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Factors involved in specific transcription by mammalian RNA polymerase II: Identification of general transcription factor TFIIG

(*in vitro* transcription/eukaryotic promoter/basal-level transcription/transcription initiation/preinitiation complex)

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ABSTRACT **We have identified and partially characterized another human general transcription factor, TFIIG. Using a reconstituted *in vitro* system comprised of purified RNA polymerase II, TFIIB, TFIID, TFIIE, and TFIIF, we found that TFIIG was essential for specific initiation from all class II genes tested. In this system TFIIA could partially replace TFIIG; however, even at saturating concentrations of TFIIA, addition of TFIIG further stimulated transcription. Since the chromatographic properties of TFIIG differed significantly from those of TFIIA, we concluded that TFIIA and TFIIG are distinct but functionally related transcription factors. Heparin challenge assays showed that TFIIG is required for the assembly of a functional preinitiation complex. However, it must act after template commitment by TFIID, since this step did not require, and was unaffected by, either TFIIG or TFIIA.**

Considerable attention is now being paid to the general transcription factors and steps involved in the initiation of RNA synthesis by RNA polymerase II (reviewed in refs. 1 and 2), since they must represent the ultimate targets for the regulation of transcription initiation by the diverse group of gene- and cell type-specific factors (reviewed in refs. 3–5). In order to elucidate the mechanism of transcription initiation in mammalian cells, various cell-free transcription systems from human cells were developed, including first an S100 extract (6) and, subsequently, both whole cell (7) and nuclear (8) extracts. Following the initial resolution of multiple transcription factors (9), the further fractionation of all these systems (10–18), as well as the application of RNA polymerase II affinity chromatography methods (19, 20), led to the identification and partial or complete purification of an expanded number of ubiquitous factors that are generally required for specific initiation by RNA polymerase II on DNA templates containing minimal or core promoter sequences (usually a common TATA element and associated initiation-site sequences). The human factors originally have been designated TFIIA, TFIIB, TFIID, TFIIE, and TFIIF, and related studies have described analogous factors in rat liver (21–24).

The first step in promoter activation involves TATA-box recognition by TFIID (16, 25), which also plays a major role in the formation of a template-committed complex at the promoter (22, 26–30). These interactions in turn are a prerequisite for the subsequent stepwise assembly of RNA polymerase II and the other general factors (TFIIB, TFIIE, and TFIIF) into a functional preinitiation complex (13, 14, 24, 26, 27, 30–35). Some reports have indicated that the general factor TFIIA is important either for stable binding of TFIID (and template commitment) or for the maximal level of transcription initiation (14, 26, 27, 33), while other reports have failed to show effects of the TFIIA fraction on one or both of these parameters (12, 24, 28–30, 32, 35). Thus, the role of TFIIA in transcription initiation is still poorly understood. In addition, and possibly related, there are reported variations in the number of required factors and in their order of interaction in the various studies of preinitiation-complex assembly and function (14, 24, 27, 33). These apparent discrepancies most likely relate to the use of different assay conditions, heterologous factors, and, perhaps most importantly, impure and cross-contaminated activities.

Clearly, more detailed and definitive studies have been hampered by the lack of a highly purified, reconstituted transcription system, and it remains important to determine, for example, the exact number and mechanisms of action of the general factors. To this end, we have further purified each of the previously identified factors, and in so doing, have discovered a general factor that we have designated TFIIG. TFIIG is essential for transcription in the absence of TFIIA and is involved in the formation of a functional preinitiation complex that is resistant to heparin, but, despite an apparent functional relationship to TFIIA, is not required for template commitment.

MATERIALS AND METHODS

DNA Templates. Plasmids used as templates contained guanine-free cassettes (12) linked downstream of promoter sequences as follows: pML(C_2AT) and pML(C_2AT)Δ−50, sequences from −404 to +10 and from −50 to +10 of the adenovirus type 2 (Ad2) major late (ML) promoter, respectively (12); pML(C_2AT)Δ−53sh, sequence from −53 to +10 of the Ad2 ML promoter (attached to a 50-nucleotide-shorter guanine-free cassette) (30); p2.5, sequence from −59 to −21 of the histone H2B promoter (36); pH4 (C_2AT)Δ−50 and pH4 (C_2AT)Δ−130, sequences from −50 to +8 and from −130 to +8 of the human histone H4 promoter, respectively (37).

