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Purification of the *Drosophila* RNA polymerase II general transcription factors

(*in vitro* transcription/TFIIH/TFIIE/TFIIF/TFIID)

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ABSTRACT **We describe a fractionation and purification scheme for the *Drosophila* RNA polymerase II general transcription factors. *Drosophila* TFIIE, TFIIF, TFIIH, and RNA polymerase II have been purified to greater than 50% homogeneity from *Drosophila* embryo nuclear extracts. TFIID has been purified 80-fold and is not significantly contaminated with any of the other general factors. This is the first reported identification and purification of *Drosophila* TFIIH and TFIIE. Further analysis shows that, similar to their mammalian counterparts, *Drosophila* TFIIH is composed of eight polypeptides sized between 30 and 100 kDa, and *Drosophila* TFIIE is composed of two polypeptides sized at 34 and 60 kDa. When all of these fractions are combined with recombinant *Drosophila* TFIIB, a highly purified *in vitro* transcription system is generated that has not previously been available in *Drosophila*. The TFIID fraction can be replaced with recombinant *Drosophila* TBP to give a transcription system that is nearly free of contaminating proteins.**

Detailed molecular and genetic studies of *Drosophila* development have identified complex transcriptional regulatory systems that serve as models for gene regulation in higher organisms (reviewed in refs. 1 and 2). Many of the genes identified in these studies are transcription factors that are thought to act by regulating the basal transcriptional machinery. Identifying the interactions between these regulatory proteins and the RNA polymerase II general transcription factors should thus provide a mechanistic foundation for understanding these complex transcription systems. For example, the subunits of *Drosophila* TFIID [TATA-binding protein (TBP) and the TBP-associated factors] have recently been identified and cloned (3–5). By expressing these subunits as recombinant proteins, subsequent experiments have shown that regulatory factors, such as NTF-1, can activate transcription through interactions with the individual subunits of TFIID (5). A further example is provided by studies on the *Drosophila even-skipped* protein that show that the *even-skipped* protein represses transcription by inhibiting the action of TFIID (6).

Extensive fractionation of human, rat, and yeast extracts has led to the identification of six factors required for initiation of transcription by RNA polymerase II. These factors, termed TFIIB, TFIID, TFIIE, TFIIF, TFIIH, and RNA polymerase II, have been purified to near homogeneity and, in many cases, have been cloned and expressed as recombinant proteins (reviewed in ref. 7). Prior to this study, however, there was no procedure for purifying all six of the *Drosophila* general transcription factors. In particular, *Drosophila* TFIIH had not been identified, and *Drosophila* TFIIE had not yet been purified as an individual activity. Existing procedures only yielded high-purity fractions of RNA polymerase II, TFIIF, and TFIIB (8–11). Although TFIIB and TFIID can be replaced by recombinant *Drosophila* proteins (5, 10), the expression and assembly of the nine subunits of TFIID is difficult (5).

We present a procedure for purifying the *Drosophila* general transcription factors that we developed for studying repression by the *even-skipped* protein (6). Because recombinant *Drosophila* TFIIB and TBP were available when we developed this procedure, our goal was to obtain highly purified and concentrated fractions of *Drosophila* TFIIE, TFIIF, TFIIH, and RNA polymerase II, and a fraction of TFIID that was not contaminated by the other general transcription factors.

