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RNA Polymerase II (Pol II)-TFIIF and Pol II-Mediator Complexes: the Major Stable Pol II Complexes and Their Activity in Transcription Initiation and Reinitiation

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Protein purification and depletion studies were used to determine the major stable forms of RNA polymerase II (Pol II) complexes found in *Saccharomyces cerevisiae* nuclear extracts. About 50% of Pol II is found associated with the general transcription factor TFIIF (Pol II-TFIIF), and about 20% of Pol II is associated with Mediator (Pol-Med). No Pol II-Med-TFIIF complex was observed. The activity of Pol II and the purified Pol II complexes in transcription initiation and reinitiation was investigated by supplementing extracts depleted of either total Pol II or total TFIIF with purified Pol II or the Pol II complexes. We found that all three forms of Pol II can complement Pol II-depleted extracts for transcription initiation, but Pol II-TFIIF has the highest specific activity. Similarly, Pol II-TFIIF has a much higher specific activity than TFIIF for complementation of TFIIF transcription activity. Although the Pol II-TFIIF and Pol II-Med complexes were stable when purified, we found these complexes were dynamic in extracts under transcription conditions, with a single polymerase capable of exchanging bound Mediator and TFIIF. Using a purified system to examine transcription reinitiation, we found that Pol II-TFIIF was active in promoting multiple rounds of transcription while Pol II-Med was nearly inactive. These results suggest that both the Pol II-Med and Pol II-TFIIF complexes can be recruited for transcription initiation but that only the Pol II-TFIIF complex is competent for transcription reinitiation.

An essential step in transcription initiation by RNA polymerase II (Pol II) is the formation of a preinitiation complex (PIC) in which Pol II and the general transcription factors are stably bound at the promoter (22, 29). Formation of the PIC involves the binding of activator, recruitment of chromatin remodeling factors and transcription coactivators, and ultimately the stable recruitment of Pol II and general transcription factors. After initiation of transcription in vitro, most of the general factors, as well as activators and coactivators, can be left behind at the promoter in the Scaffold complex (35, 42). This complex then serves to recruit Pol II and the missing general factors, TFIIB and TFIIF, for multiple rounds of transcription.

One important unresolved question is what forms of Pol II are used for the initiation and reinitiation reactions? Pol II has been isolated both as a purified enzyme and in a number of stable complexes with other factors. Initially, Young and colleagues isolated a complex from *Saccharomyces cerevisiae* termed holoenzyme, containing Pol II, Mediator, most general transcription factors, and the chromatin remodeling factor Swi/Snf (27, 41). Because this complex was suggested to contain most Mediator found in extracts, and since Mediator is essential for basal and activated transcription, it was proposed that this was the predominant form of Pol II used for initiation. However, subsequent studies found little or none of this complex. Instead, Mediator was isolated predominantly in a stable complex with Pol II but lacking general transcription factors, in a complex termed Pol II-Med or holopolymerase (13, 23, 25, 28). Several recent studies have questioned whether the Pol II-Med form is predominantly used for initiation in vivo. Upon gene induction at several regulated yeast promoters, cross-linking of Mediator to promoters was observed to occur before Pol II cross-linking, suggesting that at some promoters Pol II and Mediator are recruited separately (1, 3, 8). In agreement with this, the *Drosophila melanogaster* Mediator can be recruited to heat shock loci in the absence of Pol II (32).

In human cells, Pol II has been isolated in two large complexes, one containing Mediator, Swi/Snf, and acetyl transferases, and the other containing general factors and Mediator (6, 7). However, these human complexes may represent minor forms of Pol II, since most Pol II is not extracted from nuclei during standard nuclear extract preparations (7). Also, in contrast to results seen with *S. cerevisiae*, most Mediator is not stably associated with Pol II in extracts from higher eukaryotes (12, 15, 16, 33, 38–40).

Pol II is also known to bind several general transcription factors, as well as factors involved in transcription elongation. From biochemical studies, Pol II binds TFIIF, TFIIB, and TFIIE, with Pol II-TFIIF displaying the strongest interaction (4, 5, 10, 31). Additionally, substoichiometric amounts of Pol II were found after affinity purification of elongation factors such as Spt 4, -5, or -6, TFIIS (Dst1), and the PAF complex (21, 24, 37). Another complex termed Elongator was originally thought to copurify with Pol II (30), although this has not been observed in more recent studies (19, 21).

To investigate the activity of Pol II complexes in initiation and reinitiation, we first determined the fraction of Pol II found in stable complexes in nuclear extracts from *S. cerevisiae*, and then we assayed the activity of these Pol II complexes in initiation and in a purified reinitiation system in vitro. We found that the Pol II-TFIIF and Pol II-Med complexes were the predominant stable Pol II complexes isolated from ex-

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TABLE 1. Yeast strains used in this work

Strain	Parental strain	Epitope tag
SHY349	BY4705	SRB5-Flag3::KanMx (25a)
SHY384	BJ5460	TFG1-Flag3::KanMx
SHY386	BJ5460	SUA7-Flag3::KanMx
SHY391	BY4705	TFG1-Flag1-TAP::TRP1
SHY407	BY4705	RPB9-Flag1-TAP::TRP1
SHY433	BY4705	Rpb11-Flag3::KanMx
SHY660	BY4705	RPB9-HA6::TRP1

tracts. In extracts under transcription conditions, however, these complexes are dynamic, and Pol II can readily exchange associated TFIIF and Mediator factors. To measure activity in reinitiation, we employed a purified system in which the Pol II-TFIIF and Pol II-Med complexes are stable. We found that only the Pol II-TFIIF complex can function efficiently in transcription reinitiation, demonstrating that the Pol II-Med complex is initiation specific.

MATERIALS AND METHODS

Yeast strains and antibodies. Yeast strains (Table 1) with either a triple Flag tag, a six-hemagglutinin (HA) epitope tag, or a single Flag epitope followed by the tandem affinity purification (TAP) tag at the C terminus of coding regions were derivatives of either BY4705 (2) or BJ5460 (17). These strains were constructed by homologous recombination using the vectors pBS1479, p3FLAG-KanMX, or pYM3, as described elsewhere (11, 20, 36). The following antibodies were used: anti-Flag M2 (Sigma); 8WG16, H14, and H5 anti-HA (Covance); YN18 (Santa Cruz); and anti-Rpb3 rabbit polyclonal antiserum (this work). Other polyclonal antisera have been previously described (34, 42).

Nuclear extracts and WCE. Flag and HA epitope-tagged yeast strains were grown in yeast extract-peptose-dextrose medium, and nuclear extracts were prepared as described on the Hahn laboratory website (www.fhcrc.org/labs/hahn). For whole-cell extract (WCE) preparation, 12-liter cultures of TAP-tagged yeast strains were grown in yeast extract-peptose-dextrose to an A_{600} of 3 to 4 at 30°C. Cells were washed in 150 ml of cold lysis buffer without dithiothreitol (DTT) [50 mM Tris (pH 7.9), 0.4 M $(NH4)_2SO4$, 10 mM $MgSO_4$, 20% glycerol, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM benzamidine, 0.3 μg of leupeptin/ml, 1.4 μg of pepstatin/ml, and 2 μg of chymostatin/ml]. Cells were resuspended in 1.5 times the volume of cells (60 to 90 ml) in lysis buffer plus 2 mM DTT and passed twice through a microfluidizer (model M110-S; Microfluidics Corp.) at ~20,000 lb/in^2 to lyse the cells. Disrupted cells were centrifuged at 10,000 rpm for 15 min using a GSA rotor. The supernatant was centrifuged using an SW28 rotor at 28,000 rpm for 60 min and subsequently dialyzed three times for 2 h each time using 1 liter of dialysis buffer [20 mM HEPES (pH 7.6), 10 mM MgSO4, 1 mM EGTA, 20% glycerol, 75 mM $(NH_4)_2SO_4$, 1 mM DTT, and protease inhibitors]. Protein concentrations were determined using the Bio-Rad protein assay.

Purification of proteins and protein complexes. The Pol II-Med complex was purified from the Srb5-Flag3 strain by Flag affinity and gel filtration chromatography as previously described (25). For this work, only the form with the complete Mediator complex (Pol II-Med) was used for transcription assays.