Buffers. Buffer A contained 20 mM Tris·HCl (pH 7.9 at 4°C), 10 mM 2-mercaptoethanol, 20% (vol/vol) glycerol, 0.2 mM EDTA, and 0.5 mM phenylmethylsulfonyl fluoride. Buffer B contained 20 mM Tris·HCl (pH 7.8 at 20°C), 10 mM 2-mercaptoethanol, 10% (vol/vol) glycerol, and 0.5 mM EDTA. Buffer C contained 20 mM Hepes (pH 7.9 at 20°C), 10 mM 2-mercaptoethanol, 10% (vol/vol) glycerol, and 0.5 mM EDTA. The concentrations of KCl in all the buffers are indicated in parentheses (M).

Purification of Transcription Factors. HeLa cell nuclear extracts (8) were dialyzed against buffer A to a salt concentration of 0.1 M KCl. The dialyzed sample was loaded onto a phosphocellulose (Whatman P11) column equilibrated with buffer A containing 0.1 M KCl [buffer A(0.1)]. The column was washed with the same buffer, followed by successive elutions with buffers A(0.3), A(0.5), and A(0.85).

The flowthrough fraction of the P11 column contained TFIIA and was loaded onto a DEAE-cellulose (Whatman

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Abbreviations: Ad2, adenovirus type 2; ML, major late; ssDNA, single-stranded DNA.

DE52) column. After two step washes with buffer A(0.1) and buffer A(0.15), the TFIIA activity was eluted with buffer A(0.3). The fractions containing TFIIA activity were rechromatographed on P11 and DE52 columns under the same conditions described above.

The 0.5 M KCl fraction of the P11 column contained TFIIB, TFIIE, and TFIIF (17) and was dialyzed against buffer A to a salt concentration of 0.1 M KCl. The dialyzed sample was applied to a DE52 column equilibrated with buffer A(0.1). TFIIB activity appeared in the flowthrough fraction, while TFIIE and TFIIF activities were coeluted with buffer A(0.3). The flowthrough fraction was applied to a single-stranded DNA (ssDNA)-agarose column equilibrated with buffer A(0.1) and eluted with a 5-column-volume linear gradient of 0.1–0.6 M KCl in buffer A. The peak of TFIIB activity was eluted at 0.3 M KCl. The 0.3 M KCl fraction of the DE52 column, which contained TFIIE and TFIIF, was dialyzed against buffer B until it reached a conductivity equivalent to that of buffer B(0.1). The dialyzed sample was centrifuged at 15,000 × *g* for 5 min, and the supernatant was loaded onto a Bio-Gel DEAE-5PW HPLC column (Bio-Rad; 21.5 × 150 mm) equilibrated with buffer B(0.1). Proteins were eluted with a 100-ml linear gradient from 0.1 to 0.4 M KCl in buffer B at a flow rate of 1.0 ml/min at 20°C. The activities of TFIIE and TFIIF were eluted at 0.2 and 0.3 M KCl, respectively. The TFIIF was further purified with a TSK G5000-PW HPLC column (Beckman; 7.5 × 600 mm) equilibrated with buffer B(0.5) containing 0.01% (vol/vol) Nonidet P-40; the activity was eluted with an apparent molecular mass of 220 kDa. After dialysis against buffer B(0.1), the fraction was loaded onto a DEAE-5PW HPLC column (7.5 × 75 mm) equilibrated with the same buffer and eluted at 0.3 M KCl.

The 0.85 M KCl fraction of the P11 column, containing TFIID and TFIIG, was dialyzed against buffer A(0.1) and applied to a DE52 column equilibrated with the same buffer. The flowthrough fraction of the DE52 column was applied to a Bio-Gel SP-5PW HPLC column (Bio-Rad; 7.5 × 75 mm) equilibrated with buffer C(0.1). The column was washed with the same buffer, followed by successive elutions with buffer A(0.3) and buffer A(0.5). The TFIIG activity was eluted at 0.3 M KCl. Human TFIID was prepared as follows. After dialysis against buffer A(0.1) containing 0.1% (vol/vol) Tween 40, the 0.3 M KCl fraction of the DE52 column was loaded onto a ssDNA-agarose column with the same buffer and eluted by a 5-column-volume linear gradient of 0.1 M to 0.4 M KCl. The TFIID activity was eluted at ≈0.3 M KCl.