MATERIALS AND METHODS

Purification of the RNA Polymerase II General Transcription Factors from *Drosophila* Embryo Extracts. Detailed versions of the following procedure will be provided upon request. *Drosophila* embryo nuclear extract (800 mg) from 0- to 12-hr old embryos (12, 13) was applied to a 30-ml heparin–agarose column (14) pre-equilibrated in 0.1 M KCl in HEMGN (HEMGN contains 25 mM Hepes/K^+, pH 7.6/12.5 mM $MgCl_2$/0.1 mM EDTA/10% glycerol/1 mM DTT/0.01% Nonidet P-40) at a flow rate of 1 ml/min. After washing the column with 0.1 M KCl in HEMGN, the bound protein was eluted with 0.4 M KCl in HEMGN. The heparin–agarose elute (≈340 mg) was fractionated on an 800-ml Sephacryl S-300 gel-filtration (S300) column equilibrated with 0.1 M KCl in HEMGN, as described in ref. 12. The S300 eluate fractions (7 ml each) were separately assayed for TFIID, TFIIF/H, TFIIE, and TFIIB using *in vitro* transcription reactions (described below) lacking the appropriate transcription factor. In the transcription assays, the amount of each fraction used to detect each activity was as follows: 1 μl for TFIID, 0.25 μl for TFIIE, 1 μl for TFIIF/H, 0.5 μl for TFIIB, and 1 μl for RNA polymerase II. TFIID eluted with the column void at 286–320 ml (the peak of the column void is at ≈295 ml). TFIIF and TFIIH eluted at 321–370 ml. TFIIE eluted at 398–468 ml. TFIIB eluted at 501–573 ml. RNA polymerase II eluted from the S300 column in a region that overlaps with TFIID and TFIIF/H. Pooled fractions containing the peak of the TFIID, TFIIF/H, and TFIIE activities were individually purified by MonoQ anion exchange chromatography. Each pool was loaded onto an 8-ml MonoQ 10/10 column (Pharmacia) pre-equilibrated with 0.1 M KCl in HEMGN at a flow rate of 1 ml/min. After washing the column with 40 ml of 0.1 M KCl in HEMGN, bound protein was eluted from the column using a 160-ml gradient from 0.1 M KCl to 0.4 M KCl in HEMGN, followed by a 60-ml gradient from 0.4 M KCl to 0.6 M KCl in HEMGN (for purification of TFIIE, the bound protein was eluted with a 133-ml gradient from 0.1 M KCl to 0.35 M KCl). The eluate was collected in 1.7-ml fractions. To determine which MonoQ

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Abbreviations: TBP, TATA-binding protein; S300, Sephacryl gel filtration.

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fractions contain the peak of transcription factor activity, transcription assays were performed using an appropriate amount of each column fraction (0.25 μl for TFIIH, 0.25 μl for TFIIF, 0.5–1 μl for TFIID, and 0.1 μl for TFIIE). The TFIIH activity eluted at ≈0.20 M KCl in 5–6 fractions. The TFIIF activity eluted at ≈0.24 M KCl in 2–3 fractions. TFIID eluted over a broad region from 0.29 to 0.33 M KCl (about 11–14 fractions). The TFIIE activity eluted in 2–3 fractions at ≈0.25 M KCl. RNA polymerase II, present in both the TFIID and the TFIIF/H S300 pools, eluted from the MonoQ column as a discrete UV absorbance peak at 0.44 M KCl in 1-2 fractions. To confirm that this peak contains RNA polymerase II, transcription assays were performed using 1 μl of protein.

TFIIE, TFIIF, and TFIIH were each further purified on a 1 ml Mono 5/5 column (Pharmacia). The MonoQ fractions containing the appropriate transcription factor activity were diluted with HEMGN to a final salt concentration of 0.1 M KCl in HEMGN. Using a flow rate of 0.13 ml/min, the diluted protein was loaded onto a MonoS 5/5 column (Pharmacia) pre-equilibrated with 0.1 M KCl in HEMGN. After loading the sample, the column was washed with 5 ml of 0.1 M KCl in HEMGN. For TFIIE, the bound protein was eluted with a 14-ml gradient from 0.1 M KCl to 0.35 M KCl in HEMGN. For TFIIF, the bound protein was eluted with a 23.3-ml gradient from 0.1 M KCl to 0.45 M KCl in HEMGN. For TFIIH, the bound protein was eluted with a 16.6-ml gradient from 0.1 M KCl to 0.35 M KCl in HEMGN. During the elution, 0.25 ml fractions were collected. To find the peak of each activity from the MonoS column, *in vitro* transcription assays were performed using 0.08 μl of each fraction for TFIIE, 0.1 μl of each fraction for TFIIF, and 0.05 μl of each fraction for TFIIH. TFIIE eluted in 3–4 fractions at ≈0.21 M KCl. TFIIF eluted at ≈0.32 M KCl in ≈5–6 fractions. TFIIH eluted in ≈6 fractions at ≈0.24 M KCl. These MonoS fractions were highly concentrated, and maximum activity was achieved with ≈0.025 μl of the pooled TFIIE fractions, 0.1 μl of the pooled TFIIF fractions, and 0.1 μl of the pooled TFIIH fractions.

