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A Novel RNA Polymerase III Transcription Factor Fraction That Is Not Required for Template Commitment*

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Giorgio Dieci, Laura Duimio, Federico Coda-Zabetta, Karen U. Sprague‡, and Simone Ottonello§

From the Institute of Biochemical Sciences, University of Parma, 43100 Parma, Italy and the ‡Institute of Molecular Biology, University of Oregon, Eugene, Oregon 97403

We have identified and partially characterized a novel class III transcription factor fraction (TFIIIE) from yeast nuclear extracts. TFIIIE is functionally distinct from the standard yeast transcription factor fractions, TFIIIB and TFIIIC. It is also different from either of the TFIIIB subfractions, B′ and B″. TFIIIE is essential for specific transcription of both tRNA and 5 S RNA genes, its activity is sensitive to proteinase K, and it exhibits an apparent sedimentation coefficient of 4.0 S when analyzed on glycerol gradients. In the case of a tRNA gene, TFIIIE does not play a role in the formation of stable preinitiation complexes containing TFIIIB and TFIIIC. It is required for single as well as multiple rounds of transcription, however. Thus, TFIIIE is involved in the utilization of stable transcription complexes, but its action is not restricted to reinitiation events.

Multiple transcription factors are required to reconstitute specific *in vitro* transcription of tRNA and 5 S RNA genes by RNA polymerase III. The general view that has emerged from studies on yeast, as well as other organisms, is that two transcription factors, TF[1]IIIB and TFIIIC, plus RNA polymerase III are sufficient for transcription of tRNA genes *in vitro*. This view was initially appealing because it fit with the idea that tRNA gene promoters consist of two discrete elements, the so called A and B boxes (reviewed by Geiduschek and Tocchini-Valentini, 1988). Despite the simplicity of this "two sites/two factors" model, and intense work with the class III transcription systems from various organisms, it has not been possible to reconstitute *in vitro* transcription of class III genes with homogeneously purified components. Work with human and silkworm systems suggested to us an explanation for this difficulty; namely, that the classical factors, TFIIIB and TFIIIC, should be viewed as fractions, each of which contains multiple chromatographically similar, but functionally distinct transcription factors. More highly resolving fractionation methods would be expected to separate the different transcriptionally active polypeptides within these fractions.

Our view of the class III transcription machinery was derived from the fact that fractionation of both human and silkworm systems has revealed the existence of additional transcription factors. Specifically, the TFIIIC fraction from human cells was resolved into two distinct components, TFIIIC1 and TFIIIC2 (Yoshinaga *et al.*, 1987). Both components are required for transcriptional activity in the presence of TFIIIB and polymerase. Fractionation of the silkworm class III transcription machinery yielded three functionally distinct transcription factor fractions, TFIIIC, TFIIID, and TFIIIR, that must be added to polymerase and TFIIIB to reconstitute transcription *in vitro* (Ottonello *et al.*, 1987; Young *et al.*, 1991a). The functional relationship between the silkworm and human fractions has not yet been established. Although the silkworm TFIIIC and TFIIID fractions resemble human TFIIIC1 and TFIIIC2 in that, in each case, the two factors together protect the full intragenic promoter from DNase I digestion (Yoshinaga *et al.*, 1987; Young *et al.*, 1991b), there are clear differences between the silkworm and human components. Whereas human TFIIIC2 by itself binds stably to part of the intragenic promoter, neither of the silkworm fractions, when tested alone, can do so (Dean and Berk, 1988; Ottonello *et al.*, 1987; Young *et al.*, 1991b). In addition, the RNA-containing silkworm factor, TFIIIR, has not been identified in the human system.

The evidence for complexity in the class III transcription machinery in higher eukaryotes contrasts with the picture that has emerged from most of the fractionation studies in yeast. Work in several laboratories has reinforced the idea that in yeast, tRNA transcription is governed by two DNA-binding transcription factors, TFIIIB and TFIIIC (reviewed in Gabrielsen and Sentenac, 1991). Although recent cross-linking experiments reveal multiple polypeptides that contact tRNA gene promoters (Bartholomew *et al.*, 1990, 1991), these individual proteins are thought to associate so tightly that they behave as subunits of a single functional entity (Gabrielsen *et al.*, 1989; Johnson and Wilson, 1989; Parsons and Weil, 1990). No splitting of TFIIIC activity has been observed. Recently, yeast TFIIIB has been subdivided into two functionally distinct components (B′ and B″; Kassavetis *et al.*, 1991). Although these two components are generally considered to be subunits of TFIIIB, this result suggests that the yeast system might be more complex than was previously suspected. This interpretation is supported by the recent discovery that TATA-binding protein (TBP) is part of the standard yeast class III transcription apparatus (Cormack and Struhl, 1992; Schultz *et al.*, 1992).

We wished to learn whether the observed differences between the class III transcription machinery in yeast and higher eukaryotes reflect inherent differences in the nature of these machineries, or whether they could result from the use of different fractionation strategies. Specifically, our goal was to determine whether a fractionation scheme similar to

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§ To whom correspondence should be addressed. Tel.: 39-521-905646; Fax: 39-521-905665.

[1] The abbreviations used are: TF, transcription factor; PMSF, phenylmethylsulfonyl fluoride; BSA, bovine serum albumin; TBP, TATA-box binding protein.

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the one previously applied to the silkworm system would reveal the existence of components other than polymerase, TFIIIB, and TFIIIC when applied to the yeast class III transcription machinery. Here we report the isolation and characterization of such a component. It is a previously unidentified yeast transcription factor that we designate TFIIIE to distinguish it from class III transcription factors that have been reported in yeast or other organisms.

EXPERIMENTAL PROCEDURES

Materials and Buffers—Phosphocellulose (P11) and DEAE-ion exchange chromatography paper (DE81) were from Whatman, hydroxyapatite from Bio-Rad, DEAE-Sephadex and Sephacryl S-300 HR from Pharmacia. Buffer A was 50 mM Tris-HCl, pH 7.9, 20% glycerol (v/v), 0.1 mM EDTA, 0.5 mM benzamidine, 5 mM β-mercaptoethanol, 1 μM leupeptin, and 0.1 mM PMSF. Buffer B was 25 mM Tris-HCl, pH 7.9, 10% glycerol (v/v), 0.1 mM EDTA, 0.5 mM DTT, and 0.5 mM benzamidine. Buffer C was buffer B plus 5 mM $MgCl_2$. Buffer D was 25 mM Tris-HCl, pH 7.9, 20% glycerol (v/v), 10 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, and 0.5 mM benzamidine. Buffer E was 25 mM Tris-HCl, pH 7.9, 25% glycerol (v/v), 0.2 mM EDTA, 0.1 M KCl, 0.5 mM benzamidine, and 5 mM β-mercaptoethanol. Gradient buffer was 25 mM Tris-HCl, pH 7.9, 0.5 M KCl, 0.2 mM EDTA, 0.5 mM benzamidine, 0.5 mM DTT, 0.1 mM PMSF, 1 μM leupeptin, and 1 μM pepstatin A .

Plasmid DNAs—Plasmids pPM16, containing a yeast serine suppressor tRNA gene, pUCLeu3, containing a yeast $tRNA^{Leu3}$ gene and pUC9–5S, containing the yeast 5 S rRNA gene, were from P. A. Weil (Vanderbilt University School of Medicine, Nashville, TN). Plasmid pJD137, containing another member of the $tRNA^{Leu3}$ gene family, and a plasmid containing Leu3Δ20, a $tRNA^{Leu3}$ gene derivative with a 20-base pair deletion in the intervening sequence, were from J. D. Johnson (University of Wyoming, Laramie, WY). Plasmids were all propagated in *Escherichia coli* HB101 and purified by hydroxyapatite chromatography (Shoyab and Sen, 1979).