Pol II, TFIIF, and the Pol II-TFIIF complex were purified from WCE using TAP purification (36) followed by Source 15Q (Amersham) ion-exchange chromatography. For TAP tag purification, 600 mg of WCE (20 to 40 mg/ml) was diluted to 5 mg/ml using dialysis buffer (described above) and adjusted to 0.05% NP-40. The diluted protein was incubated on a roller at 4°C for 10 min, followed by centrifugation at 10,000 × *g* for 10 min to remove insoluble protein. The supernatant was collected and added to washed immunoglobulin G-Sepharose fast-flow beads (Amersham) {10 ml of a 1:1 slurry in buffer A (20 mM HEPES [pH 7.9], 10% glycerol, 0.5 mM EDTA, 300 mM potassium acetate [KOAc], 2 mM DTT, and 0.05% NP-40 with protease inhibitors described above}. After incubation for 2 h at 4°C on a roller, the slurry was centrifuged at 2,000 × *g* for 2 min. The beads were collected and washed five times with 20 ml of buffer A. The washed beads were resuspended in 4 ml of buffer A and 6 U of mutant TEV protease (US Biological) or recombinant mutant protease (26) was added per milligram of starting WCE. This slurry was incubated at 16°C for 4 h on a rotating wheel at slow speed. After protease cleavage, beads were washed three times with 1 volume of buffer A for 5 min at 4°C. The supernatant and washes were pooled. A 1-μl aliquot of 1 M $CaCl_2$ was added per milliliter of pooled protein, followed by dilution with 3 volumes of calmodulin binding buffer (20 mM Tris [pH 8], 300 mM KOAc, 1 mM magnesium acetate [MgOAc], 1 mM imidazole, 2 mM $CaCl_2$, 10% glycerol, 0.01% NP-40, 1 mM PMSF, and 2 mM DTT). Three milliliters of calmodulin agarose beads (Stratagene) washed in calmodulin binding buffer was added, and the sample was incubated on a roller at 4°C for 90 min. After 10 3-ml washes with calmodulin binding buffer, proteins were eluted from the beads twice by incubation at 4°C for 10 min with calmodulin elution buffer (20 mM Tris [pH 8], 300 mM KOAc, 1 mM MgOAc, 1 mM imidazole, 3 mM EGTA, 10% glycerol, 0.01% NP-40, 1 mM PMSF, and 2 mM DTT). The eluted proteins were analyzed by Western blotting.

The above procedure was carried out with extracts from Rpb9 and Tfg1 epitope-tagged strains to isolate Pol II and TFIIF/Pol II-TFIIF, respectively. The TAP-purified Pol II also contained TFIIF, and the Tfg1-TAP-purified fractions contained both TFIIF and Pol II-TFIIF. To isolate factors and complexes free of cross-contamination, the TAP-purified material was fractionated on a Source 15Q column (0.49 ml). The column was initially equilibrated with buffer A containing 0.01% NP-40 and the protease inhibitors Pefablock and leupeptin (Roche). The respective TAP-purified proteins Pol II and Pol II-TFIIF were filtered on Millex-GV (0.22 μm) before loading to the Source 15Q column at 0.1 ml/min at 4°C. The column was washed with 5 column volumes of buffer A at 0.5 ml/min, followed by elution with a 40-column-volume gradient with buffer B (1 M KOAc, 20 mM HEPES [pH 7.9], 20% glycerol, 2 mM DTT, 1 mM EDTA, 0.01% NP-40, Pefablock, and leupeptin). Fractions of 0.4 ml were collected and assayed by Western blotting. TFIIF eluted at ~575 mM KOAc, and Pol II-TFIIF eluted at ~675 mM KOAc. Pol II eluted from 720 to 840 mM KOAc. Fractions containing the isolated Pol II or Pol II complexes were concentrated in Centricon YM-30 and Microcon YM-10 (Millipore) and were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by silver staining.

Nonspecific transcription assays were carried out on equivalent amounts of Pol II, Pol II-Med, and Pol II-TFIIF using sonicated salmon sperm DNA. All forms of Pol II had specific activities in this assay within a twofold range, with Pol II having a 1.8-fold-higher specific activity than either Pol II-Mediator or Pol II-TFIIF.

Immune depletion of factors from nuclear extract. Anti-Flag M2 agarose beads were washed in nuclear extract dialysis buffer [20 mM HEPES-KOH (pH 7.6), 10 mM $MgSO_4$, 1 mM EGTA, 20% glycerol, 75 mM $(NH_4)_2SO_4$, 3 mM DTT, and protease inhibitors] and added to nuclear extract at a ratio of 9 μl of beads to 75 μl of Flag-tagged nuclear extract. After incubation for 3 h on a roller at 4°C, beads were removed by microcentrifugation for 1 min, and an amount of beads equal to that used initially was added to the supernatant. The extracts were incubated for an additional 3 h at 4°C. Beads were removed by centrifugation, and the doubly depleted nuclear extract was stored at −70°C.

For the experiment shown below in Fig. 1, 75 μl of nuclear extract was added to 9 μl of Flag M2 agarose beads (equilibrated as above) and incubated for 3 h at 4°C. Beads were collected and washed four times in wash buffer (20 mM HEPES [pH 7.6], 300 mM KOAc, 10% glycerol, 1 mM EDTA, 0.01% NP-40, 1 mM DTT, and protease inhibitors). Bound proteins were eluted for 1 h in an equal volume of wash buffer plus 0.05 mg of triple Flag peptide (Sigma)/ml at 4°C and eluted a second time for 10 min at 23°C. Elutions were pooled and analyzed by SDS-PAGE.

Quantitative Western analysis. Protein samples were fractionated by SDS-PAGE on 4 to 12% NuPAGE or 3 to 8% Tris-acetate gels (Invitrogen) and transferred to Immobilon (Millipore Corp). Membranes were probed using the indicated primary antibodies or antisera, then visualized with fluorescent dye-labeled secondary antibodies, and quantitated using the LI-COR Biosciences Odyssey infrared imaging system and associated software. For quantitative analysis, multiple dilutions of samples were analyzed and signals were compared to multiple dilutions of starting nuclear extracts.

In vitro transcription. Plasmid transcription and primer extension were performed as described on the Hahn laboratory website. PICs were first formed by incubation of plasmid pSH515 (containing a single Gal4 binding site upstream from a modified HIS4 promoter), Gal4-VP16, depleted or undepleted nuclear extract, and indicated factors for 30 min (34). A 250 μM concentration of nucleoside triphosphates (NTPs) was added for 3 or 40 min to give single-round or multiround transcription. Products were analyzed by primer extension.

The transcription reinitiation assay was performed on Scaffold complexes formed on immobilized HIS4 DNA (42). First, Scaffold complexes were generated using PICs formed with Gal4-VP16 and wild-type nuclear extracts by the addition of ATP (35). After washing, scaffolds were resuspended in 1× transcription buffer with the indicated factors and complexes (see Fig. 8) and 500 ng of *Hae*III-digested *Escherichia coli* DNA competitor. A 600 μM concentration of NTPs was added for either 2 min (single round) or 30 min (multiround transcription). RNA products were analyzed by primer extension, and signals were quantitated by using a PhosphorImager (Molecular Dynamics).

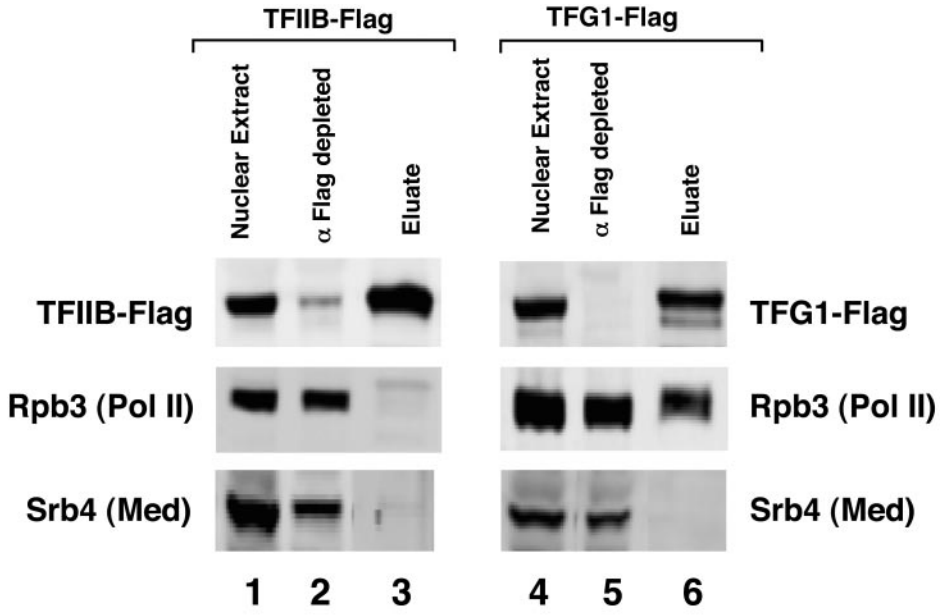

FIG. 1. Affinity purification of TFIIB and TFIIF from Flag-tagged SUA7 and TFG1 strains. Nuclear extracts made from the Flag-tagged strains were incubated with anti-Flag M2-agarose beads. Bound proteins were washed and eluted with triple Flag peptide. Comparable volumes of undepleted nuclear extract, Flag protein-depleted extract, and Flag peptide eluates were analyzed by Western blotting with the indicated antibodies.