TFIIE and TFIIH were further purified using 24 ml of Superose 6 10/30 gel-filtration column (Pharmacia) or a 120 ml of Superdex 200 16/60 gel-filtration column (Pharmacia). TFIIF was further purified using the Superdex 200 column (Pharmacia). For purifying TFIIE and TFIIH on the Superose 6 column, 200 μl of a MonoS fraction of the appropriate general transcription factor was loaded at a flow rate of 0.1 ml/min onto the column after pre-equilibration with 0.1 M KCl in HEMGN. The eluate is collected in 0.2-ml fractions. The TFIIH peak elutes from the Superose 6 column at 13 ml after sample application, and the TFIIE peak elutes at 15.2 ml after sample application. For purifying TFIIF, TFIIE, and TFIIH on the Superdex 200 column, the column was first equilibrated with 0.2 M KCl in HEMGN, and the appropriate MonoS pool (0.75 to 1.5 ml) was loaded onto the column at a flow rate of 0.5 ml/min. Eluted protein was collected in 1-ml fractions. The TFIIE peak elutes at 63 ml, the TFIIF peak elutes at 60 ml, and the TFIIH peak elutes at 51 ml after sample application.

***In Vitro* Transcription Reactions.** Transcription reactions were performed using conditions described in refs. 6 and 10. For the experiments presented here, a complete *in vitro* transcription reaction contained: 125 ng of alcohol dehydrogenase (Adh) distal template DNA (pDΔ5′-34) (15); 125 ng of Adh proximal template DNA (pPΔ5′-55) (12); 1.5 μl of TFIID, MonoQ fraction; 25 ng of recombinant *Drosophila* TFIIB; 1 μl of TFIIE, Superdex 200 fraction; 0.3 μl of TFIIF, MonoS fraction; 0.25 μl of TFIIH, MonoS fraction; and 1 μl of RNA polymerase II, MonoQ fraction. For the data presented in Figs. 3 and 4, recombinant human TFIIE (3 ng of the 34-kDa subunit, 12 ng of the 56-kDa subunit) was used in place of *Drosophila* TFIIE. Note that in setting up the transcription reactions to assay for TFIIF or TFIIH, if the transcription factors are mixed without TFIID and then are allowed to incubate on ice prior to setting up the rest of the transcription reaction, weak transcription is observed in the absence of TFIIF or TFIIH that is ≈7-fold lower than with a complete set of the general transcription factors. A similar phenomenon is observed when TBP is substituted for TFIID, but only when using supercoiled templates.

Recombinant Proteins. Published methods were used for the expression and purification of recombinant *Drosophila* TFIIB (10), recombinant *Drosophila* TBP (3, 16), and recombinant human TFIIE (17), using modifications described in ref. 6.

RESULTS

Overview of the Fractionation Procedure. Here we present a purification procedure for the *Drosophila* general transcription factors that yields fractions of TFIID, TFIIE, TFIIF, TFIIH, and RNA polymerase II. The availability of purified fractions from other systems allowed us to identify and separate six different transcriptional activities early in the development of this procedure. This permitted us to develop a highly efficient purification strategy that avoided the losses experienced in earlier procedures, where the transcriptional activities were partially divided and lost over a series of steps (8, 11).