Preparation and Initial Fractionation of Yeast Nuclear Extracts—Yeast nuclear extracts were prepared from the diploid, protease-deficient, *Saccharomyces cerevisiae* strain RH804C775D (α lys2 leu2 pep4–3/a leu2 trp1::URA3 gal2 pep4–3), which was kindly provided by H. Riezman (University of Basel, Switzerland). Cells were grown in YPD medium (0.5% yeast extract, 1% peptone, and 2% glucose) in a 10-liter fermentor to an OD_{600}= 5–8 corresponding to 1–2 × 10^8 cells/ml. Spheroplast production, lysis, nuclei isolation, and crude extract preparation were as described (Huibregtse *et al.*, 1987). A typical extract from 7 liters of culture had a protein concentration of about 15 mg/ml in a final volume of 30 ml. Nuclear extract in buffer A (15 ml) was made 5 mM in $MgCl_2$, adjusted to a final KCl concentration of 0.26 M, and applied to a 200-ml DEAE-Sephadex A-50 column (internal diameter = 30 mm) equilibrated in buffer C plus 0.26 M KCl. The column, run at a flow rate of 0.7 ml/min, was washed with 1 volume of buffer C + 0.26 M KCl. Bound material was then eluted with 2 column volumes of buffer C + 0.6 M KCl to generate fraction D-600 (Fig. 1). Flow-through material (150 mg of total protein) was applied directly to a 25-ml phosphocellulose column (internal diameter = 20 mm) equilibrated in buffer B + 0.25 M KCl and run at a flow rate of 0.4 ml/min. After washing with 1 column volume of buffer B + 0.25 M KCl, the column was step eluted with buffer B + 0.45 M KCl to generate fraction P-450 and buffer B + 0.6 M KCl to yield fraction P-600 (Fig. 1). Transcript stability assays, which were routinely carried out by incubating preformed $tRNA^{Ser}$ transcripts (5–20 fmol) with individual chromatographic fractions, showed that fraction P-450 contained variable amounts of a ribonuclease activity. Such activity was resistant to protein inhibitors of ribonucleases like RNasin (Promega) and Inhibit-ACE (5′-3′). Various types of commercially available RNAs were thus tested for their ability to competitively inhibit this nuclease activity without interfering with the transcription reaction. 16–23 S ribosomal RNA from *E. coli* (Boehringer Mannheim) was found to be effective. Accordingly, calibrated amounts of *E. coli* rRNA (7–30 μg/ml) were added to all transcription reactions containing fraction P-450. Under conditions of complete transcript stability, fraction P-450 alone was found to be transcriptionally active, but its activity was stimulated up to 3-fold by the addition of fraction D-600. All fractionation procedures were carried out at 4 °C. Fractionation of flow-though material from the DEAE column was carried out without interruption by freezing. Fractions D-600, P-450, and P-600 were stored frozen at −80 °C before subsequent fractionation. Protein concentration in the various fractions was determined by the method of Bradford (1976).

Fractionation of TFIIIB—To separate transcription components in fraction P-450, active fractions derived from 30 ml of crude extract were pooled and concentrated 6-fold by ammonium sulfate precipitation (0.45 g/ml). The resulting sample, resuspended in buffer B + 0.5 M KCl, was then loaded in aliquots (0.5 ml each) on 12.5–30% (v/v) glycerol gradients in gradient buffer. Gradients were poured into polycarbonate tubes (Sorvall, 16 × 80 mm) which were centrifuged for 5.5 h at 66,000 revolutions/minute (200,000 × g at r_{avg}) in a Sorvall T-1270 rotor at 2 °C. Gradients were fractionated from the top into 19 equal sized aliquots (0.5 ml each). Individual fractions were supplemented with acetylated BSA (0.25 mg/ml) and assayed for TFIIIB transcription activity. In at least 10 independent experiments, fractions 3–6 were found to contain TFIIIB activity. TFIIIB-containing fractions were dialyzed against buffer E and stored at −80 °C in small aliquots. As determined by single round transcription assays (see below), a typical preparation of TFIIIB, starting from 30 ml of crude extract, yielded 1.7 pmol of active component and 2.3 mg of total protein in a final volume of 7 ml. These values correspond to a 16-fold increase of specific activity with a 6% recovery of total activity and a 250-fold reduction in total protein with respect to the crude extract.

Variable amounts of a nuclease activity which was present in fraction P-450 partly overlapped with TFIIIB upon gradient fractionation. When an amount of preformed $tRNA^{Ser}$ transcripts (10 fmol), corresponding to approximately one-fourth of the average output of an *in vitro* transcription reaction, was incubated in the presence of fraction TFIIIB under transcription reaction conditions, the recovery of undegraded transcripts varied from 85 to 25%. Transcript stability assays were thus carried out on each preparation of TFIIIB to determine the amount of *E. coli* rRNA that was needed to obtain a 100% recovery of preformed transcripts after incubation in the presence of TFIIIB for 20 min at 20 °C under transcription reaction conditions. This amount of rRNA varied for different preparations of TFIIIB, ranging from 200 to 800 ng/reaction and was always included in transcription reaction mixtures (see below).

Fractionation of TFIIIC and RNA Polymerase III—Fractions 12–19 from the same gradients that yielded fraction TFIIIB were pooled and supplemented with acetylated BSA (0.25 mg/ml). Pooled material was then concentrated 10-fold by ammonium sulfate precipitation (2.5 mg of total protein, 3 ml), dialyzed against buffer D plus 0.075 M KCl, and applied to a DEAE-Sephadex A-25 column (15 ml, 15-mm diameter) in buffer D plus 0.05 M $(NH_4)_2SO_4$ at a flow rate of 0.25 ml/min. After washing with 1 column volume of buffer A plus 0.1 M KCl, bound proteins were eluted with a 100-ml linear gradient of $(NH_4)_2SO_4$ (0.05–0.675 M) in buffer D at a flow rate of 0.35 ml/min. Individual fractions (1 ml each) were supplemented with acetylated BSA (0.25 mg/ml) and assayed for TFIIIC and RNA polymerase III transcription activity (see below). TFIIIC activity eluted at 0.11 M $(NH_4)_2SO_4$, whereas RNA polymerase III activity eluted at 0.35 M $(NH_4)_2SO_4$. TFIIIC-containing fractions were pooled, concentrated 5-fold by ammonium sulfate precipitation (0.45 g/ml), dialyzed against buffer E, and stored in small aliquots at −80 °C. Fractions containing RNA polymerase III activity were treated in the same way, except that they were concentrated approximately 5-fold by ultrafiltration (Amicon, YM5 membranes). As determined by single round transcription assays, a typical preparation starting from 30 ml of crude extract contained 1.4 pmol of TFIIIC in a final volume of 1.4 ml (0.3 mg of total protein) and 2 pmol of RNA polymerase III in a final volume of 3.6 ml (0.3 mg of total protein). In the case of TFIIIC, these values correspond to a 26-fold increase of specific activity with a 1.5% recovery of total activity and a 1800-fold reduction in total protein with respect to the crude extract. In the case of RNA polymerase III, the corresponding values were: 40-fold, 2%, and 2000-fold. In the course of fractionation, RNA polymerase III was detected by a nonspecific polymerization assay which was carried out at 25 °C for 15 min in a 25-μl reaction mixture containing 10 mM Tris-HCl, pH 7.9, 2 mM $MnCl_2$, 100 mM $(NH_4)_2SO_4$, 10% glycerol (v/v), 20 μg/ml α-amanitin, 750 μM ATP, 50 μM UTP, 1.5 μCi of [α-^{32}P]UTP (New England Nuclear, 800 Ci/mmol), and 50 μg/ml poly(d(A-T)). One unit of nonspecific polymerizing activity corresponds to the incorporation of 1 pmol of UMP into RNA/min. The final purification and activity recovery determined by this assay closely matched the corresponding values obtained by measuring single rounds of specific transcription (see "Results").

Fractionation of TFIIIA—TFIIIA activity in fraction P-600 was detected on the basis of the specific requirement of this fraction for 5 S RNA gene transcription. As outlined in Fig. 1, TFIIIA activity in fraction P-600 was further purified by glycerol gradient sedimentation

which was carried out as previously described for fraction P-450. TFIIIA activity was maximum in a gradient fraction corresponding to a sedimentation coefficient value of about 3 S. As a further proof of the identity of gradient-purified material, we found that a highly purified preparation of yeast TFIIIA (kindly supplied by P. A. Weil; Wang and Weil, 1989) could functionally substitute for our fraction in the reconstitution of *in vitro* 5 S RNA gene transcription (data not shown).