Immune precipitation. Anti-HA monoclonal antibody (Covance) was bound to protein G Dynabeads (Dynal) at a ratio of 6 μg of antibody to 1 μl of beads. The antibody was cross-linked to beads with 0.2 M triethanolamine, 0.003% NP-40, and 20 mM dimethyl pimelimidate for 30 min at room temperature. After cross-linking, beads were washed with 1× phosphate-buffered saline, 0.1% bovine serum albumin, and 0.003% NP-40. For analysis of subunit exchange, RPB9-HA nuclear extract was diluted in transcription buffer as described above for a standard plasmid in vitro transcription reaction, except that DNA was omitted and nucleotides (and the ATP regenerating system) were added only where indicated. Pol II-TFIIF or Pol II-Med was also added where indicated. Reactions were incubated for 20 min at room temperature. For immune precipitation of Pol II-HA and associated factors, 12 μl of HA-linked Dynabeads from above was added and incubated for 40 min at room temperature. Beads were washed five times with 1× transcription buffer containing 0.1% bovine serum albumin and 0.05% NP-40. The bound proteins were eluted in 50 mM Tris (pH 8.3), 1 mM EDTA, and 0.15% SDS for 10 min at 37°C. The eluted proteins were analyzed by SDS-PAGE and Western blotting.

Mass spectrometry analysis of the Pol II-TFIIF complex. Proteins in the TAP-tagged purified fraction were denatured by the addition of urea to 8 M, and the sample was diluted fivefold by the addition of TE (20 mM Tris [pH 8.3], 1 mM EDTA) containing 8 M urea. The sample was concentrated, and buffer was exchanged to TE containing 8 M urea in a Centricon 10 device (Amicon). After 37°C incubation for 30 min, proteins were reduced and alkylated by incubation with 2 mM DTT at 37°C for 30 min, followed by incubation with 10 mM iodoacetamide for 20 min at room temperature. The urea concentration was adjusted to 6 M by the addition of TE, and proteins were digested with 100 ng of endoprotease Lys-C (Boehringer Mannheim) for 3 h at 37°C. The urea concentration was adjusted to 1.25 M by addition of TE, and digestion was continued by the addition of modified trypsin (Promega) to a final enzyme-to-substrate ratio of 1:25 (wt/wt) for 16 h at 37°C. The sample was diluted twofold by the addition of 0.1% trifluoroacetic acid (TFA), and the pH was adjusted to ~3 with TFA. The sample was applied to a 1-ml mixed-bed cation-exchange cartridge (Oasis), equilibrated in 0.1% TFA. The column was washed with 5 ml of 0.1% TFA, followed by 5 ml of 80% acetonitrile–0.1% TFA. Peptides were eluted with 1 ml of 10% ammonium hydroxide–90% methanol. The sample was dried under reduced pressure and resuspended in 0.5% acetonitrile–0.1% TFA.

Peptides were analyzed by microcolumn high-performance liquid chromatography (HPLC) coupled to electrospray ionization tandem mass spectrometry as described previously (25). Briefly, a 100- by 365-μm fused silica capillary (Polymetrics Inc.) with a tapered tip was packed to a length of 10 cm with a 5-μm C_{18} reversed-phase resin (Monitor). The sample was directly loaded onto the microcolumn by helium pressurization of the sample in a stainless steel bomb. The mobile phase for HPLC consisted of buffer A (0.5% acetic acid, 0.005% heptafluorobutyric acid) and buffer B (99.5% acetonitrile, 0.5% acetic acid, 0.005% heptafluorobutyric acid). A precolumn split was used to deliver a flow rate of 300 nl/min through the column. The HPLC pump was programmed to ramp solvent B from 0.5 to 40% in 80 min. Electrospray voltage was set at 1.8 kV. Tandem mass spectra were automatically acquired with an ion tap mass spectrometer (ThermoFinnigan LCQ) and searched against an *S. cerevisiae* protein database with SEQUEST (9). Data were filtered and organized using INTERACT (14). Tandem mass spectra for all high-scoring peptides were manually inspected to ensure that the match was correct.

RESULTS

Pol II-TFIIF and Pol II-Med are the predominant stable forms of Pol II complexes in nuclear extracts. Previously, Pol II, TFIIF, and TFIIB were found to dissociate from the promoter upon initiation, leaving the remaining general factors and Mediator in the Scaffold complex (42). For transcription reinitiation, Pol II, TFIIB, and TFIIF must be recruited to the Scaffold complex. Since Mediator is a stable component of the Scaffold complex, it seemed unlikely that Pol II-Med would be the form of Pol II recruited by the Scaffold complex for reinitiation. Instead, Pol II either alone or in a complex with TFIIF and/or TFIIB might be recruited for reinitiation. To measure the fraction of Pol II in complex with either TFIIB or TFIIF, we first characterized the amount of stable Pol II general transcription factor complexes that could be isolated from nuclear extracts. Yeast strains were created with a triple Flag epitope integrated at the C terminus of the gene encoding TFIIB (SUA7) or the large subunit of TFIIF (TFG1). These strains grew normally, and transcription activity in vitro was identical with that of extracts made from wild-type strains. TFIIB and TFIIF were isolated from nuclear extract by Flag affinity purification. Immune complexes were washed with 300 mM potassium acetate buffer, bound protein was eluted with Flag peptide, and fractions were assayed for the copurification

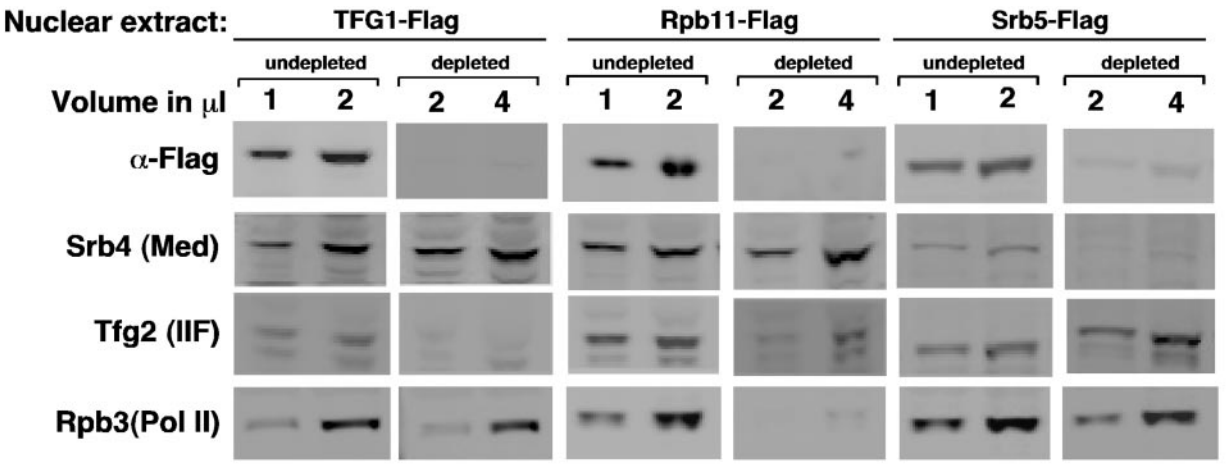

FIG. 2. Analysis of Flag-depleted nuclear extracts. Nuclear extracts made from Flag-tagged Tfg1, Rpb11, and Srb5 strains were depleted with anti-Flag M2-agarose beads as described in Materials and Methods. The indicated volumes of undepleted and Flag-depleted nuclear extracts were analyzed by SDS-PAGE and Western blotting.

of Pol II (Fig. 1). Both TFIIB and TFIIF were efficiently depleted from extracts and recovered by elution from the Flag antibody beads. However, very little Pol II copurified with TFIIB under these conditions (Fig. 1, lane 3). In contrast, a significant fraction of Pol II copurified with TFIIF (Fig. 1, lane 6), as expected from previous studies showing that TFIIF has a higher affinity for Pol II than TFIIB does (5). Pol II and some TFIIB have previously been found to coimmunoprecipitate (6a, 31); however, this was observed under conditions where the immune complexes were washed with lower-stringency buffers. In agreement with previous results where only small amounts of TFIIF copurified with the Pol II-Med complex (25), no detectable Mediator copurified with TFIIF, suggesting that the Pol II-Med and Pol II-TFIIF complexes are distinct. To test if these results were specific for purification in KOAc, the affinity purification was repeated using equivalent concentrations of KCl. Consistent with the above results, affinity-purified TFIIF contained only trace amounts of Mediator and affinity-purified Mediator contained no detectable TFIIF (data not shown).