Our purification procedure is outlined in Fig. 1. Nuclear extracts are initially fractionated by heparin–agarose and S300 chromatography. The identities of the transcription factors in the S300 column fractions were initially assigned based on their ability to be substituted in a transcription system containing recombinant *Drosophila* TFIIB, recombinant human TFIIE, human fractions containing TFIIF and TFIIH (a gift of Leigh Zawel and Danny Reinberg), and *Drosophila* TFIID and RNA polymerase II (11) (data not shown). Based on transcriptional activity, the eluate fractions from the S300 column are grouped into four pools consisting of TFIID, TFIIF/H, TFIIE, and TFIIB. Both the TFIID and the TFIIF/H S300 pools contain large amounts RNA polymerase II. The TFIID, TFIIF/H, and TFIIE S300 pools are purified using MonoQ anion exchange chromatography to yield individual fractions of TFIID, TFIIE, TFIIF, TFIIH, and RNA polymerase II (Fig. 1; see *Materials and Methods* for details). The TFIIE, TFIIF,

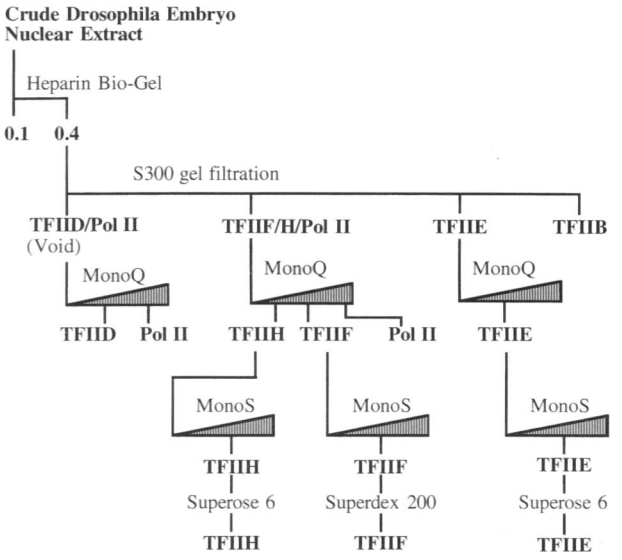

FIG. 1. Overview of the method for purification of the RNA polymerase II general transcription factors from *Drosophila* embryo nuclear extracts. The different columns used during the purification are indicated together with the approximate position at which each factor elutes.

Table 1. Summary of the yield of the *Drosophila* RNA polymerase II general transcription factors from 920 mg of nuclear extract protein

Fraction	mg Protein,	Overall-fold purification	Total activity*	Yield
TFIIH MonoS	0.12	1,600	40,000	21%
TFIIH Superdex	0.06	3,200	40,000	21%
TFIIE Mono S	0.01	14,000	26,000	15%
TFIIE Superdex	0.001	21,000	4,000	2.4%
TFIIF MonoS	0.04	4,900	40,000	21%
TFIIF Superdex	0.004	7,400	6,000	3.2%
Pol II MonoQ	0.76	660	6,600	55%
TFIID MonoQ	4.6	80	22,000	39%

*One activity unit is the amount of protein that yields maximum transcription in the presence of the other five transcription factors (when titering a particular transcription factor activity, the other transcription factors were kept at the volumes described in the *Materials and Methods*).

and TFIIH are further purified using MonoS anion exchange and gel-filtration chromatography (Fig. 1).

An important feature of this purification procedure is that highly purified and concentrated fractions are obtained with minimal loss of activity. Consequently, one preparation purifies enough of these factors for at least 6000 transcription reactions, with the limiting reagent being RNA polymerase II. After the MonoQ column TFIID and RNA polymerase II are purified 80-fold and 660-fold, respectively, with overall yields of 39 and 55% (Table 1). After the MonoS column, TFIIE, TFIIF, and TFIIH are purified 1,500- to 14,000-fold with overall yields of 15 to 21% (Table 1), and are purified to within 25%-50% of homogeneity (data not shown).