Fractionation of TFIIIE—TFIIIE activity in the D-600 fraction was initially identified on the basis of its ability to stimulate $tRNA^{Ser}$ gene transcription catalyzed by fraction P-450. Based on this crude assay, active fractions from the DEAE column were pooled and concentrated 4-fold by ultrafiltration. An aliquot of concentrated material (9 ml, 9 mg of total protein) was then applied to a Sephacryl S-300 HR column (430 ml, 25-mm diameter) in buffer B plus 0.5 M KCl, at a flow rate of 0.3 ml/min. A peak of TFIIIE activity, which eluted at a position corresponding to an apparent molecular weight of 30,000, was detected by transcriptional complementation of partially purified TFIIIB, TFIIIC, and RNA polymerase III. Active fractions were pooled, supplemented with acetylated BSA (0.25 mg/ml), concentrated 4-fold by ultrafiltration, and dialyzed against buffer E before being frozen and stored at −80 °C in small aliquots. As determined by single round transcription assays, a typical preparation of TFIIIE from 30 ml of crude extract yielded 3.9 pmol of active component in a final volume of 11 ml containing 0.55 mg of total protein. Such values correspond to a 90-fold increase of specific activity, an 8% recovery of total activity, and a 1000-fold reduction in total protein with respect to the crude extract. When fraction D-600 was subjected to an analytical rate zonal sedimentation in glycerol gradients, a peak of TFIIIE activity was recovered at a position corresponding to a sedimentation coefficient of about 4.0 S.

Reconstitution of in Vitro Transcription Activity—Transcription assays were carried out in a final volume of 37 µl, up to 30% of which was contributed by fractions of the transcription machinery. The standard assay contained 15 mM Tris-HCl, pH 7.9, 120 mM KCl, 6 mM $MgCl_2$, 10% (v/v) glycerol, 5.4–22 µg/ml of *E. coli* rRNA, 600 µM each of ATP, GTP, and CTP, 25 µM UTP, 5 µCi of $[\alpha\text{-}^{32}P]$UTP (800 Ci/mmol), and 8 µg/ml (80 fmol) of supercoiled plasmid DNA. Transcription reactions were incubated at 20 °C for 20 min and terminated by the addition of 10 µl of a solution containing 4% (v/v) SDS and 5 mg/ml of carrier RNA (Sigma type R-6625 T6). After extraction with an equal volume of phenol-chloroform (1:1), RNA products were ethanol precipitated, resuspended in 25 µl of loading buffer (10 M urea, 0.05% (w/v) each of bromphenol blue and xylene blue cyanol), and resolved by electrophoresis on a 10% polyacrylamide gel as previously described (Ottonello *et al.*, 1987). Radioactive RNA bands were detected by autoradiography and were quantitated by scintillation counting.

During purification, the activities of individual transcription components were detected by complementation of the activities present in appropriate subsets of the full transcription apparatus. The amounts of components used in these assays were determined under conditions of multiple rounds of transcription by titration of individual fractions against each other in the presence of an excess amount of template DNA. TFIIIB was chosen as a reference component, and each of the other fractions was titrated against it. The amount of each fraction used in transcription experiments was that amount needed to saturate the transcription rate when TFIIIB was limiting and the other two components were in excess. As determined by single round transcription assays (see below), these titrated amounts were within the following ranges: 0.6–0.9 fmol of TFIIIB, 1.0–1.5 fmol of polymerase, 1.0–1.5 fmol of TFIIIC, and 0.8–1.0 fmol of TFIIIE.

The purity of individual fractions with respect to the other components of the transcription apparatus was determined for each preparation by a series of transcription reactions in which individual components were systematically omitted (see for example, Fig. 2). These assays showed that the extent of cross-contamination among the four fractions required for tRNA gene transcription was very low. The level of contaminating polymerase, TFIIIB, or TFIIIC in each of our four fractions was undetectable (<1%). Contaminating TFIIIE was also undetectable in fractions TFIIIC and RNA polymerase III, whereas low levels of TFIIIE (3–7%) cross-contaminated fraction TFIIIB.

The experiments reported here were carried out with four independent preparations of transcription components. The stability of all fractions to incubation under the conditions used for transcription and DNA-binding experiments was tested. No deleterious effects of such incubations were detected. Concentrated TFIIIC, TFIIIE, and polymerase in buffer E were stable for at least 12 months at −80 °C. TFIIIB retained full transcriptional activity for about 6 months of storage at −80 °C. Usually, fraction aliquots were used only once after thawing.

Single Round Transcription Assays—Assays in which RNA polymerase III is limited to one round of transcription were carried out as previously described (Kassavetis *et al.*, 1989). Heparin (0.3 mg/ml, from Fluka) was added to transcription complexes that had been stalled at position +12 of the $tRNA^{Ser}$(UAA) gene by omission of CTP (Klekamp and Weil, 1982; Olson *et al.*, 1981). Upon addition of CTP, the 12-mer oligonucleotide was quantitatively converted into a full-length transcript in the presence of heparin (data not shown). In agreement with previous data (Kassavetis *et al.*, 1989), the time required for one round of transcription ranged from 25 to 35 s. Quantitation of the activity of partially purified fractions by single-round transcription assays was carried out under conditions in which template DNA and the complementing fractions were in excess and transcription was linearly dependent upon addition of the component being tested. Similarly, the concentration of individual components in the crude extract was determined by adding increasing amounts of extract to different subsets of the apparatus and measuring transcription activity within the linear range of response.

DNA Binding Assays—Template exclusion assays were carried out under standard *in vitro* transcription conditions following a previously described experimental protocol (Ottonello *et al.*, 1987). Reaction conditions during preincubations were similar to those of the final reaction except for the absence of nucleoside triphosphates. Preincubations were carried out in a 20-µl volume for the experiments reported in Table I and Fig. 8. In both cases the final reaction volume was 37 µl, and additions to the preincubated reaction mixtures were made simultaneously. The various factor fractions, DNA templates, and times of incubation used for these reactions are specified in the legends to Table I and Fig. 8. Conditions of factor limitation appropriate for the template-exclusion experiments were determined by quantitation of individual activities in single round transcription assays. For electrophoretic DNA binding assays, a 250-base pair fragment containing the $tRNA^{Leu3}$ gene was prepared from plasmid pUCLeu3 by digestion with *Pst*I and *Eco*RI (Promega). The fragment was labeled at the *Eco*RI site with $[\alpha\text{-}^{32}P]$dATP and the Klenow fragment of DNA polymerase I (Promega). After labeling and isolation, fragment concentration was determined by ethidium bromide staining of a sample run on a 4% polyacrylamide gel. Binding reaction mixtures contained 1 fmol of TFIIIC, 8 fmol of labeled fragment, and 2.5 µg of poly(d(I-C)) (Boehringer Mannheim) in a final volume of 20 µl. The final concentrations of buffer components were 10 mM Tris-HCl, pH 7.9, 80 mM KCl, 5 mM $MgCl_2$, 10% (v/v) glycerol, and 3 mM dithiothreitol. Reaction mixtures were incubated at 20 °C for 30 min. Bound complexes were then resolved from free DNA by electrophoresis on a 3.5% polyacrylamide gel and quantitated by scintillation counting of excised gel slices as previously described (Young *et al.*, 1991b).

RESULTS

Transcription by Yeast RNA Polymerase III Requires at Least Three General Factors—To study the yeast class III transcription machinery, we developed a fractionation scheme similar to one that has revealed additional components in the *Bombyx mori* system. Using this scheme, we have resolved the yeast machinery necessary for transcription of tRNA genes into four fractions. The fractions were generated by the standard chromatographic methods outlined in Fig. 1. During purification, the activity of RNA polymerase III was followed by its ability to catalyze nonspecific transcription (Ottonello *et al.*, 1987). This direct assay established that fraction 1 contains RNA polymerase III. The activities of transcription factors were detected by their ability to complement the appropriate subsets of the transcription machinery and reconstitute specific transcription of a yeast $tRNA^{Ser}$(UAA) gene. On the basis of the evidence presented below, we conclude that two of the transcription factor fractions we have resolved correspond to the fractions commonly called TFIIIB and TFIIIC. Therefore, we will refer to these fractions (2 and 3) as TFIIIB and TFIIIC, respectively. Since fraction 4 contains a component that has not been described previously we have named it TFIIIE to distinguish it from the class III transcription factor fractions already known in yeast or other systems.