To quantitate the amount of Pol II in complex with TFIIF or Mediator, nuclear extracts were made from strains containing a triple Flag tag on genes encoding Tfg1, the Pol II subunit Rpb11, or Mediator subunit Srb5. These strains all grew normally and gave active transcription extracts. Extracts were depleted with anti-Flag-conjugated beads under the conditions used above, and the amounts of Pol II, TFIIF, and Mediator before and after depletion were quantitated (Fig. 2 and Table 2). Depletion of TFIIF removed about 50% of Pol II, suggesting that half of Pol II in extracts was in the Pol II-TFIIF complex. Depletion of Pol II suggested that about 70% of TFIIF was in the Pol II-TFIIF complex. Depletion of Mediator suggested that about 20% of Pol II was in the Pol II-Med complex, and depletion of Pol II suggested that this complex represented about 40% of the Mediator in extracts. These results are in contrast to some previous studies of factor copurification, which suggested that nearly all Mediator is in a complex with Pol II (22, 28). Together, our results show that the Pol II-TFIIF and Pol II-Med complexes can account for about 70% of Pol II in extracts, although there exists a significant amount of free TFIIF and Mediator (~30 and ~60% of the total factors, respectively).

Activity of Pol II, TFIIF, Pol II-Med, and Pol II-TFIIF in transcription. For measurement of the activity of these Pol II complexes in transcription, the factors were first purified using a combination of affinity and ion-exchange or gel filtration chromatography. To purify Pol II and TFIIF, a TAP tag (36) was integrated at the C terminus of the genes encoding the Pol II subunit Rpb9 and the TFIIF subunit Tfg1. These strains grew normally and gave active transcription extracts. Factors were initially purified by immunoglobulin G and calmodulin affinity chromatography. Mass spectrometry analysis of the calmodulin-purified Pol II fractions revealed the presence of TFIIF, the elongation factor Spt5, and Pab1, which is involved in mRNA 3′ end processing (18), as well as ribosomal subunits and other proteins not obviously related to transcription. Some Spt5 was expected to copurify with Pol II, since substoichiometric amounts of Pol II copurified with the elongation factors Spt4, -5, and -6 (21, 24). To test if Spt4, Spt5, or Pab1 were specific components of the PIC, extracts were made from strains with triple Flag tags integrated at the SPT4, SPT5, or PAB1 genes. These extracts were used to form PICs on immo-

TABLE 2. Quantitation of factors present in Flag-depleted nuclear extract used for complementation of Pol II and TFIIF activities[a]

Nuclear extract	Factor	% Factor remaining after depletion
Tfg1-Flag	IIF	2
	Pol II	50
	Mediator (Srb4)	97
Rpb11-Flag	IIF	26
	Pol II	7
	Mediator (Srb4)	56
Srb5-Flag	TFIIF	90
	Pol II	82
	Mediator (Srb4)	10

[a] A quantitation of the results of Fig. 2 is shown.

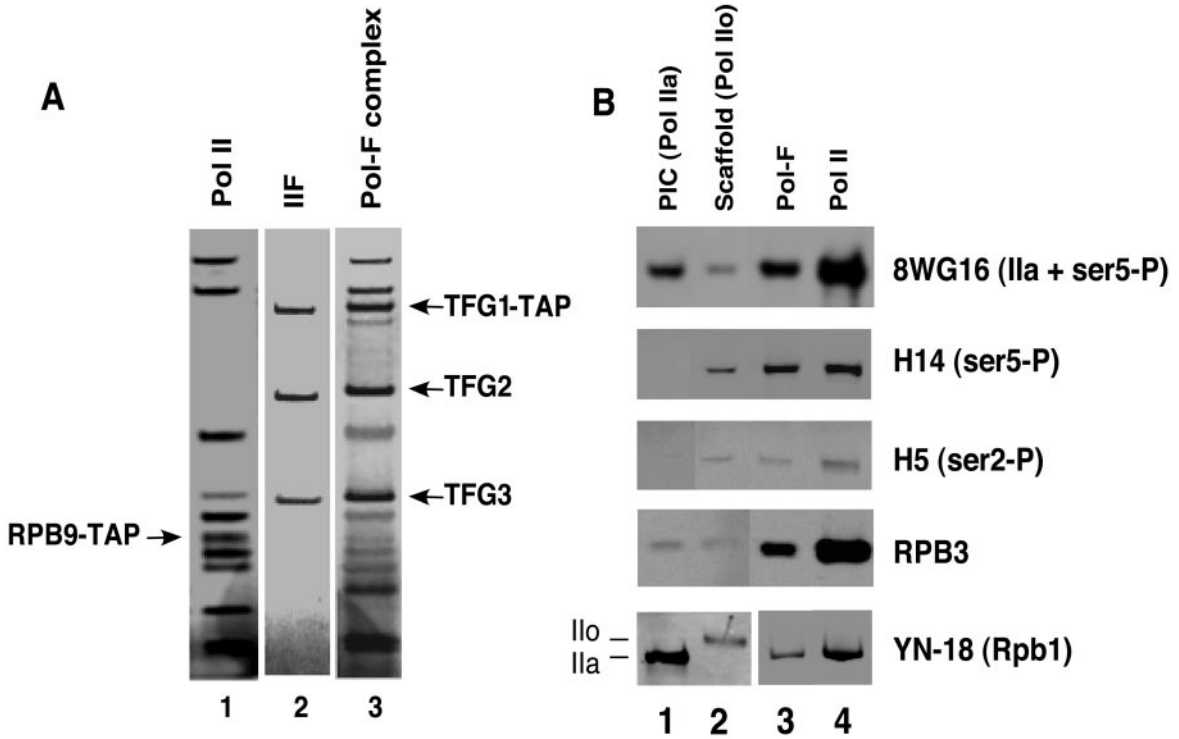

FIG. 3. Purified factors used for transcription. (A) Purified Pol II, TFIIF, and the Pol II-TFIIF complex were separated on a 4 to 12% NuPAGE gel (Invitrogen) run in morpholineethanesulfonic acid buffer and visualized by silver stain. (B) Western blot to monitor the phosphorylation state of the Pol II CTD. In the top four rows, proteins were separated on 4 to 12% NuPAGE gels, which do not separate differently phosphorylated forms of Pol II. In the lower row, samples were separated on a 3 to 8% Tris-acetate gel, which separates the IIo and IIa forms of Pol II. Blots were probed with the indicated antibodies.

bilized promoter templates or control nonpromoter templates. This analysis demonstrated that these three proteins were not components of the PIC (data not shown). Further, extracts made from strains with mutations in either Spt4 or Spt5 had levels of single- and multiround transcription activity within twofold of that from wild-type extracts (data not shown). Finally, to test if these factors were recruited to promoters during transcription reinitiation, PICs were formed using the Scaffold complex supplemented with Spt4, Spt5, or Pab1-Flag-tagged extracts. These PICs also showed no evidence of specific factor incorporation. From these experiments, we conclude that the small amounts of Spt5 and Pab1 which copurify with Pol II are likely the remnants of an elongation complex and are not active in either transcription initiation or reinitiation.

To further purify the Pol II complexes and to separate Pol II, TFIIF, and the Pol II-TFIIF complex, these calmodulin-purified samples were further fractionated by Source Q ion-exchange chromatography. Figure 3A shows purified Pol II, TFIIF, and the Pol II-TFIIF complex analyzed by SDS-PAGE and silver stain. These complexes appeared nearly free of any contaminating factors. The Pol II-Med complex was purified using Flag affinity purification and gel filtration as previously described (25). The concentration of these purified factors was compared with undepleted nuclear extracts using quantitative Western blot analysis (Table 3). For simplicity, we defined the amounts of TFIIF, Pol II, and Mediator in a standard in vitro transcription reaction mixture (90 μg of nuclear extract) as 1,000 U of each factor.