Transcription Is Dependent on All of the General Transcription Factors. Presented in Fig. 2*A* are *in vitro* transcription experiments showing that, with reactions containing TBP, transcription is dependent on all six general transcription factors. Removal of any factor eliminates transcription from the *Adh* distal and *Adh* proximal template DNAs (Fig. 2*A*, compare lane 1 with lanes 2–7). In Fig. 2*B*, an experiment performed with TFIID shows that transcription from the two template DNAs only occurs in the presence of all six transcription factor fractions (lanes 8, 14, and 16). Removal of TFIIB, TFIID, TFIIE, RNA polymerase II (Fig. 2*B*, lanes 9–12), or TFIIF (Fig. 2*B*, lane 13) completely eliminates transcription. A slight amount of transcription occurs in the absence of the TFIIH fraction (lane 15).

TFIIH. TFIIH has previously been identified using human, yeast, and rat transcription systems. Presented here is the first biochemical identification of TFIIH from *Drosophila*. As shown in Fig. 3, elution of eight polypeptides from the final gel-filtration column (Fig. 3*A*) closely follows the profile of TFIIH transcriptional activity (Fig. 3*B*). The size distribution of these eight polypeptides ranges from ≈30 to 100 kDa (Fig. 3*A*), closely resembling the size range of the eight polypeptides seen with human and rat TFIIH (18, 19) and the nine polypeptides present in yeast TFIIH (20).

The *Drosophila haywire* gene (21) shares 66% sequence identity with the human *ERCC3* gene, which encodes the largest subunit of human TFIIH (22). Based on this homology, *haywire* has been predicted to encode a subunit of *Drosophila* TFIIH (22). Two features of the data presented in Fig. 3 suggest that this prediction is correct. First, the size of the largest major polypeptide in the TFIIH fraction (Fig. 3*A*) approximately corresponds with the predicted size of the expected molecular mass of the 801-amino acid *haywire* gene product (21). Second, in a western blot, antibodies recognizing bacterially produced *haywire* protein (Fig. 3*C*, lane 8) also recognize the largest TFIIH subunit (Fig. 3*C*, lane 9).

TFIIE. The purification of TFIIE on a Superose 6 column is presented in Fig. 4. A protein gel of fractions from the column demonstrate that elution of two polypeptides (Fig. 4*A*) closely follows the elution of TFIIE transcriptional activity (Fig. 4*B*). These two polypeptides are ≈34 and ≈60 kDa in size, and are similar in size to the subunits of human and yeast TFIIE (17, 23, 24). This fraction is identified as TFIIE because it can be replaced by recombinant human TFIIE in transcription reactions (data not shown).

TFIIF and RNA Polymerase II. TFIIF (Fig. 4*C*, lane 10) and RNA polymerase II (Fig. 4*C*, lane 9) are also highly purified