Purification of yeast TFIID (38) overexpressed in *Escherichia coli* will be published elsewhere. RNA polymerase II was prepared from HeLa cell nuclear pellets by the method of Bitter (39) and further purified by HPLC on a DEAE-5PW column according to Payne *et al.* (40).

Transcription Assays. Standard transcription mixtures (25 μl) contained 12 mM Tris·HCl (pH 7.9), 40 mM Hepes (pH 8.4), 60 mM KCl, 12% (vol/vol) glycerol, 8 mM $MgCl_2$, 100 μM ATP and UTP, 25 μM [α-^{32}P]CTP, 0.5 μg of pML(C_2AT)Δ−50 (12), unless otherwise indicated, and the following amounts of transcription factors: TFIIA, 8.0 μg; TFIIB, 0.8 μg; yeast or human TFIID, 0.05 or 0.5 μg, respectively; TFIIE, 0.8 μg; TFIIF, 1.0 μg; TFIIG, 0.8 μg; RNA polymerase II, 0.4 μg. Since all templates contained guanine-free cassettes, 0.1 mM 3′-*O*-methylguanosine triphosphate was present in the reaction mixture (12). After incubation for 60 min at 30°C, the activity was measured by autoradiography of polyacrylamide gel-fractionated transcripts.

RESULTS

TFIIG Is Required for *in Vitro* Transcription. To further investigate transcription initiation mechanisms, we undertook a more extensive purification of the general initiation factors (summarized in Fig. 1). In the process of purifying TFIIF, and using purified RNA polymerase II, TFIIB, TFIID, and TFIIE as a complementation system (see Fig. 1), we found that we could no longer reconstitute transcription activity with TFIIF purified past the second round of HPLC on DEAE-5PW. However, transcriptional activity could be restored by adding a flowthrough fraction (asterisk in Fig. 1) from a DE52 column onto which the P11 0.85 M KCl fraction had been loaded (data not shown). These results suggested the existence of a previously unidentified basic factor, which we have designated TFIIG. We further purified this activity by HPLC on an SP-5PW column (see Fig. 1). The transcriptional activity observed with TFIIF purified through the first DEAE-5PW HPLC column (double asterisk in Fig. 1) was not completely dependent upon this fraction of TFIIG (that is, it was stimulated only 2- to 4-fold), because a minor fraction of

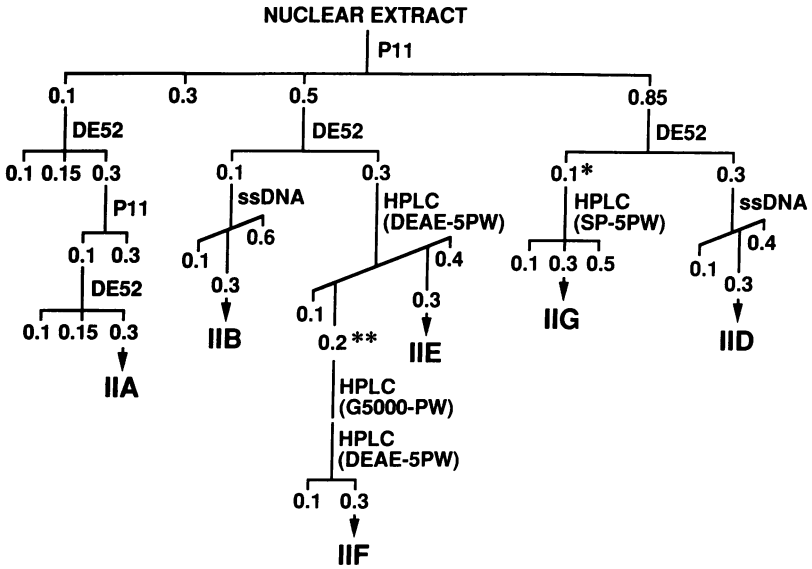

FIG. 1. Schematic representation of the chromatographic separation of HeLa general transcription factors. The samples, in buffers containing 0.1 M KCl, were loaded onto the indicated columns and eluted either stepwise (horizontal lines) or with gradients (oblique lines). The numbers represent the molar concentrations of KCl. For details, see *Materials and Methods*. For explanation of asterisks, see *Results*.