The phosphorylation state of the Pol II C-terminal domain (CTD) in the purified Pol II and Pol II-TFIIF complex was examined by probing with antibodies reactive to phosphorylation at Ser2 or Ser5 (Fig. 3B). For comparison, antibody reactivity was compared with Pol II from a purified PIC (the IIa nonphosphorylated form) and from an isolated Scaffold complex (the Pol II that remains in the scaffold is in the IIo phosphorylated form) (25a). From the reactivity observed with both phosphate-specific antibodies, the Pol II and Pol II-TFIIF preparations contained some Ser2 and Ser5 CTD phosphorylation. A similar analysis showed that the Pol II-Med fraction did not react with either the Ser2-P- or Ser5-P-specific antisera (data not shown). To estimate the extent of CTD phosphorylation in Pol II and Pol II-TFIIF, the samples were separated by SDS-PAGE on a Tris-acetate gel, which separates the IIo and IIa forms, and analyzed by Western blotting (Fig. 3B, lower row). The blot was probed with antisera against the N terminus of Rpb1 that reacts equally well with both phosphorylated and nonphosphorylated forms. This analysis showed that Pol II and

TABLE 3. Concentration of TFIIF, Pol II, Pol II-IIF, and Pol II-Med complexes used for in vitro transcription[a]

Purified protein	IIF (U/μl)	Med (U/μl)	Pol II (U/μl)
IIF	100		
Pol II			220
Pol II-IIF	25		55
PolII-Med	<1	125	55

[a] The amount of each factor present in 90 μg of a typical nuclear extract (the amount used in a standard in vitro transcription reaction) was defined as 1,000 U.

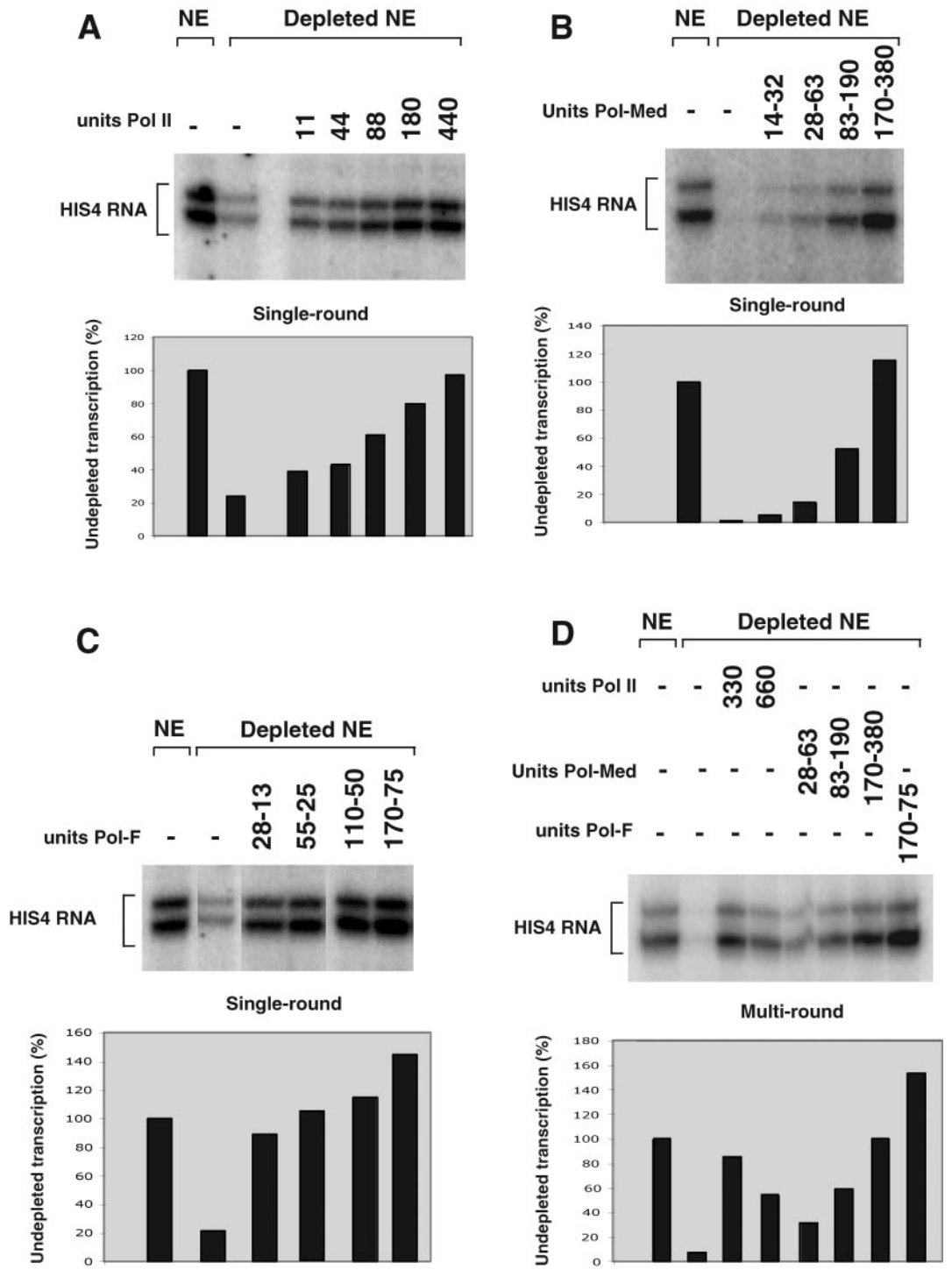

FIG. 4. Transcription activity of different Pol II forms in a Pol II-depleted nuclear extract. Single- and multiround transcription assays were performed as described in Materials and Methods. Units of each factor added to the depleted extract and transcription from an undepleted extract are shown for comparison. (A) Complementation with purified Pol II. (B) Complementation with purified Pol II-Med. (C) Complementation with Pol II-TFIIF complex. (D) Multiround transcription complementation with the indicated factors. Quantitation of transcription assays is shown.

Pol II-TFIIF complex are not heavily phosphorylated, as Rpb1 in these fractions migrated similarly to the IIa form seen in purified PICs.

To measure the activities of these Pol II complexes in transcription, we used the depleted extracts described above, supplemented with either purified Pol II, Pol II-Med, or Pol II-TFIIF. Figure 4 shows the activity of these factors in complementing a nuclear extract depleted for total Pol II (depleted of free Pol II, Pol II-Med, and Pol II-TFIIF). As noted above, these extracts contain TFIIF and Mediator at ~30 and ~60%

