Mediator is required for activated transcription in a
Schizosaccharomyces pombe in vitro system

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RNA polymerase II (RNAPII) requires a set of general transcription factors – TFIIA, TFIIB, TFIID, TFIIE, TFIIF and TFIIH – to initiate transcription from a gene promoter in vitro. General transcription factors have been isolated from Saccharomyces cerevisiae, rat, human and Drosophila, and their corresponding cDNAs have been cloned. In this report, we describe a reconstituted in vitro transcription system that consists of the following preparations of factors from the yeast Schizosaccharomyces pombe: affinity-purified RNAPII, TFIIH, and recombinant TBP, TFIIB, TFIIE and TFIIF. We show that this system can support basal transcription from the adenovirus major late promoter when purified RNAPII is used and activated transcription when the RNAPII holoenzyme (RNAPII plus the Mediator proteins) is included in the reaction. In contrast, the TATA binding protein-associated factors had no effect on transcriptional activation in our Sc. pombe system. These results indicate that Sc. pombe uses the same set of general transcription factors as other eukaryotes and that the Mediator is involved in activated transcription.

The ability to achieve basal levels of transcription from protein-encoding genes in eukaryotes requires RNA polymerase II (RNAPII) and a set of additional proteins called general transcription factors (GTFs). The GTFs have been purified to homogeneity from HeLa cells, rat liver, Drosophila and the yeast Saccharomyces cerevisiae, and have been named TFIIA, TFIID, TFIIE, TFIIF, TFIIB and TFIIH [1]. The cDNAs that encode these factors have been isolated, and their amino acid sequences show a high degree of evolutionary conservation. These findings indicate that the transcriptional machinery is highly conserved among eukaryotes.

An in vitro transcription assay that consists of purified RNAPII and recombinant GTFs can carry out basal transcription but cannot respond to gene-specific transcriptional activators. Activated transcription requires additional multiprotein complexes named coactivators. The main coactivators required for activated transcription in in vitro systems are the TFIID complex and the Mediator [2]. Recent work suggests that the TFIID complex, which contains the TATA binding protein (TBP) and other TBP-associated factors (TAFs), plays an important role in facilitating activation by gene-specific transcription factors as well as in recognition of the TATA box and other core promoter sequences (necessary for both basal and activated transcription) [3]. Mediator is a large multiprotein complex that is brought to promoters by DNA-bound, gene-specific transcriptional regulatory proteins and helps these proteins to communicate with factors bound to the core promoter. Mediator is required for transcription in vivo and for optimal levels of both basal and activated transcription in vitro in nuclear extracts from human cells [4,5]. Components of both the TFIID complex and Mediator are conserved from yeast to humans.

The yeast Schizosaccharomyces pombe can be genetically manipulated and has served as an excellent model for the study of cell division cycle control and DNA repair and recombination. The Sc. pombe genome has been fully sequenced and annotated and contains the smallest number of protein-coding genes [4] of any eukaryotic genome sequenced to date [6]. Evidence suggests that the mechanism of transcription initiation by Sc. pombe RNAPII is more similar to that of higher eukaryotes than that of S. cerevisiae. In Sc. pombe, transcription initiation occurs 25–30 bp downstream from the TATA box region of the core promoter, whereas in S. cerevisiae, it occurs 40–120 bp downstream from the TATA box. Also, transcription initiation from mammalian promoters that have been introduced into Sc. pombe occurs at the same sites as it does in mammalian cells [7].

On the basis of these observations, we have begun to study mechanisms of transcriptional activation in Sc. pombe in order to compare these processes with those of higher eukaryotes. Given the observed similarities, findings from the Sc. pombe system can probably be extrapolated to higher eukaryotes. Studies of RNAPII transcription in Sc. pombe has been hampered by the lack of a reconstituted in vitro transcription system that responds to transcriptional activator proteins. Here we describe an in vitro transcription system that contains the following preparations from Sc. pombe: affinity-purified RNAPII, highly purified TFIIH, and recombinant TBP, TFIIB, TFIIE and TFIIF.

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Abbreviations: RNAPII, RNA polymerase II; GTF, general transcription factor; TBP, TATA binding protein; TAF, TBP-associated factor; CTD, C-terminal domain; Ad-MLP, adenovirus major late promoter.

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We have also purified the RNAPII holoenzyme RNAPII plus the Mediator complex from *Sc. pombe* and demonstrated that it is able to stimulate basal transcription and support activated transcription in the absence of TAFs.

**Materials and methods**

**Cloning of *Sc. pombe* TFIIE, TFIIF, TFIIB and the p52 subunit of TFIIH**

To identify homologs of TFIIE, TFIIF, TFIIB and TFIIH, we searched the *Sc. pombe* genome sequence using the BLASTp program and the amino acid sequences of human and *S. cerevisiae* TFIIE, TFIIF and TFIIB, and of the p62 subunit of human TFIIH. We identified homologs of both subunits of TFIIE, TFIIF and TFIIB, and of the Tfb1 subunit of TFIIH. The cDNAs encoding both subunits of TFIIE, the smallest subunit of TFIIH, and Tfb1 subunit of TFIIH were amplified by PCR from a cDNA library of *Sc. pombe*. The oligonucleotides used in the PCR amplifications were designed from the nucleotide sequences obtained from the *Sc. pombe* genome sequence database. The primer that was complementary to the N-terminal sequences of the various genes contained an *NdeI* recognition site, and the primer that was complementary to the C-terminal sequences contained a *BamHI* recognition site. The PCR products were digested with *NdeI* and *BamHI* and cloned in-frame into the *NdeI* and *BamHI* sites of the bacterial expression vector pET15b (Novagen), which adds a hexahistidine tag at the N terminus of the protein. Positive clones were sequenced using the Sequenase version 2 kit (USB). The cDNA encoding the largest subunit of TFIIF was synthesized by the GeneScript Corporation and cloned in-frame into the *NcoI* and *BamHI* sites of the bacterial expression vector pET19b (Novagen).