TFIIG copurified with TFIIF through this step (data not shown).

Using this collection of highly purified general factors, we examined the requirements for accurate transcription from the Ad2 ML promoter (Fig. 2*A*). In the absence of the TFIIA fraction, TFIIG was required, along with RNA polymerase II, TFIIB, TFIID, TFIIE, and TFIIF, for the synthesis of a full-length transcript from the Ad2 ML promoter. Thus, TFIIG performs an essential function in this reconstituted transcription system. Importantly, the factors used here showed no appreciable cross-contamination (with the exception noted below) and were free of RNA polymerase II activity, since the full complement of factors was necessary for efficient transcription (Fig. 2).

The complete homologous system without TFIIF or TFIIG gave barely detectable amounts of transcript (Fig. 2*A*, lanes 7 and 8), but this was no longer seen when *E. coli*-expressed yeast TFIID was used instead of human TFIID (Fig. 2*B*, lanes 7 and 8). In all other respects, yeast TFIID acted in the same manner as human TFIID in the reconstituted system (compare Fig. 2 *A* and *B*). Therefore, to avoid any complications arising from low levels of contaminating TFIIF or TFIIG in the human TFIID fraction, we used the yeast TFIID for the following experiments.

TFIIG Further Stimulates TFIIA-Saturated Transcription. The requirement for TFIIA in transcription initiation has been controversial. Various studies have indicated either an absolute requirement (14, 17), a stimulatory effect (26, 27), or no effect at all (25, 32). In our present reconstituted system, TFIIA prepared as shown in Fig. 1 was not required, and had no effect, in the presence of TFIIG (Fig. 2). In the absence of TFIIG, however, transcription was dependent upon added TFIIA, although the activity at a saturating level of TFIIA was 2- to 4-fold less than that observed with TFIIG (Fig. 3, compare lanes 1–5 and 8).

The observation that TFIIA could substitute at least partially for TFIIG led us to question the identity of TFIIG. However, the chromatographic behavior of TFIIG is substantially different from that of TFIIA. As indicated in Fig. 1,

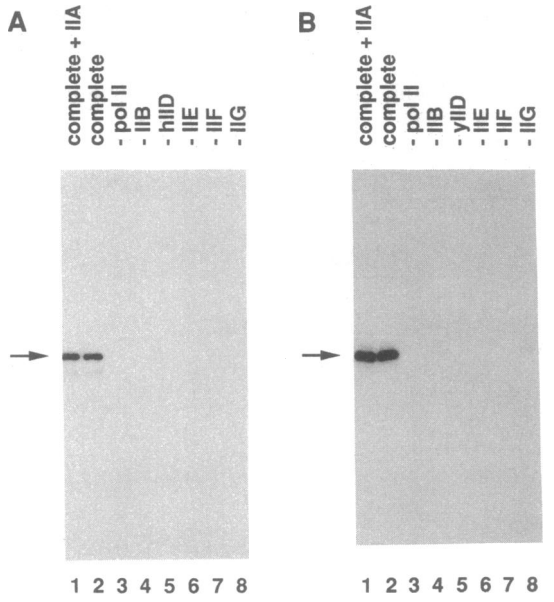

FIG. 2. Factors required for accurate transcription from the Ad2 ML promoter. The complete system (lane 2) contained RNA polymerase II (pol II), TFIIB, TFIIE, TFIIF, TFIIG, and either human TFIID (hIID) (*A*) or yeast TFIID (yIID) (*B*). Additions of TFIIA to the complete systems (lanes 1) or omissions of the factors (lanes 3–8) were as indicated. Arrow indicates the position of the specific transcript from pML(C_2AT)Δ−50.