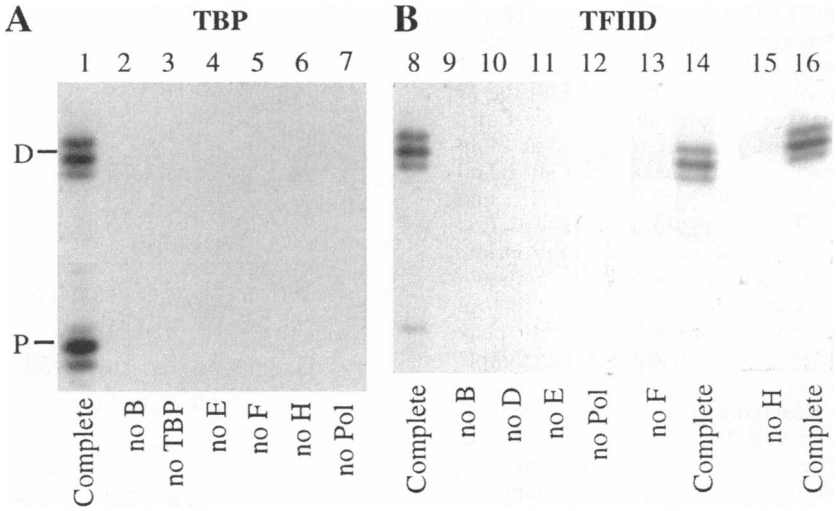

FIG. 2. Transcription is dependent upon the presence of each general transcription factor. S1 nuclease analysis of RNA transcribed from the *Adh* distal and the *Adh* proximal promoters. Indicated to the left of lane 1 are the products for correct transcriptional initiation from the *Adh* distal (D) and *Adh* proximal (P) promoters. (*A*) Transcription reactions performed with TBP. The complete system (lane 1) contains recombinant TBP (300 ng), recombinant TFIIB (25 ng), TFIIE (Superdex 200 fraction), TFIIF (MonoS fraction), TFIIH (MonoS fraction), and RNA polymerase II (MonoQ fraction). In other reactions, individual fractions were removed as indicated (lanes 2–7). (*B*) Transcription reactions performed with TFIID. The complete system (lanes 8, 14, and 16) is identical to that in *A* except that TFIID was substituted for TBP. In other reactions (lanes 9–12, 13, and 15), individual fractions were removed as indicated. Note that with TFIID, transcription from the proximal promoter is weaker than that from the distal promoter. The template DNAs for *B* were supercoiled. The template DNAs for *A* were linearized by digestion with *Nde*I, which cuts at position −270 of pDΔ5′-34 (*Adh* distal) and at position +310 of pPΔ5′-55 (*Adh* proximal). Other details of the transcription reactions are described in the *Material and Methods*.