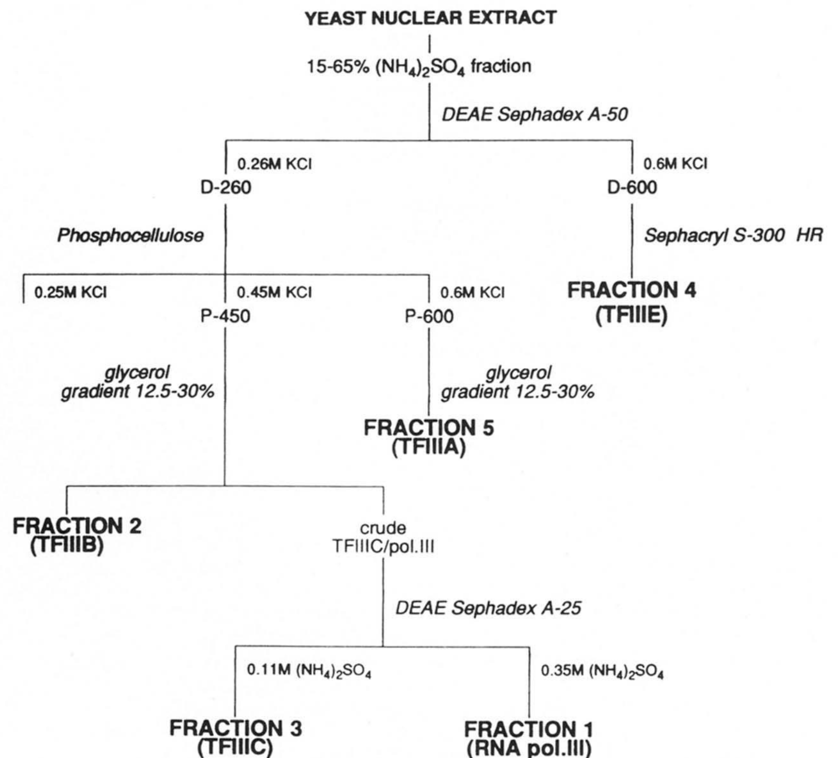

FIG. 1. **Resolution and partial purification of RNA polymerase III transcription components from yeast nuclear extract.**

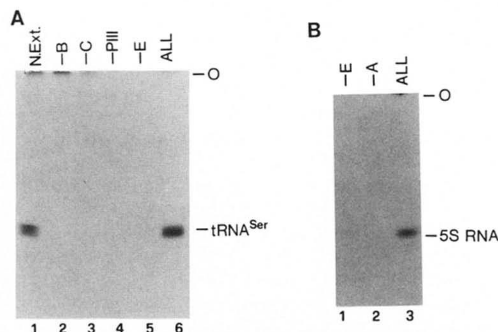

FIG. 2. **Transcription of tRNA and 5S RNA genes requires TFIIIE.** *Panel A*, transcription was carried out in the presence of the fully reconstituted transcription apparatus (*lane 6*) or by a partially reconstituted system lacking TFIIIB (*lane 2*), TFIIIC (*lane 3*), RNA polymerase III (*lane 4*), or TFIIIE (*lane 5*). Transcription reactions contained 80 fmol of the $tRNA^{Ser}$(UAA) gene as template and internally calibrated amounts of each fraction as described under "Experimental Procedures." Transcripts synthesized by a crude nuclear extract (*N.Ext*) (1 μl) are shown for comparison in *lane 1*. The position of transcripts (*$tRNA^{Ser}$*) after resolution on a polyacrylamide gel is shown. The amounts of radioactivity (Cerenkov counts/minute) in $tRNA^{Ser}$ transcripts were (left to right): 3620, 11, 28, 14, 309, 5239. *Panel B*, transcription reactions were carried out as in *panel A* using 130 fmol of a yeast 5 S RNA gene as template. Transcription was catalyzed by a fully reconstituted system supplemented with TFIIIA fraction (*lane 3*) and by subsets of components lacking TFIIIE (*lane 1*) or TFIIIA (*lane 2*). The position of transcripts (5 S RNA) after resolution on a polyacrylamide gel is shown. The amounts of radioactivity (Cerenkov counts/minute) were (left to right) 271, 23, 3515.

We use the word "factors" simply to indicate that the activities in each fraction correspond to functionally distinct components. Since none of the factors has been purified to homogeneity, however, each of these activities could represent the sum of multiple components separable by higher resolution fractionation. Fig. 2*A* shows that all four fractions are required for transcription of a tRNA gene, and Fig. 2*B* shows that the novel factor, TFIIIE, must be added to TFIIIA plus the other three fractions to reconstitute transcription of a 5 S RNA gene. The requirement for TFIIIE on both templates is independent of changes in the concentration (70–175 mM) or type (potassium acetate instead of potassium chloride) of salt in the transcription reaction mixture. Neither is it affected by changes in the reaction temperature (15–30 °C) or the time interval (10–45 min) used for the transcription assay.

We have examined the possibility that the requirement for TFIIIE might be a trivial consequence of dilution of one of the known components, or of irreversible inhibition in the *in vitro* system. To test the possibility that the TFIIIE fraction might actually supply an additional portion of one of the other components, and allow transcription by overcoming a concentration threshold, we measured specific transcription in reactions where individual fractions were systematically omitted and the amounts of the remaining fractions, including TFIIIE, were varied (data not shown). These experiments established that TFIIIE was not functionally interchangeable with any of the other factor fractions. In addition, TFIIIE was distinguished from RNA polymerase III on the basis of direct measurements of the nonspecific transcription rate on a synthetic poly(d(A-T)) template. By this test, our polymerase fraction exhibited a specific activity of 700 units/mg corresponding to a 2000-fold enrichment over the starting extract with respect to total protein. In contrast, TFIIIE catalyzed no detectable transcription by itself and did not alter the nonspecific transcription activity of the RNA polymerase III fraction.

To test the possibility that the TFIIIE fraction might be required merely to provide an inhibitor of a negative activity (a nuclease or a protease, for instance), we used a preincubation protocol (Ottonello *et al.*, 1987) that allowed potential inhibitors to act in the absence of TFIIIE, and then asked whether transcription could be rescued by the subsequent addition of TFIIIE. As shown in Fig. 3*A*, incubation in the absence of TFIIIE does not permanently damage the template or the transcription machinery. Fig. 3*B* rules out the possibility that TFIIIE simply protects the transcript from degradation. Although a small amount (<10%) of the primary $tRNA^{Ser}$ transcript is converted into a shorter product upon incubation

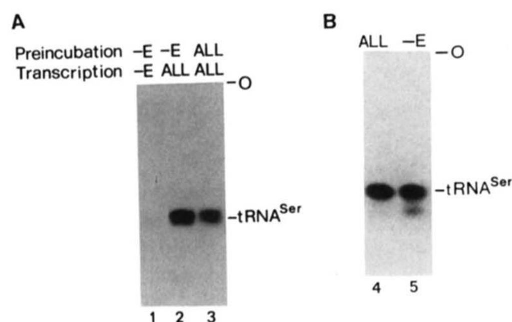

FIG. 3. **TFIIIE does not act by inhibiting an irreversible negative activity.** *Panel A*, preincubation of a subset of the transcription apparatus lacking TFIIIE in the presence of template DNA (tRNA^{Ser} gene) does not impair transcriptional activity. Transcripts in *lane 3* came from reactions in which all four transcription components were present during both preincubation and transcription. Transcripts in *lane 2* came from a reaction in which the TFIIIE fraction was present during transcription only. TFIIIE was never added to the reaction whose products are shown in *lane 1*. Preincubations were carried out under standard *in vitro* transcription conditions for 20 min. Transcription was then initiated by adding [α-^{32}P] UTP to all reaction mixtures and TFIIIE where appropriate. The position of transcripts (*tRNA^{Ser}*) after resolution on a polyacrylamide gel is shown. The amounts of radioactivity (Cerenkov counts/minute) in tRNA^{Ser} transcripts were (left to right) 308, 5030, and 4510. *Panel B*, stability of tRNA^{Ser} transcripts in the absence of TFIIIE. Isolated ^{32}P-labeled tRNA^{Ser} transcripts (4 fmol) were incubated for 20 min under transcription reaction conditions in the presence of all four fractions (*lane 4*) or in the presence of a subset of the apparatus lacking TFIIIE (*lane 5*). The position of the incubated transcripts (*tRNA^{Ser}*) after resolution on a polyacrylamide gel is shown.

in the absence of TFIIIE, the total molar amount of transcript was unaffected.