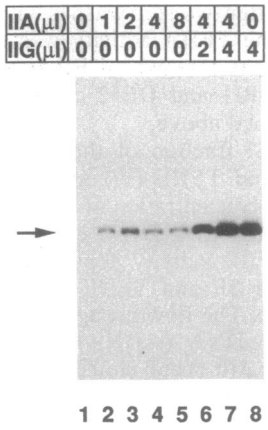

FIG. 3. Further stimulation by TFIIG of TFIIA-saturated transcription. Reaction mixtures contained the indicated amounts of TFIIA (4.0 μg/μl) (lanes 2–5), TFIIG (0.4 μg/μl) (lane 8), or both (lanes 6 and 7), as well as the standard amounts of RNA polymerase II, TFIIB, TFIIE, TFIIF, and yeast TFIID. Arrow indicates the position of the specific transcript from pML(C_2AT)Δ−50.

TFIIG binds to P11 and SP-5PW columns, while TFIIA does not; and TFIIA binds to a DE52 column while TFIIG flows through. These findings suggest that TFIIG is not identical to TFIIA. To further test the interchangeability of TFIIA and TFIIG, we added increasing amounts of TFIIG to a complementation system (−TFIIA, −TFIIG) that had been saturated with TFIIA. As shown in Fig. 3, addition of TFIIG further stimulated transcription even when TFIIA was saturating. This confirms that TFIIA and TFIIG are not functionally equivalent and that TFIIG is a distinct basic transcription factor.

TFIIG Is Required for Transcription of Various Class II Promoters. To determine whether TFIIG is indeed a general transcription factor, we tested it in transcription reactions with various promoters (Fig. 4). TFIIG was essential both for the core Ad2 ML promoter (lanes 1 and 2) and for the core

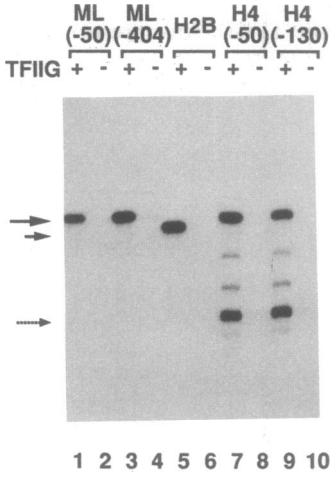

FIG. 4. Requirement of TFIIG for transcription from various promoters. Reaction mixtures contained RNA polymerase II, TFIIB, TFIIE, TFIIF, and yeast TFIID, with or without TFIIG. The DNA templates used were pML(C_2AT)Δ−50 (lanes 1 and 2), pML(C_2AT) (lanes 3 and 4), p2.5 (lanes 5 and 6), pH4(C_2AT)Δ−50 (lanes 7 and 8), and pH4(C_2AT)Δ−130 (lanes 9 and 10). The long and short solid arrows indicate the specific 400- and 380-nucleotide transcripts derived, respectively, from the ML/H4 and H2B promoters. The dashed arrow indicates the transcript started after a TATA-like sequence located in the guanine-free cassette of the H4 promoter. The film for lanes 7–10 was exposed to the dried gel for a 10-fold longer time than the film in lanes 1–6.

histone H2B promoter (lanes 5 and 6), which were transcribed with about equal efficiencies. Although the core histone H4 promoter was transcribed only about 1/10th as efficiently as these promoters, there was still a TFIIG requirement (lanes 7 and 8; see Fig. 4 legend for an explanation of the other bands). As expected, the complete Ad2 ML (lanes 3 and 4) and histone H4 (lanes 9 and 10) promoters showed no upstream sequence-dependent transcription in this system and still required TFIIG. These results show that TFIIG is generally required for basal transcription of various TATA-containing promoters *in vitro*.

TFIIG Is Required for the Formation of a Functional Preinitiation Complex. Previous studies have shown that the previously identified general transcription factors assemble into a preinitiation complex whose formation can be monitored by a heparin challenge assay; addition of heparin after coincubation of factors and DNA has no effect on subsequent initiation and elongation following nucleotide addition, whereas addition of individual factors in the presence of heparin inhibits further complex assembly and function (13, 14, 17).