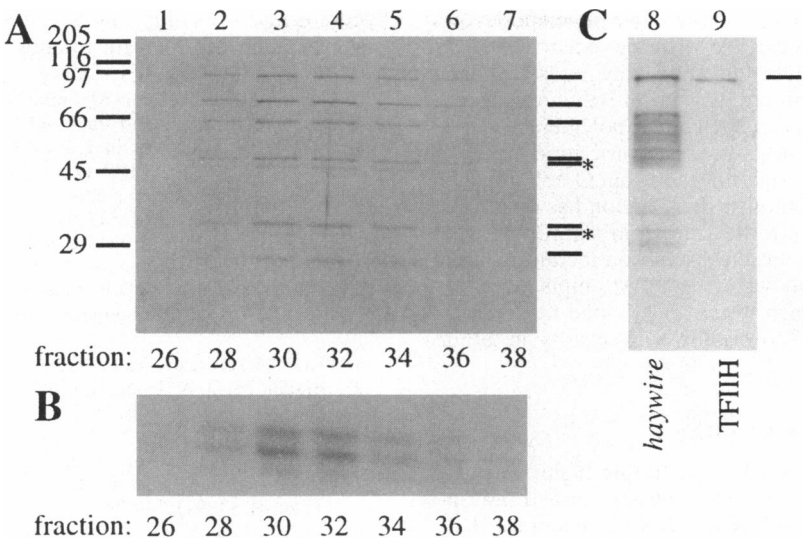

FIG. 3. Final purification of the general transcription factor TFIIH on a 24-ml Superose 6 gel-filtration column. (*A*) Silver-stained SDS/12% polyacrylamide gel of column fractions. Lanes 1–7: each lane contains 20 μl of the column fraction indicated at the bottom of the panel. The migration of molecular mass standards is indicated to the left of lane 1. The lines to the right of lane 7 indicate the eight polypeptides that coelute with TFIIH transcriptional activity. The asterisks indicate two polypeptides that silver stain with a light brown color that is difficult to visualize with black and white photography. (*B*) Assay for TFIIH transcriptional activity. S1 nuclease analysis of RNA produced from the *Adh* distal promoter *in vitro*. Each reaction contained 0.01 μl of each column fraction as indicated by the numbers at the bottom of the panel. (*C*) Western blot analysis performed using an antibody for the *haywire* gene product. Lane 8 contains an extract from bacteria expressing a histidine-tagged haywire fusion protein. Lane 9 contains 5 μl of TFIIH (Superose column fraction 32). The line to the right of lane 9 indicates the expected size of the haywire gene product. The smaller polypeptides recognized by the antibody in lane 8 are probably either proteolyzed fragments of the full-length recombinant *haywire* product or crossreacting bacterial proteins.

with this fractionation procedure. For TFIIF, the elution of two polypeptides present in the TFIIF fraction (Fig. 4*C*, lane 9) closely follows the profile of TFIIF transcriptional activity (data not shown). The sizes of these two polypeptides are consistent with the published sizes of 34 and 85 kDa for the subunits of *Drosophila* TFIIF (9, 25, 26). The sizes of the polypeptides present in the RNA polymerase II fraction (Fig. 4*C*, lane 9) are in agreement with the previously published data for *Drosophila* RNA polymerase II (9, 11, 27) except for two polypeptides of ≈68 and ≈76 kDa in size (indicated by asterisks in Fig. 4*C*) that are consistently present in our preparations of RNA polymerase II (data not shown). Given that previous descriptions of RNA polymerase II in both *Drosophila* and other eukaryotes lack any polypeptides in this