Since the fractionation method we employed is substantially different from the one typically applied to the yeast transcription machinery, we undertook a direct comparison between our fractions and the known yeast components TFIIIB, TFIIIC, and RNA polymerase III. The comparison was based on two kinds of experiments. First, we examined our fractions for specific properties that have previously been attributed to the TFIIIB, TFIIIC, and RNA polymerase III fractions from yeast. Our TFIIIC fraction exhibits the ability to interact stably with a tRNA gene as assessed either by two-step template exclusion experiments (Table I) or gel retardation assays (Fig. 4). In agreement with previous data, our TFIIIC was the only fraction that was capable of such interaction on its own. Our polymerase fraction displayed the properties (*e.g.* insensitivity to high concentrations of α-amanitin and a salt optimum for nonspecific transcription at 100 mM ammonium sulfate) previously ascribed to the yeast enzyme (Schultz and Hall, 1976). Moreover, the sedimentation coefficients corresponding to the transcriptional activities in our TFIIIB (3 S), TFIIIC (11 S), and polymerase (17 S) fractions are remarkably close to the previously reported values for these components from yeast (3.7 S, 12 S, and 16 S, respectively: Klekamp and Weil, 1987; Stillman *et al.*, 1985; Sentenac and Hall, 1982).

As a second method for comparing fractions, we carried out a systematic exchange in which each of our fractions was tested for its ability to substitute functionally for one of the well characterized standard fractions (kindly supplied by P. A. Weil and G. A. Kassavetis). As shown in Fig. 5*A*, fractions 1–3 from our system were able to substitute effectively for the standard RNA polymerase III, TFIIIB, and TFIIIC fractions, respectively, prepared by the Weil laboratory. Under identical conditions, TFIIIE was unable to substitute for any of these standard fractions. In the reciprocal exchange experiment (Fig. 5*B*), TFIIIE was provided in all of the complementing

TABLE I

Binding of transcription components to tRNA genes

Binding reactions were carried out under standard *in vitro* transcription conditions following a previously described experimental protocol (Ottonello *et al.*, 1987). Leu3 corresponds to the wild type tRNA^{Leu3} gene in plasmid pJD137. The requirement for TFIIIE with this gene is identical to the TFIIIE requirement with the tRNA^{Ser} gene used in other experiments (data not shown). Leu3Δ20 is a derivative of the tRNA^{Leu3} gene with a 20-base pair deletion in the intervening sequence (Raymond and Johnson, 1983). Each reaction mixture contained amounts of Leu3 (120 fmol) and of Leu3Δ20 (70 fmol) that were previously determined to give a 1:1 ratio of transcripts from the two genes under simultaneous addition conditions. The time allowed for binding to gene 1 was 15 min. Each experiment consisted of binding reactions carried out in parallel for every individual fraction and for the complete set of components. The data are expressed as ratios of the transcription rates for gene 1 and gene 2. They represent the average of three experiments which differed by no more than 10% of the mean.

Preferential transcription of gene 1	
Gene 1 = Leu3Δ20	
Leu3Δ20 + all components	4.5
Leu3Δ20 + TFIIIB	1.2
Leu3Δ20 + TFIIIC	4.0
Leu3Δ20 + TFIIIE	0.9
Leu3Δ20 + RNA pol III	1.2
Gene 1 = Leu3	
Leu3 + all components	3.5
Leu3 + TFIIIB	1.0
Leu3 + TFIIIC	3.0
Leu3 + TFIIIE	1.1
Leu3 + RNA pol III	1.2
Simultaneous addition of both genes	
Leu3 + Δ20 + all components	1.0

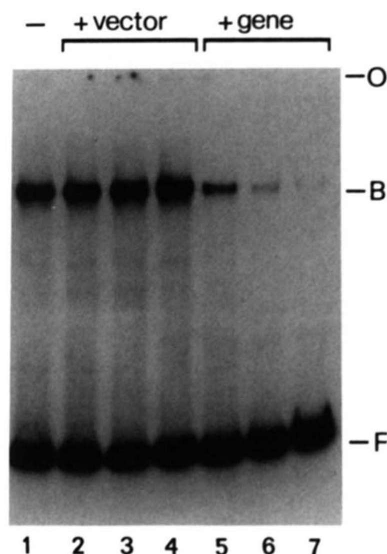

FIG. 4. **Gel Retardation Assay with the TFIIIC fraction.** Bound fragment generated by the TFIIIC fraction in the absence of competitor is shown in *lane 1*. Complexes that are detectable in the presence of increasing amounts of either vector (pBR322) DNA alone or gene-containing (tRNA^{Ser}) DNA are shown in *lanes 2–4* and *5–7*, respectively. Mole ratios of competitor to ^{32}P-labeled fragment were 5, 25, or 100 in *lanes 2–4* and *5–7*, respectively. At these competitor/fragment ratios, gene-containing competitor reduced the number of bound complexes to 24, 10, and 2% of the values observed with no competitor, respectively . At these same ratios, vector DNA did not reduce the number of bound complexes at all. The position of free (*F*) and bound (*B*) DNA fragments are indicated.

subsets of our fractions, and the results confirm the functional equivalence between the standard fractions and our fractions 1–3. The ability of the standard fractions to support transcrip-

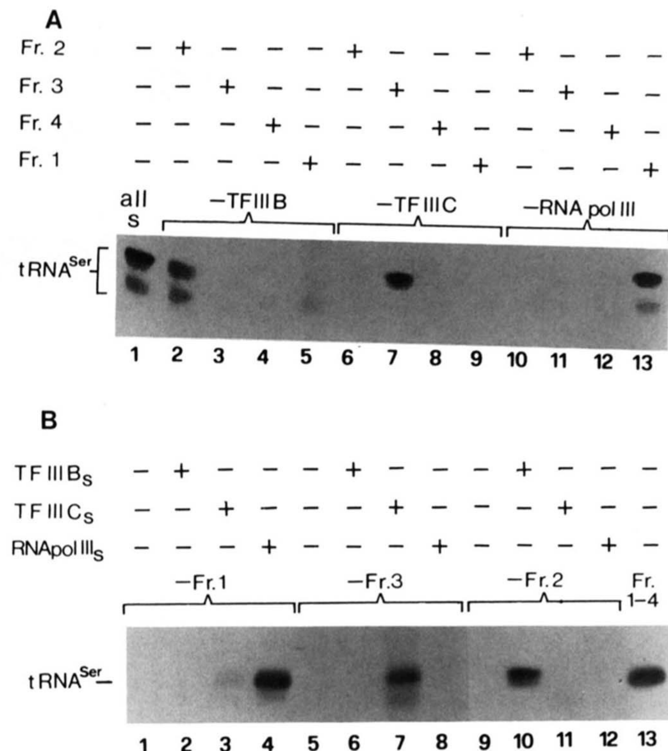

FIG. 5. **Fractions 1–3 substitute functionally for standard fractions containing RNA polymerase III, TFIIIB, and TFIIIC.** *Panel A*, replacement of standard fractions by fractions 1–3. Transcripts in *lane 1* were synthesized by a system reconstituted with standard yeast fractions from the Weil laboratory (*s*) TFIIIB, TFIIIC, and RNA polymerase III. Various subsets of these components lacking TFIIIB (*lanes 2–5*), TFIIIC (*lanes 6–9*), or RNA polymerase III (*10–13*) were supplemented with our four fractions of yeast nuclear extract. Transcripts in *lanes 2, 7,* and *13* came from reactions supplemented with fractions 2, 3, and 1, respectively. *Panel B*, replacement of fractions 1, 2, or 3 by standard fractions. Transcription reactions were carried out in the presence of our four fractions of yeast nuclear extract (*lane 13*) or with various subsets of them lacking fraction 1 (*lanes 1–4*), fraction 3 (*lanes 5–8*), or fraction 2 (*lanes 9–12*), respectively. Fraction 4 was present in every transcription reaction. Transcripts in *lanes 4, 7,* and *10* came from reactions supplemented with standard RNA polymerase III, TFIIIC, and TFIIIB, respectively. All transcription reactions contained 80 fmol of the tRNA^{Ser} gene as template and were carried out under standard conditions (20 min at 20 °C) using internally calibrated amounts of both sets of fractions. The position of transcripts (*tRNA^{Ser}*) after resolution on polyacrylamide gels is shown.

tion without the addition of TFIIIE (Fig. 5*A*) is not surprising since other experiments (not shown), in which TFIIIE was deliberately omitted from the complementing fractions, revealed variable amounts of TFIIIE activity in each of the standard fractions.