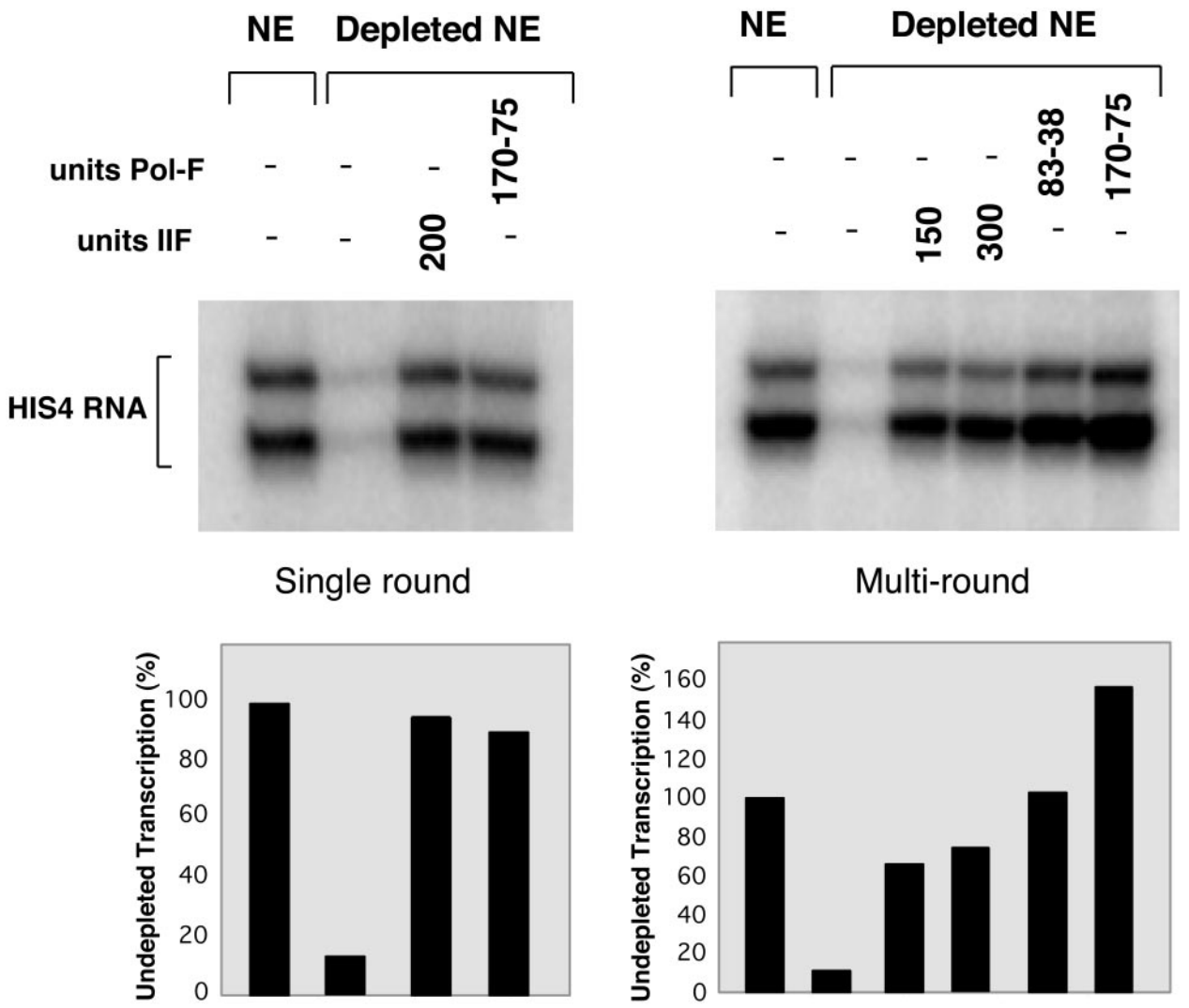

FIG. 5. Transcription activity of TFIIF and Pol II-TFIIF in a TFIIF-depleted nuclear extract. Units of each factor added to the depleted extract are indicated. Single- and multiround transcription assays are indicated, and transcription from undepleted extract is shown for both assays. Quantitation of transcription assays is shown below.

of the level of undepleted extracts. A total of 180 to 440 U of purified Pol II complemented single-round transcription assays using the Pol II-depleted extract (Fig. 4A). Similar levels of the Pol II-Med complex (~170 U of Pol II and 380 U of Mediator) were required to restore transcription to the Pol II-depleted extracts (Fig. 4B). The Pol II-TFIIF complex had the highest specific activity in the single-round assay, with only 28 U of Pol II and 13 U of TFIIF giving near-complete complementation (Fig. 4C). Increasing the concentration of Pol II-TFIIF to 170 U of Pol II gave a 1.5-fold increase in transcription compared to undepleted extracts.

Similar trends were seen in multiround transcription assays. High levels of purified Pol II (330 U) could not completely complement the depleted extract, and increasing or decreasing the amount added reduced transcription (Fig. 4D and data not shown). In contrast, lower levels of Pol II-Med (170 U of Pol II, 380 U of Mediator) nearly complemented the depleted extract. Finally, the Pol II-TFIIF complex was the most active, with 170 U of Pol II and 75 U of TFIIF giving a 1.5-fold increase in transcription compared to undepleted extracts. In titration experiments, 28 to 55 U of Pol II in the Pol II-TFIIF complex was able to restore undepleted levels of transcription to this extract (data not shown).

Activities of Pol II-TFIIF and purified TFIIF were assayed in extracts depleted for total TFIIF (depleted of TFIIF and Pol II-TFIIF). As shown in Table 2, this depleted extract contained normal amounts of Mediator and about 50% of the normal level of Pol II. Addition of various concentrations of TFIIF to this depleted extract showed that normal single-round transcription was restored with ~200 U of purified TFIIF (Fig. 5 and data not shown). In contrast, much lower levels of TFIIF were required when the Pol II-TFIIF complex was used for complementation (75 U of TFIIF and 170 U of Pol II) (Fig. 5 and data not shown). This discrepancy in the activity of TFIIF versus Pol II-TFIIF was even greater in multiround transcription assays (Fig. 5). A total of 300 U of purified TFIIF complemented the TFIIF-depleted extract to only 70% of normal activity in multiround transcription. In contrast, only 38 U of

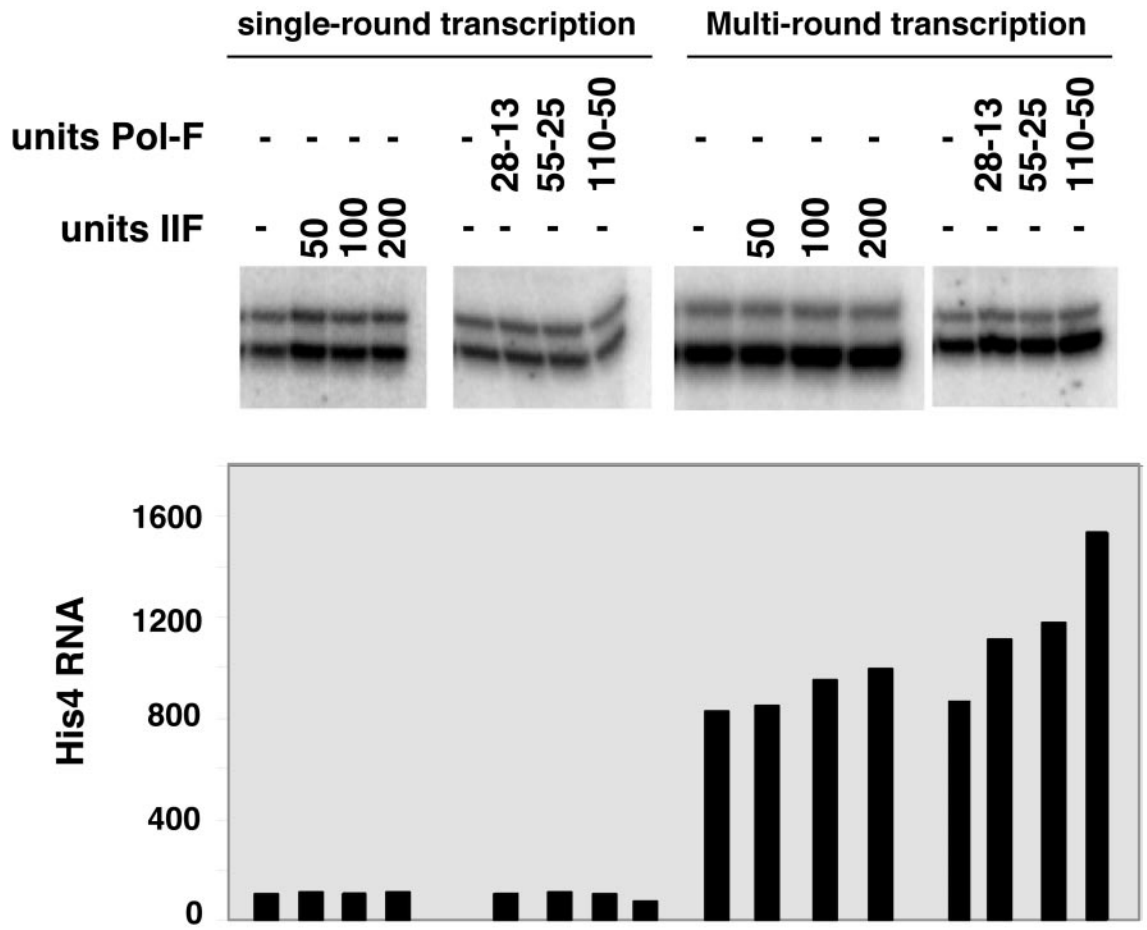

FIG. 6. Factors limiting for transcription in nuclear extract. Nuclear extract made from a Flag-tagged Tfg1 strain was supplemented with the indicated amounts of purified TFIIF or Pol II-TFIIF in single- or multiround in vitro transcription assays. Quantitation of transcription assays is shown below.

TFIIF in the Pol II-TFIIF complex fully complemented this same extract. Further, doubling the amount of Pol II-TFIIF complex to 75 U of TFIIF gave a 1.6-fold increase in transcription over undepleted extract. As expected, Pol II-Med failed to complement a TFIIF-depleted extract, as it lacks detectable TFIIF (data not shown).

To test if these factors were limiting in undepleted nuclear extract, purified TFIIF and Pol II-TFIIF were added to wild-type nuclear extract and tested in both single- and multiround transcription assays (Fig. 6). In these assays, both Pol II-TFIIF and TFIIF gave only a slight increase in single-round transcription when added to normal extracts. In contrast, Pol II-TFIIF (50 U of TFIIF and 110 U of Pol II) stimulated multiround transcription 1.8-fold compared to normal extract. However, 50 U of purified TFIIF had no effect, and only limited stimulation was observed with 100 and 200 U of TFIIF.