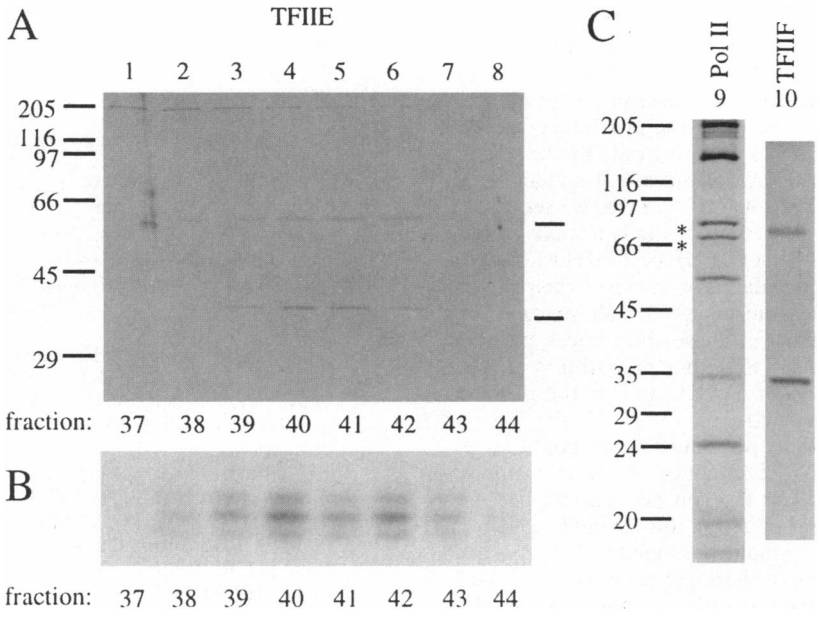

FIG. 4. (*A*) Purification of the general transcription factor TFIIE on a Superose 6 gel-filtration column. Silver-stained SDS/12% polyacrylamide gel of column fractions. Lanes 1 to 8 contain 9 μl of each column fraction, as indicated at the bottom of the panel. The lines to the right of lane 10 indicate the two polypeptides that coelute with TFIIE transcriptional activity. The migration of molecular mass standards are indicated to the left of lane 1. (*B*) Assay for TFIIE transcriptional activity. S1 nuclease analysis of RNA produced from the *Adh* distal promoter *in vitro*. Each reaction contained 0.03 μl of the column fraction indicated by the number at the bottom of the panel. (*C*) Purification of RNA polymerase II and TFIIF. Lane 9: a silver-stained SDS/12% polyacrylamide gel of RNA polymerase II (5 μl) purified on a MonoQ anion exchange column. The asterisks to the left of lane 9 indicate two probable contaminants that coelute with the RNA polymerase II. Lane 10: a silver-stained SDS/12% polyacrylamide gel of TFIIF (20 μl) purified on a Superdex 200 gel-filtration column. The migration of molecular mass standards are indicated to the left of lane 9.

size range, (7, 9, 27), we believe that these two polypeptides are contaminants. Although it may be a fortuitous occurrence that these two polypeptides coelute with RNA polymerase II, these data could also be interpreted to indicate that these two polypeptides physically interact with RNA polymerase II.

TFIID. Compared to the other general transcription factors, TFIID elutes in a broad peak from the MonoQ column (see *Materials and Methods*). Although this fraction has only been purified 80-fold, it appears to be free from significant contamination by the other general transcription factors purified here (discussed above and shown in Fig. 2). Attempts to further purify TFIID resulted in high losses and yielded little purification (data not shown), consistent with results in other systems.

DISCUSSION

We have developed a method for generating highly purified and concentrated fractions of the *Drosophila* general transcription factors TFIIE, TFIIF, TFIIH, and RNA polymerase II, as well as a TFIID fraction that is free from significant contamination by the other general transcription factors. This method is the first to identify *Drosophila* TFIIH and the first to purify *Drosophila* TFIIE as a separate activity. In addition, this fractionation scheme, unlike its predecessors, yields a complete set of the *Drosophila* general transcription factors in a single purification procedure without large losses of activity. Further examination of the TFIIE and TFIIH fractions indicate that the subunit composition of these two factors is similar in number and size to their human, rat, and yeast counterparts. Additional data strongly support the prediction that the *Drosophila haywire* gene encodes the largest subunit of TFIIH (Fig. 4; refs. 21 and 22). Although TFIIB was not purified beyond the S300 column in this procedure due to the availability of recombinant *Drosophila* TFIIB, we predict that the endogenous S300 TFIIB can be easily purified using standard chromatographic techniques. *Drosophila* TFIIA (28) is not accounted for in our procedure because it is not essential for basal transcription.

Previous studies have shown that transcription of some promoters does not require all of the general transcription factors when using supercoiled template DNA (16, 29, 30). Specifically, it has been shown that transcription from one of the promoters used in this paper, the *Adh* proximal promoter, can occur in a minimal system containing only the *Drosophila* proteins TBP, TFIIB, and RNA polymerase II and a subunit of human TFIIF (16). Our results differ in that we see a strict requirement for TFIIE, and we observe only a weak amount of transcription in the absence of TFIIF or TFIIH when using a supercoiled DNA template (data not shown) (when a linear template is used, a strict requirement for all six factors is observed, as seen in Fig. 2*A*). These differences probably reflect our use of *Drosophila* TFIIF, our preparation of RNA polymerase II, the addition of TFIIH, and/or the different concentrations of template DNA.

The purification procedure presented here should be extremely useful in studying the interactions between transcriptional regulatory proteins and the general transcription factors. Although it is likely that all of the *Drosophila* general transcription factors will eventually be cloned and expressed as recombinant proteins, some of these factors consist of a large number of subunits, and reconstituting activity may be difficult. In addition, the recombinant proteins would lack any possible posttranslational modifications. Therefore, it may often prove to be more efficient and practical to purify and use native protein, and certainly it will be essential to use native protein to ensure that any activities observed with recombinant proteins are relevant to endogenous activity.

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