The preceding exchanges show that TFIIIE is different from the activities that distinguish the members of one set of standard yeast fractions. Since it is not clear that yeast fractions resolved by different laboratories are identical (for instance, compare Klekamp and Weil, 1987; Kassavetis *et al.*, 1991; Buratowski and Zhou, 1992), we also determined the relation of TFIIIE to the standard yeast fractions prepared by the Geiduschek laboratory (from G. A. Kassavetis). In experiments not shown, we found that TFIIIE activity was present in the Geiduschek TFIIIB fraction (purified to the stage of Cibacron Blue-Sepharose chromatography; Kassavetis *et al.*, 1989) but was absent from both the RNA polymerase III and the TFIIIC fractions. The Geiduschek TFIIIB fraction supplied both TFIIIB and TFIIIE activities in our system. Since this TFIIIB fraction has recently been resolved into two functionally distinct components (B′ and B″; Kassavetis *et al.*, 1991), we considered the possibility that TFIIIE might correspond to one of these subfractions. We addressed this point by determining the ability of either the B′ or B″ fraction to supply TFIIIE activity to our system. As shown in Fig. 6*A*, each of these fractions individually supplies comparable amounts of TFIIIE activity (35–50% of the activity of saturating TFIIIE). As shown in Fig. 6*B*, however, neither fraction alone supplies substantial TFIIIB activity. Thus, TFIIIE is distinct from either of the transcriptional activities that distinguish the B′ and B″ fractions from each other. This conclusion is reinforced by the reciprocal exchange (Fig. 6*C*), which shows that TFIIIE does not supply either B′ or B″ activity.

General Properties of TFIIIE—The experiments described above show that the yeast class III transcription machinery consists of at least four readily separable components. Three of these correspond to previously identified activities: RNA polymerase III, TFIIIB, and TFIIIC. Since the fourth, TFIIIE, is a novel activity, we wished to determine its properties. Our analyses were carried out with a TFIIIE fraction that had been purified at least 90-fold with a 1,000-fold reduction of

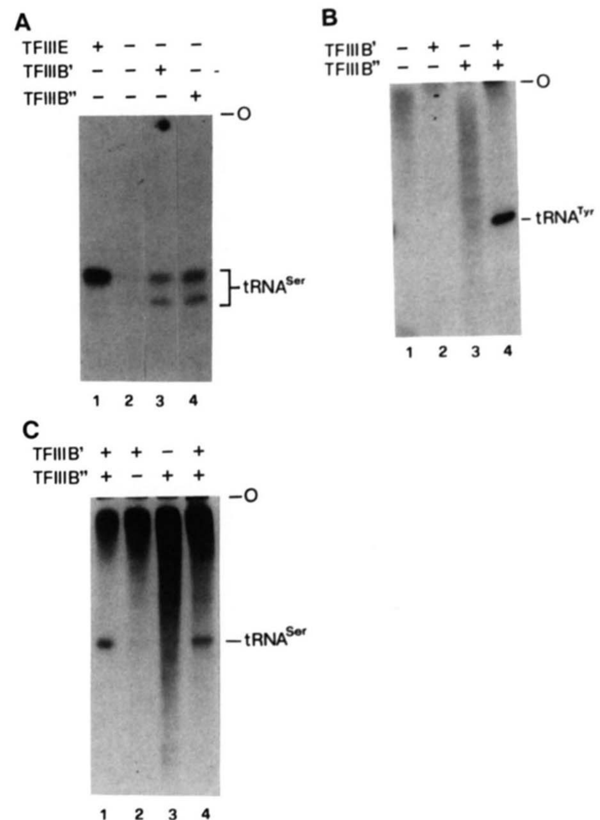

FIG. 6. **TFIIIE is neither B′ nor B″.** *Panel A*, transcription reactions were carried out in the presence of our reconstituted system containing either a saturating amount of TFIIIE (*lane 1*) or no TFIIIE (*lanes 2–4*). Transcripts in *lanes 3* and *4* were synthesized in reaction mixtures supplemented with either B′ (4 µl, *lane 3*) or B″ (4 µl, *lane 4*). The position of transcripts (*tRNA^{Ser}*) after resolution on a polyacrylamide gel is shown; the amounts of radioactivity (Cerenkov counts/minute) were (left to right): 4675, 398, 2020, and 2728. All the lanes shown come from the same exposure of a single gel. *Panel B*, transcription reactions were carried out in the presence of either a complete set of standard fractions from the Geiduschek laboratory (*lane 4*) or subsets of fractions lacking both B′ and B″ (*lane 1*), B″ (*lane 2*) or B′ (*lane 3*). The *SUP4* tRNA^{Tyr} gene carried on plasmid pTZ1 was used as template. Reaction conditions were as described by Kassavetis *et al.* (1989). *Panel C*, transcription reactions were performed in the presence of a subset of our fractions comprising TFIIIC, TFIIIE, and RNA polymerase III, plus B′ and B″ (*lanes 1* and *4*), B′ only (*lane 2*) or B″ only (*lane 3*).

total protein with respect to the starting extract. Size exclusion chromatography on Sephacryl S-300 HR yielded an apparent molecular weight estimate for TFIIIE of 30,000, and glycerol gradient sedimentation gave an apparent sedimentation coefficient for TFIIIE of 4.0 S. Assuming that the active component of TFIIIE is a globular protein of average partial specific volume and density of hydration, this value would correspond to an apparent molecular weight of about 60,000. The reason for the discrepancy between the size estimates obtained by gel filtration and glycerol gradient sedimentation is not yet clear.

A class III transcription factor with chromatographic properties similar to TFIIIE has recently been discovered in *B. mori* and shown to consist of RNA rather than protein (Young *et al.*, 1991a). We were, therefore, particularly interested in establishing the chemical nature of the transcriptional activity in fraction TFIIIE. TFIIIE activity is destroyed by treatment with either heat (65 °C, 10 min) or protease (Fig. 7), but not by treatment with micrococcal nuclease (not shown). Thus, we conclude that TFIIIE activity is conferred by one or more polypeptides.

TFIIIE Is Not Required for Template Commitment—To determine how TFIIIE contributes to transcription activity, we first asked whether it influences the formation of a template committed complex. We used preincubation experiments to determine whether TFIIIE alone binds template stably, or whether it influences template binding by other components. In these experiments, one template (gene 1) was incubated with a limiting amount of TFIIIE, and then a second template (gene 2) was added along with the remainder of the transcription apparatus. After a second incubation during which transcription was allowed to proceed, the RNA products of the two genes were resolved by gel electrophoresis and separately quantitated. Stable binding of a transcriptional component to the first template was revealed by preferential transcription from this template. This kind of experiment has been widely used to demonstrate template commitment by TFIIIC (Lassar *et al.*, 1983; Ruet *et al.*, 1984). Table I shows that although the TFIIIC fraction binds stably to the first template, the TFIIIE fraction does not. In agreement with the results of previous studies (Lassar *et al.*, 1983; Baker and Hall, 1984), neither TFIIIB nor polymerase was able to bind stably in the absence of TFIIIC.