The Pol II-TFIIF and Pol II-Med complexes are dynamic. The results presented in Fig. 4D showing that Pol II-Med could complement Pol II-depleted extracts for multiround transcription were surprising. Since Mediator is a stable component of the Scaffold complex, we expected that Pol II-Med would only function in single-round transcription. One possible explanation for this behavior could be that the Pol II-Med complex is unstable when present in extracts under transcription conditions. If unstable, Pol II-Med could dissociate into free Pol II and Mediator and Pol II could potentially associate with the free TFIIF present in the extract. To test this possibility, purified Pol II-Med with a Flag epitope-tagged Srb5 subunit was added to a transcription reaction lacking DNA along with an extract made from a strain with a six-HA epitope tag on the Pol II subunit Rpb9. Extracts were incubated 40 min at room temperature either with or without NTPs. Pol II and associated proteins were then purified by immune precipitation with HA antibody beads. Figure 7, lanes 1 to 3, shows that purification of the HA-tagged Pol II also purified the Flag-tagged Mediator added to the reaction as the Pol II-Med complex. This demonstrates that under transcription conditions using nuclear extracts, the Pol II-Med complex is unstable. This instability seems unrelated to transcription or phosphorylation, as the reaction occurred without promoter DNA and equally well with or without nucleotide addition. A control precipitation without HA antibody showed the specificity of this analysis (Fig. 7, lane 3). A similar stability test was carried out for the Pol II-TFIIF complex (Fig. 7, lanes 4 to 6). Again, it was seen that the Pol II-TFIIF complex was unstable under these conditions in a transcription- and nucleotide-independent fashion.

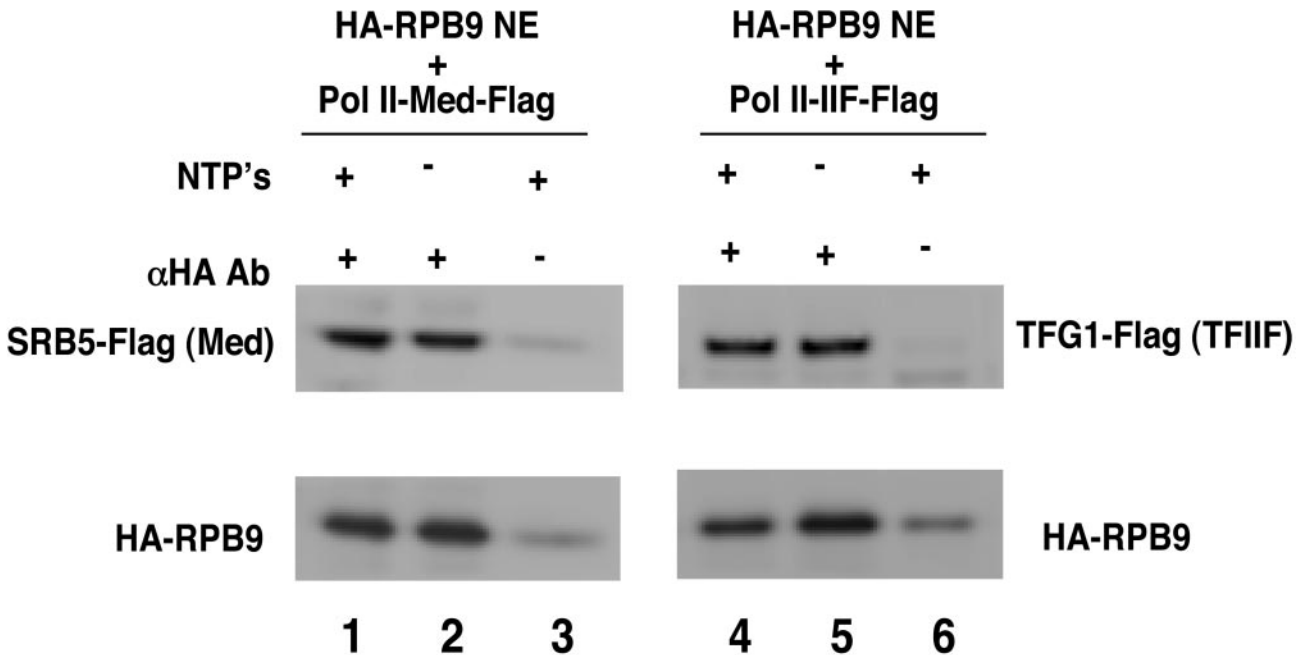

FIG. 7. Pol II-Med and Pol II-TFIIF complexes are dynamic in nuclear extract. Purified Pol II-Med or Pol II-TFIIF was incubated with nuclear extract from an Rpb11-HA-tagged strain under transcription conditions without DNA template and in the presence or absence of NTPs as indicated. Reactions were incubated at 25°C for 20 min and immune precipitated with HA antibody. Precipitated proteins were analyzed by SDS-PAGE and Western blotting with Flag M2 and anti-HA antibodies.

The Pol II-Med complex does not function for reinitiation in a purified system. To test if the Pol II-Med and Pol II-TFIIF complexes could function in transcription reinitiation, we turned to a purified transcription system. Magnetic beads with immobilized HIS4 promoter DNA, the activator VP16, and wild-type nuclear extract were used to generate PICs. These washed PICs were converted to Scaffold complexes by the addition of ATP (35, 42). This treatment dissociates Pol II, TFIIF, and TFIIB. After washing, these immobilized Scaffold complexes were incubated with either no added factors or TFIIB plus the following: Pol II and TFIIF, Pol II-Med and TFIIF, or Pol II-TFIIF. Nucleotides were added, and transcription was assayed by primer extension (Fig. 8). When nucleotides were added for 2 min to limit reinitiation to about a single round, a striking difference was observed between the activity of the Pol-Med complex and that of Pol II-IIF or Pol II plus TFIIF (Fig. 8, left panel). Pol II-Med gave only about a twofold increase in transcription compared to adding nucleotides to purified Scaffold complex with no added factors. In contrast, Pol II plus TFIIF and Pol II-IIF gave about 10-fold higher levels of transcription. This inactivity of Pol II-Med in the reinitiation assay was magnified when nucleotides were added for 30 min to generate multiple rounds of transcription (Fig. 8, right panel). Under these conditions, about 10-fold greater activity was seen for Pol II-TFIIF compared to Pol II-Med plus TFIIF. The highest levels of transcription were obtained using high levels of purified Pol II and TFIIF. Since the purified Pol II-TFIIF complex was not as concentrated as the purified Pol II and TFIIF, we were not able to test the activity of the Pol II-TFIIF complex under comparably high concentrations of TFIIF. From the results of Fig. 8, we conclude that the Pol II-Med complex does not function in the reinitiation assay and that it appears to be an initiation-specific complex. Also, purified Pol II and TFIIF seem to function nearly the same as the Pol II-TFIIF complex in this reinitiation assay. Finally, in this purified system, TFIIF does not appear able to exchange with Mediator in the Pol II-Med complex, suggesting that other components in the nuclear extracts promote this exchange.

DISCUSSION

Upon gene induction, an essential step in the transcription cycle is the stable recruitment of Pol II and the general transcription factors to promoters. It is well established that Pol II is recruited to the PIC with the CTD in the nonphosphorylated state. However, an unresolved question has been whether Pol II is recruited as part of a multifactor complex and, if so, which factors are prebound to Pol II. Originally, it was proposed that yeast Pol II bound as part of a holoenzyme containing Pol II, Mediator, many general factors, and some chromatin remodeling factors. Other studies using affinity chromatography to purify yeast Mediator have demonstrated that there is very little if any of this large complex in yeast extracts (reference 25 and this work). Instead, yeast Mediator is largely bound to Pol II in the absence of other factors. In addition, several recent studies have questioned whether Mediator binds to promoters as a complex with Pol II. Cross-linking studies at several regulated promoters in yeast and *Drosophila* have suggested that Mediator can be recruited to promoters before Pol II.

