA polymerase II, in agreement with studies demonstrating an interaction between TFIIIE and RNA polymerase II.

**DISCUSSION**

In this study, we report the purification to apparent homogeneity of TFIIIE. Transcription factor IIE copurified with polypeptides of 34 and 56 kDa and eluted from gel filtration columns performed in high salt (0.8 M KCl) with an apparent mass of approximately 200 kDa. These results suggest that TFIIIE exists in solution as a tetramer composed of two 56- and two 34-kDa polypeptides. However, we observed that the 56-kDa polypeptide was sufficient for low levels of activity and that addition of the 34-kDa polypeptide resulted in stimulation of transcription. Studies analyzing TFIIID, the TATA-binding protein, have indicated that recombinant yeast and the TFIIH protein preparation, the only factor not present in the transcription assay but present in the transcription assay (see "Discussion"). Another less likely possibility is that the 34-kDa component of TFIIIE, capable of transmitting activation through TFIIID and the DNA-binding assay but present in the transcription assay (see "Discussion"). Another less likely possibility is that the 34-kDa component of TFIIIE, can bind stably to the complex; however, any change (in conformation, charge, or mass) induced to the DABPolF complex is such that it cannot be resolved using the mobility shift assay.

The results presented above demonstrate that TFIIIE enters the transcription cycle after RNA polymerase II, in agreement with studies demonstrating an interaction between TFIIIE and RNA polymerase II.

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