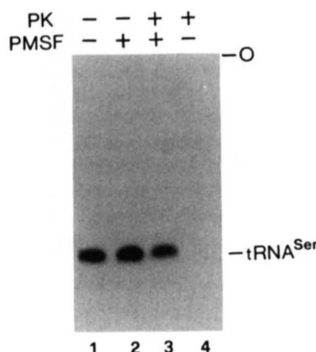

FIG. 7. **Sensitivity of TFIIIE activity to proteinase K.** The TFIIIE fraction was incubated with proteinase K (*lanes 3* and *4*) or with an equal amount of BSA (*lanes 1* and *2*) in the presence of PMSF (*lanes 2* and *3*) or of an equal amount of the PMSF solvent, 40% ethanol (*lanes 1* and *4*). Protease treatment was with 1 mg of proteinase K/50 mg of protein for 3 min at 20 °C. Protease digestion was stopped by the addition of PMSF to the *+PK,−PMSF* reaction (*lane 4*), while an equal amount of 40% ethanol was added to the other reaction mixtures (*lanes 1–3*). The transcriptional activity of the treated fractions was determined in a standard TFIIIE complementation assay. The position of the resulting transcripts (*tRNA^{Ser}*) after resolution on a polyacrylamide gel is shown.

Although the preceding experiments showed that TFIIIE does not bind detectably to template DNA by itself, it is possible that TFIIIE could bind the template in the presence of other factors or could influence the stability of other factor-template complexes. We performed two experiments to determine whether TFIIIE acts in this fashion. One experiment was to determine whether TFIIIE resembles TFIIIB in being able to bind template stably in the presence of TFIIIC, even though it does not bind in the absence of TFIIIC (Kassavetis *et al.*, 1989, 1990). This experiment was done by incubating two different tRNA genes separately with saturating TFIIIC and adding a limiting amount of TFIIIE to one of the incubation mixtures. After further incubation, the two mixtures were combined, the rest of the transcription machinery was added, and transcription was allowed to proceed. Fig. 8, *lane 3*, shows that inclusion of TFIIIE with TFIIIC during the preincubation period did not result in any preferential transcription from the first template. The ratio of gene1/gene2 transcripts was identical to that of a control reaction in which TFIIIE was added to both genes simultaneously (Fig. 8, compare *lanes 1* and *3*).

The second experiment was to determine whether the stability of a TFIIIB-TFIIIC-template complex is affected by TFIIIE. This experiment was done by including both TFIIIB and TFIIIE (along with saturating TFIIIC) in one of the preincubation mixtures to ask whether TFIIIE influences the ability of TFIIIC to sequester TFIIIB. To make this experiment as sensitive as possible, we chose a template, the yeast tRNA^{Leu3} gene, that has a relatively low affinity for TFIIIB. This property is shown in *lane 1* of Fig. 8. When limiting

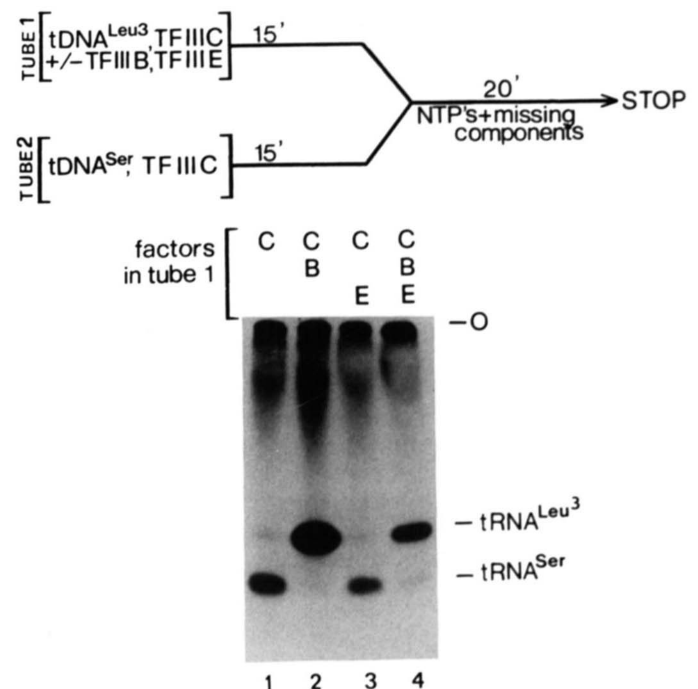

FIG. 8. **TFIIIE is not involved in template commitment.** The tRNA^{Leu3} gene in pUCLeu3 plasmid (150 fmol) was preincubated in the presence of different subsets of transcription components containing a saturating amount of TFIIIC (*lane 1*), TFIIIC plus a limiting amount of TFIIIB (*lane 2*), TFIIIC plus a limiting amount of TFIIIE (*lane 3*), or TFIIIC plus limiting TFIIIB and excess TFIIIE (*lane 4*). The tRNA^{Ser} gene (70 fmol) was separately preincubated with saturating TFIIIC. After a 15-min preincubation at 20 °C, the two reaction mixtures were combined, NTPs and missing components were added, and transcription was allowed to proceed for 20 min under standard conditions. The position of transcripts (*tRNA^{Leu}*, *tRNA^{Ser}*) after resolution on a polyacrylamide gel is shown. The ratios of tRNA^{Leu3}/tRNA^{Ser} transcripts were (left to right) 0.12, 11.7, 0.1, and 10.0.

TFIIIB is allowed to interact simultaneously with calibrated amounts of $tRNA^{Leu3}$ and $tRNA^{Ser}$ gene, the $tRNA^{Leu3}$ gene competes very poorly. The ratio of $tRNA^{Leu3}/tRNA^{Ser}$ transcripts is 0.1. The weak competition by the yeast $tRNA^{Leu3}$ gene has been documented previously and attributed to non-optimal spacing of the intragenic promoter (Baker and Hall, 1984). *Lane 4* of Fig. 8 shows that preincubation of the $tRNA^{Leu3}$ gene with TFIIIB plus TFIIIC and TFIIIE overcomes the disadvantage of the $tRNA^{Leu3}$ gene. The ratio of $tRNA^{Leu3}/tRNA^{Ser}$ transcripts under these conditions is 10. TFIIIE does not contribute to the ability of the $tRNA^{Leu3}$ gene to sequester TFIIIB, however. A comparison of *lanes 2* and *4* shows that the ratio of transcripts is unchanged by the presence or absence of TFIIIE during preincubation with TFIIIB. These experiments do not address the possibility that TFIIIE might influence the rate of association between the other factors and the template, but we have not observed any effects of TFIIIE with a preincubation time as short as 5 min. While carrying out these preincubation experiments, we noticed a reduction of $tRNA^{Leu3}$ gene transcription when this template was preincubated with TFIIIC, TFIIIB, and TFIIIE instead of TFIIIC and TFIIIB only (compare *lanes 2* and *4* in Fig. 8). Since we observed a similar pattern when using the $tRNA^{Ser}$ gene as template (Fig. 3*A*), this effect is not specific for the $tRNA^{Leu3}$ gene. It appears to depend on the presence of TFIIIE in incomplete preincubation mixtures, and it may reflect a requirement for a particular order of factor addition.

TFIIIE Is Required for a Single Round of Transcription—Since TFIIIE is required for transcription under conditions where each template is transcribed multiple (~40) times (see "Experimental Procedures"), our analysis does not distinguish between a role for TFIIIE in the first round of transcription and a role in subsequent rounds only. To distinguish between these possibilities, we asked whether TFIIIE is required under conditions where transcription is limited to a single round. Heparin was used to prevent reinitiation of transcription (Kassavetis *et al.*, 1989), and experiments described under "Experimental Procedures" demonstrated that these conditions did not interfere with transcript elongation or termination on the $tRNA^{Ser}$ gene. Fig. 9 shows that TFIIIE is required for a single round of transcription. TFIIIE must, therefore, participate in the initial formation and/or utilization of active transcription complexes. Its action is not limited to reinitiation events.