With the present set of factors, a high level of transcription was observed in the complete reaction without heparin (Fig. 5, lane 1). As expected, transcription was completely abolished in a control reaction with heparin added at time zero (lane 3), whereas a nearly normal level of transcription was observed when heparin was added after a 45-min preincubation of the DNA with RNA polymerase II, TFIIB, TFIID, TFIIE, TFIIF, and TFIIG (lane 2). In confirmation of previous studies of the role of individual factors (13), addition of TFIIB 1 min after heparin addition (i.e., at 46 min), rather than at 0 min, resulted in complete elimination of transcription (lane 5). Similarly, no transcription was observed when TFIIG was added after heparin addition (lane 7), indicating that TFIIG, like the other general factors, is a component of the heparin-resistant preinitiation complex.

TFIIG Is Not Necessary for and Does Not Stimulate Template Commitment. Previous studies have indicated that TFIIA might facilitate TFIID interactions under some assay conditions. Given the further demonstration (above) that TFIIA and TFIIG are in part functionally interchangeable, we used a template challenge assay (24, 26, 27, 30) to investigate whether TFIIG (or TFIIA) participates in formation of a template-committed complex. This analysis was performed with both yeast (lanes 1–7) and human (lanes 8–14) TFIID according to the protocol indicated in Fig. 6. DNA templates I and II each contained the core Ad2 ML promoter, but their respective transcripts were distinguished by the use of guanine-free cassettes of different length.

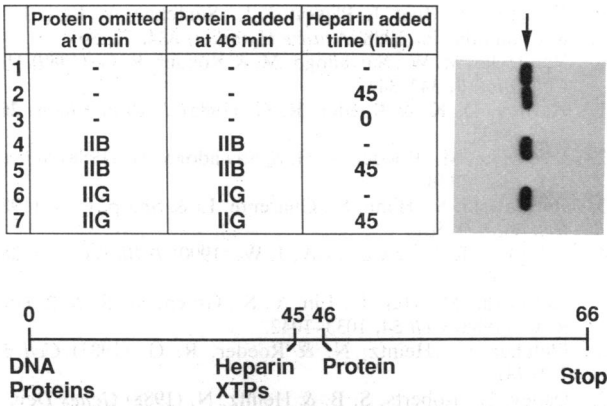

FIG. 5. TFIIG is required for the formation of a heparin-resistant preinitiation complex. Reaction mixtures contained TFIIB, yeast TFIID, TFIIE, TFIIF, TFIIG, and RNA polymerase II. All or a subset of the factors were preincubated for 45 min at 30°C, at which time ribonucleoside triphosphates (XTPs) were added, and the transcription mixture was incubated for an additional 21 min. Heparin (5 μg/ml) was added either at 0 min (lane 3) or at 45 min (lanes 2, 5, and 7). The indicated factors were omitted from the initial incubation and subsequently added at 46 min (TFIIB, lanes 4 and 5; TFIIG, lanes 6 and 7). Arrow indicates the position (electrophoresis from left to right) of the specific transcripts from pML(C_2AT)Δ−50.

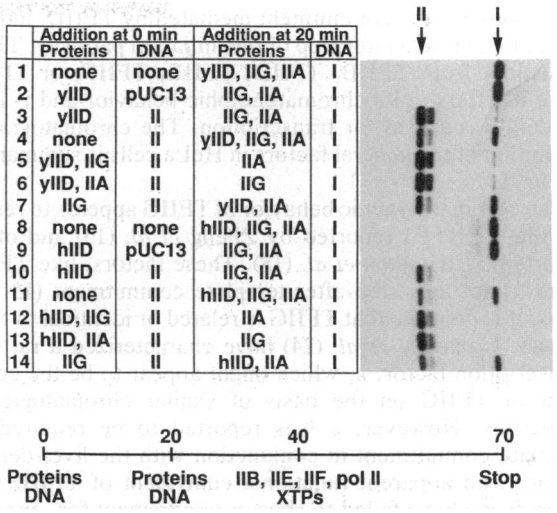

FIG. 6. TFIIG is not involved in template commitment. Reaction mixtures contained TFIIA, TFIIB, yeast or human TFIID (yIID or hIID), TFIIE, TFIIF, TFIIG, and RNA polymerase II. Subsets of the factors were incubated with DNA templates (500 ng of each) under standard reaction conditions according to the protocol at the bottom of the figure. DNA I is pML(C_2AT)Δ−50 and DNA II is pML(C_2AT)Δ−53sh; arrows indicate the corresponding specific transcripts (electrophoresis from right to left). Quantitation of the transcripts by liquid scintillation counting showed that template commitment by TFIID (lanes 3, 5, 6, 10, 12, and 13) resulted in a >10-fold preferential transcription of DNA II relative to DNA I, whereas DNA I and DNA II were transcribed equally in the absence of template commitment (lanes 4, 7, 11, and 14).