To address the function and activity of yeast Pol II complexes, we used affinity chromatography to identify and purify the major stable forms of Pol II in yeast nuclear extracts. We found that about half of Pol II was bound to TFIIF and about

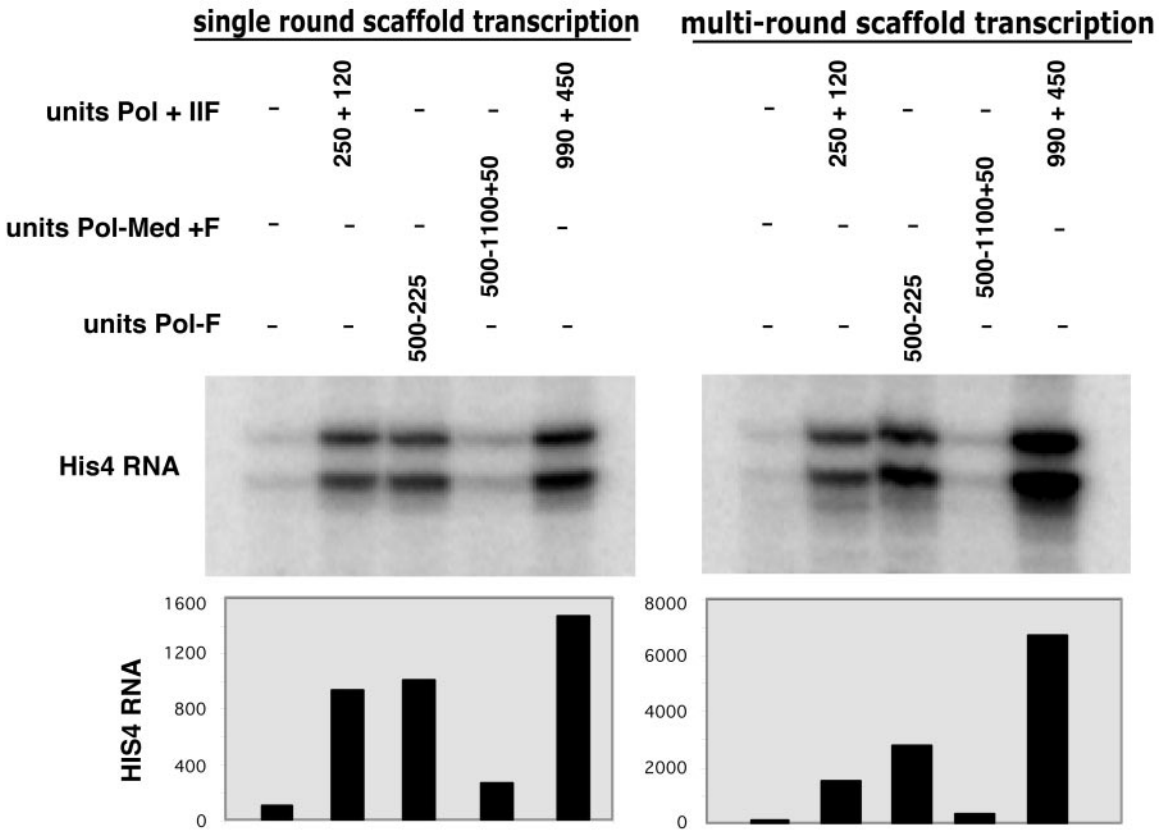

FIG. 8. Pol II-Med does not function in transcription reinitiation. Scaffold complexes were generated by ATP treatment of PICs formed on immobilized promoter templates. Immobilized scaffolds were complemented with TFIIB and the indicated amounts of Pol II and TFIIF, Pol II-Med and TFIIF, or Pol II-TFIIF. Single- and multiround transcription assays were as described in Materials and Methods. Shown are results of RNA analysis by primer extension and quantitation of primer extension products.

20% of Pol II was bound to Mediator. These complexes represented about 70 and 40% of the total TFIIF and Mediator, respectively. It seems that other forms of yeast Pol II previously identified are either not stable under the conditions used here or represent a minor fraction of the total Pol II in yeast extracts. Interestingly, we found no significant levels of a complex containing Pol II, Mediator, and TFIIF. Since each factor binds Pol II separately, this suggests that TFIIF and Mediator compete for binding to free Pol II. Since both factors bind to Pol II when in the PIC, it is likely that in the PIC interaction with other general factors or a general conformational change in Mediator or TFIIF allows both TFIIF and Mediator to interact with Pol II simultaneously.

To examine the function of these two stable Pol II complexes, we used them to supplement nuclear extracts depleted of either total Pol II (extracts missing Pol II-Med, Pol II-TFIIF, and Pol II) or total TFIIF (extracts missing both TFIIF and Pol II-TFIIF). In single- and multiround transcription assays, purified Pol II as well as Pol II-Med and Pol II-TFIIF could each complement a Pol II-depleted extract. Strikingly, Pol II-TFIIF had the highest specific activity in transcription with about 7 times more activity than Pol II and about threefold more activity than Pol II-Med in single-round transcription assays. The fact that these Pol II-depleted extracts are partially depleted for TFIIF cannot explain the higher activity of the Pol II-TFIIF complex. First, the Pol II-depleted extracts still contain ~300 U of TFIIF, which is enough to allow complementation by both Pol II and Pol II-Med. Second, the amount of TFIIF added in the form of Pol II-TFIIF for complete complementation was quite low (13 to 25 U) compared to the amount of TFIIF remaining in the depleted extract. This strikingly higher activity of Pol II-TFIIF was also shown in assays of TFIIF function. Pol II-TFIIF had about 2.5-fold higher activity than TFIIF in single-round assays and greater than 8-fold higher activity in multiround assays performed in TFIIF-depleted extracts.

Paradoxically, we found significant levels of multiround transcription when Pol II-Med was used to supplement Pol II-depleted extracts. If transcription reinitiation proceeds through the Scaffold complex intermediate as proposed, it seemed unlikely that Pol II-Med would function in the reinitiation assay, since Mediator is a stable component of the Scaffold complex. Using a purified reinitiation system containing purified Scaffold complexes, we found that this was indeed the case. Both Pol II alone (supplemented with TFIIF) and the Pol II-TFIIF complex functioned efficiently in this assay. In contrast, the Pol II-Med complex showed little activity in the reinitiation assay.

An explanation for this apparent discrepancy is provided by the observation that the Pol II-Med and Pol II-TFIIF com-

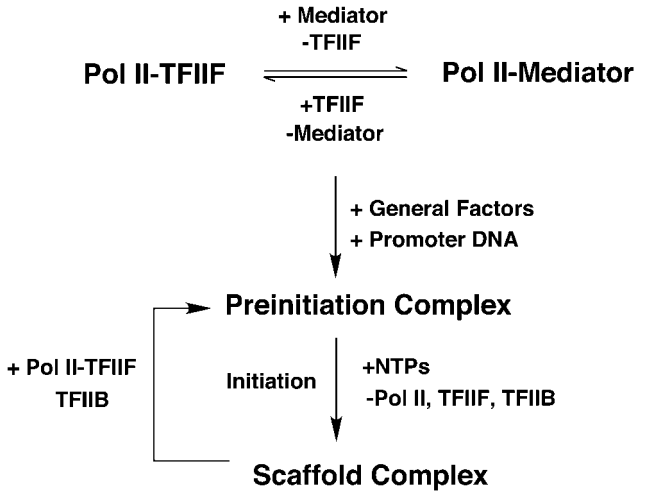

FIG. 9. Model for action of Pol II complexes in transcription initiation and reinitiation. Pol II-TFIIF and Pol II-Med complexes are dynamic, and Pol II in these complexes can exchange Mediator and TFIIF. Both Pol II complexes likely function in PIC formation. After initiation and Scaffold complex formation, Pol II-TFIIF and TFIIB are recruited for subsequent PIC formation and reinitiation.

plexes are unstable under transcription conditions using extracts, with a single polymerase capable of exchanging Mediator and TFIIF. This behavior is in contrast to the results of protein purification studies where both Pol II-Med and Pol II-TFIIF could be isolated as stable complexes. This factor exchange does not seem to depend on transcription or phosphorylation, as it is independent of both nucleotide addition and promoter DNA. Although the reason for this instability is still unknown, possible contributing factors include the transcription reactions being carried out at room temperature and the high levels of free Mediator and TFIIF in extracts competing for binding to Pol II. In contrast, protein isolation is typically done at 4°C, and once a complex is purified, free TFIIF and Mediator are absent. Therefore, the depletion results most likely represent a snapshot of the equilibrium between the different Pol II complexes in the nuclear extract at 4°C.

Based on previous findings and our new results, we propose the following model for initiation (Fig. 9). Since Pol-Med and Pol II-TFIIF are dynamic and were shown to function in transcription, both forms can likely be recruited to form the first PIC. Given that most Pol II is in the Pol II-TFIIF complex and that this complex has higher specific activity than either Pol II or Pol II-Med, it is suggested that Pol II-TFIIF is the more frequently recruited form of Pol II. This model agrees with the few regulated yeast promoters studied by chromatin immunoprecipitation assays, where Mediator was observed to crosslink before Pol II. Specific activators or promoters, however, could potentially influence the specific form of Pol II recruited. After initiation at promoters where the Scaffold complex is stable, both Pol II and Pol II-TFIIF can be recruited for multiple rounds of transcription. Since Pol II-TFIIF seems the predominant form of Pol II and because it has much higher activity in extracts compared to Pol II alone, we suggest that Pol II-TFIIF is the major form of Pol II recruited during transcription reinitiation. By this model, there is no unique form of Pol II used for initiation. Further studies in vivo and in vitro may reveal particular activators or promoters that influence the specific form of Pol II recruited during initiation.

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