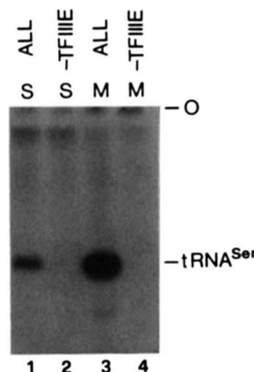

FIG. 9. **TFIIIE is required for a single round of transcription.** TFIIIB (0.8 fmol), TFIIIC (1.5 fmol), RNA polymerase III (1.5 fmol), and, where indicated, TFIIIE (1 fmol) were preincubated for 10 min in the presence of the $tRNA^{Ser}$ gene (80 fmol). A mixture of nucleoside triphosphates lacking CTP was added, and the reaction mixtures were further incubated for 5 min to allow formation of a 12-mer ternary complex. Reaction mixtures were then supplemented with CTP, and transcription was allowed to proceed for 4 min in the presence (*lanes 1* and *2*) or in the absence (*lanes 3* and *4*) of heparin (0.3 mg/ml). The position of transcripts ($tRNA^{Ser}$) after resolution on a polyacrylamide gel is indicated. The amounts of radioactivity (Cerenkov counts/minute) in $tRNA^{Ser}$ transcripts were (left to right) 1183, 81, 8285, and 408.

DISCUSSION

We have identified and partially characterized a novel yeast class III transcription factor designated TFIIIE. This factor is required for transcription of tRNA and 5 S RNA genes, and it does not substitute for the activity of any of the previously described fractions of the yeast class III transcription machinery TFIIIB, TFIIIC, or RNA polymerase III. TFIIIE resembles the known yeast transcription factors in that it is required for single as well as multiple rounds of transcription. It differs from these, however, in its mode of interaction with the template. It does not bind stably to the template either by itself or in the presence of TFIIIC. Neither does it influence the sequestration of TFIIIB by TFIIIC. Moreover, within the time range we have explored, TFIIIE does not influence the rate of association of TFIIIB with TFIIIC and the template.

The existence of TFIIIE fits with other data indicating that polymerase III transcription is more complex than the two sites/two factors model for tRNA transcription originally suggested. These data include the fact that sequences far outside the A and B blocks contribute promoter activity (Wilson *et al.*, 1985; Geiduschek and Tocchini-Valentini, 1988; Young *et al.*, 1991b), and the evidence for additional transcription factors in silkworm (Ottonello *et al.*, 1987; Young *et al.*, 1991a), human (Yoshinaga *et al.*, 1987; Oei and Pieler, 1990; White *et al.*, 1992), and yeast (Kassavetis *et al.*, 1991; Cormack and Struhl, 1992; Schultz *et al.*, 1992) systems. Does TFIIIE resemble any of these novel transcription factors? Since TFIIIE activity is sensitive to proteinase K digestion and to heat, but resistant to nuclease treatment, it must be conferred by protein. Hence, TFIIIE is not the yeast equivalent of the silkworm transcription factor, TFIIIR, that consists entirely of RNA (Young *et al.*, 1991a). Our data do not eliminate the possibility that the yeast class III transcription machinery contains a TFIIIR-like activity, since, if such an RNA were distributed in more than one fraction, our tests would not have detected the requirement for it. The inability of TFIIIE to bind template DNA by itself, or to influence template binding by the other two transcription factors, argues that it probably does not correspond to silkworm TFIIID or to human TFIIIC1 or TFIIIC2. Silkworm TFIIID does not bind template by itself, but it is required for stable binding by either TFIIIB or TFIIIC (Ottonello *et al.*, 1987). Human TFIIIC2 alone binds template stably, and TFIIIC1 is stably sequestered in the presence of TFIIIC2 (Yoshinaga *et al.*, 1987; Dean and Berk, 1988).

An apparently stronger candidate for a TFIIIE homologue in a non-yeast system is the stimulatory activity recently identified in extracts from human cells (Oei and Pieler, 1990). This activity stimulates transcription of 5 S and tRNA genes up to 15-fold above the level observed in its absence. Cross-contamination among fractions could account for the HeLa activity appearing to be stimulatory, rather than absolutely required. Thus, this activity may actually be as critical for transcription in the human system as TFIIIE is in the yeast system. On the other hand, some evidence argues against a simple correspondence between TFIIIE and the HeLa stimulatory factor. The HeLa activity requires the presence of at least two components. Although one of these does not bind DNA, the other does, and its DNA binding activity is essential for the transcriptional stimulatory activity of the fraction as a whole. Thus, although TFIIIE could potentially correspond to the non-DNA binding component in this fraction, at present, there is not an obvious one-to-one correspondence be-

tween TFIIIE and the HeLa stimulatory fraction.

Could TFIIIE correspond to one of the polypeptides previously identified as a subunit of TFIIIB or TFIIIC? The presence of TFIIIE-sized polypeptides among these subunits makes it worth considering such a possibility. Specifically, recent cross-linking studies have revealed polypeptides of 145, 135, 95, and 55 kDa in TFIIIC and 70 and 90 kDa in TFIIIB that are in close contact with the template (Bartholomew *et al.*, 1990, 1991). On the other hand, the fact that our TFIIIC and TFIIIB fractions can substitute functionally for the corresponding standard fractions argues that we have not split either TFIIIB or TFIIIC into multiple activities. Specifically, both the transcriptional activity and the DNA binding activity of the TFIIIC fraction resolved by our fractionation scheme are indistinguishable from the properties used previously to define yeast TFIIIC. In addition, the estimate of the concentration of transcriptionally active TFIIIC molecules obtained by single round transcription assays was identical to the estimate of TFIIIC molecules competent to bind template obtained by gel retardation assays (1 fmol/μl). Thus, it is unlikely that by resolving TFIIIE, we have segregated a functionally important subunit of TFIIIC.

The situation is more complicated in the case of TFIIIB because this factor has recently been divided into two components (B′ and B″), each of which is required for tRNA gene transcription (Kassavetis *et al.*, 1991). These two components separately contain the 70-kDa (B′) and the 90-kDa (B″) polypeptides that have been identified as TFIIIB subunits by DNA cross-linking, and they appear to play distinct roles in the formation of an active transcription complex (Kassavetis *et al.*, 1991; Bartholomew *et al.*, 1991). One might imagine, therefore, that our TFIIIE and TFIIIB fractions correspond to these two TFIIIB subfractions. Direct tests of functional equivalence show, however, that neither B′ nor B″ corresponds individually to the TFIIIE or TFIIIB fractions we or Buratowski and Zhou (1992) have resolved. This result fits with the observed fractionation behavior of TFIIIE and the TFIIIB subfractions. In contrast to B′ and B″, which copurify through a series of chromatographic steps, TFIIIE is readily separable from all of the other class III transcription components at the first step in our fractionation scheme. Furthermore, by our assays, TFIIIE can be present in all of the conventionally prepared fractions of the class III transcription machinery. In the fractions resolved by the Weil laboratory, TFIIIE is not restricted to the TFIIIB fraction. Thus, unlike B′ and B″, TFIIIE does not behave as a tightly associated subunit of TFIIIB.

The TATA-binding protein, TBP, has recently been shown to be an essential component of the yeast class III machinery that transcribes tRNA and 5 S RNA genes (Cormack and Struhl, 1992; Schultz *et al.*, 1992; Kassavetis *et al.*, 1992; Huet and Sentenac, 1992). We therefore tested the possibility that TFIIIE is functionally equivalent to TBP. Using transcriptional complementation as an assay, we find that cloned yeast TBP (kindly supplied by D. Hawley) is unable to substitute functionally for TFIIIE or for any of the other fractions we have resolved. Of course, it is possible that TFIIIE activity requires TBP plus additional polypeptides, perhaps corresponding to TBP-associated factors. Western blots have failed to reveal TBP in the TFIIIE fraction, however.

The arguments presented above indicate that TFIIIE is a class III transcription factor that has not been resolved previously. It is required for an event that can occur after the template has been committed to transcription through the combined action of TFIIIB and TFIIIC. It thus resembles the subset of RNA polymerase II basal transcription factors required for transcript formation, but not for template commitment (Roeder, 1991; Conaway and Conaway, 1991; Flores *et al.*, 1992). Transcription components like TFIIIE may provide the means by which DNA-bound transcription factors are recognized and used by RNA polymerase III. Such components have the potential to regulate polymerase III transcription at the level of stable complex utilization.

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