When the two templates were mixed simultaneously with the factors (omission of first step), they were transcribed equally well (Fig. 6, lane 4). However, preincubation of the first template with yeast TFIID prevented transcription from a second template, which had not been preincubated with TFIID (lane 3). Yeast TFIID was thus sufficient for formation of an initial, stable preinitiation complex, thereby conferring template commitment. The commitment was not nonspecific, since addition of pUC13 could not titrate out the available TFIID (compare lanes 1 and 2). Preincubation of the first template with TFIIG did not affect transcription from the second template (lane 7), indicating that TFIIG by itself did not commit a template to transcription. Moreover, neither TFIIG nor TFIIA affected the level of commitment mediated by TFIID (lanes 5 and 6), nor did they affect the rate of commitment by TFIID (data not shown). These findings indicate that TFIIG does not participate in the formation of the template-committed complex. We obtained the same results with human TFIID in place of yeast TFIID (lanes 8–14), confirming that yeast and human TFIID act in the same manner in this system.

DISCUSSION

We have identified and partially characterized a general transcription factor, designated TFIIG, by using a highly purified transcription system derived from HeLa cells. TFIIG is essential for specific initiation of transcription of several TATA box-dependent class II genes and is required for the formation of a functional initiation complex. However, this factor neither participates in template commitment

nor stimulates the commitment mediated by TFIID, indicating that it acts at a later step of the initiation pathway. TFIIG is distinct from TFIIB, TFIID, TFIIE, TFIIF, or TFIIA, since it differs in its chromatographic behavior and is independently required for transcription. The chromatographic resolution of the general factors in HeLa cells is summarized in Fig. 1.

The chromatographic behavior of TFIIG appears to resemble that of BTF3 reported by Zheng *et al.* (15) and of FB reported by Kitajima *et al.* (18). These factors, like TFIIG, seem to act at a step after template commitment (15, 18). Thus, it is possible that TFIIG is related or identical to these factors. Conaway *et al.* (24) have characterized a rat liver transcription factor, ε, which might appear to be the equivalent of TFIIG on the basis of similar chromatographic properties. However, ε was reported to be required for template commitment in conjunction with the liver-derived factor τ, an apparent functional equivalent of TFIID (24), whereas we have failed to show a requirement for, or effect of, TFIIG in template commitment. In addition, human TFIIA could partially substitute for TFIIG in the reconstituted human system but not for ϵ in the reconstituted rat liver system (24), although this might be a consequence of the heterologous systems. Thus, TFIIG appears to be functionally different from ε.

The present results also indicate that TFIID, whether yeast or human, is necessary and sufficient for formation of a template-committed complex and that TFIIA, like TFIIG, does not stimulate its formation. These results confirm other studies showing the lack of a TFIIA requirement for template commitment by natural yeast (28, 29) and human (30) TFIID. However, they contrast with earlier studies that showed a requirement for TFIIA (26, 27) and that prompted our analysis of the possible roles of both TFIIA and TFIIG in this process. Although the reason for this discrepancy is not clear, this may be due to differences in the purities of the factors used in these studies or to particular assay conditions.

The observation that TFIIA can partially substitute for TFIIG raises the intriguing possibility that there may be at least two distinct classes of initiation complexes (and corresponding pathways of formation), one containing TFIIA and the other containing TFIIG. The existence of different general transcription complexes could allow for another level of regulation by other factors, since, for example, an upstream factor could stimulate transcription from one complex but not from the other. Further studies using highly purified factors in this reconstituted system, as well as cloned factors as they become available, will aid in elucidating the roles of TFIIG, TFIIA, and the other general factors in the initiation of transcription.

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