**Expression and purification of *Sc. pombe* TFIIE, TFIIF, TFIIB, TBP and the Tfb1 subunit of TFIIH**

TFIIB, TFIIE, TFIIF, and the Tfb1 subunit of TFIIH were expressed in *Escherichia coli* strain BL21 (DE3 pLysS). The bacteria were grown in TB medium (500 mL) at 37 °C to *D*$_{600}$ = 0.8. Production of the proteins was then induced with 0.5 mM isopropyl thio-β-β-D-galactoside, and the culture was incubated an additional 4 h at 37 °C. Bacteria were harvested by centrifugation and lysed with mild sonication at 4 °C in buffer A [20 mM Hapes pH 7.9, 500 mM KCl, 0.1% (v/v) NP-40, 0.1 mM phenylmethylsulfonyl fluoride]. The lysate was cleared by centrifugation, and the insoluble pellet containing the recombinant protein was washed twice with buffer A containing 0.05% (v/v) sodium deoxycholate and 0.1% (v/v) Triton X-100. Recombinant proteins were extracted from the pellet by incubation for 8 h at 4 °C in 20 mL 20 mM Hapes pH 7.9, 6 M guanadium hydrochloride, 0.1 mM phenylmethylsulfonyl fluoride. The mixture was then centrifuged to pellet the cell debris, and the soluble protein was diluted six times with dialysis buffer [20 mM Hapes pH 7.9, 10% (v/v) glycerol, 2 mM dithiothreitol, 1 mM EDTA, 100 mM potassium acetate, 0.1 mM phenylmethylsulfonyl fluoride]. The recombinant protein was incubated overnight at 4 °C, dialysed against 100 vols dialysis buffer, and centrifuged to eliminate any precipitated material. The TFIIFα (250 μg) and TFIIFβ (250 μg) preparations were mixed and incubated for 1 h at 4 °C. The mixture was loaded onto a 10-mL gel filtration column made of ACA22 resin (Sigma) and eluted from the column with dialysis buffer; 200-μL fractions were collected. Both subunits were detected in the column eluate by Western blotting with antibodies to the His-tag (Clontech). Fractions containing the TFIIF heterotetramer were used in *in vitro* transcription experiments. TFIIFα and TFIIFβ were mixed and purified by the same procedure as described for TFIIF.

**Purification of TFIIH, RNAPII and the RNAPII holoenzyme**

TFIIH and RNAPII were purified from *Sc. pombe* whole-cell extracts that were prepared from the wild-type strain 972h. Cells were grown to 100 g wet weight in 200 L of YPD media and harvested by centrifugation (10 000 g, 4 °C, 30 min). The cell pellet was washed twice with distilled water and introduced into liquid nitrogen. Cell extracts were prepared from 500 g yeast cell extracts (wet weight) that were obtained from 200 L culture. The cells were mixed with liquid nitrogen in a 4-L Waring blender and blended four times for 5 min each at maximum speed. The blending was repeated until 95% of the cells were broken as judged by viewing the cells under a light microscope. The broken cells were mixed with 750 mL buffer B (100 mM Hapes pH 7.9, 250 mM KCl, 5 mM EGTA, 10 mM EDTA, 2.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 0.5 μg/mL Pepstatin A), and the mixture was centrifugated in a Sorvall rotor at 30 000 g, 4 °C, for 4.5 min. The supernatant was pooled and precipitated with ammonium sulfate at 40% saturation, and the precipitates were recovered by centrifugation at 30 000 g, 4 °C in a Sorvall rotor. The precipitate was resuspended in 400 mL buffer C (20 mM Hapes pH 7.9, 5 mM EGTA, 1 mM EDTA, 2 mM dithiothreitol, 20% (v/v) glycerol, 1 mM phenylmethylsulfonyl fluoride), dialysed against 4 L of the same buffer. The extract was stored at −80 °C until use.

TFIIH was prepared from the yeast, cell extracts as described by Li et al. [8]. The TFIIH-containing fraction (500 μg protein) from the heparin-agarose column was loaded onto a 10 mL ACA22 gel filtration column, and eluted with dialysis buffer (200 μL fractions were collected). The ACA22 column fractions were assayed by Western blotting with antibodies to Tfb1, and the TFIIH-containing fractions were pooled and used in the transcription assays. TFIIH was free of RNAPII, GTFs, and Mediator as judged by Western blotting with antibodies to TBP, the smallest subunit of TFIIH, TFIIE, RNAPII and Srb4 (part of the Mediator complex).

RNAPII was purified from the yeast cell extracts (1 g) by chromatography on a DEAE-52 column (100 mL wet resin) (Whatman) that had been equilibrated with buffer C. The column was eluted with a 10-column volume gradient of ammonium sulfate (0.1–0.5 M in buffer C). The elution of RNAPII was detected by Western blotting with monoclonal antibodies to the C-terminal domain (CTD) of the largest subunit of RNAPII (SWG16). Fractions containing
RNAPII were pooled and dialysed against buffer C containing 500 mM potassium acetate and 0.01% (v/v) NP-40. Two milliliters of the RNAPII preparation (1 mg·mL⁻¹ protein) were incubated for 2 h at 4 °C with 500 µL of anti-CTD (SWG16) mAb that had been cross-linked to protein A–agarose. After the incubation, the resin was washed with buffer C containing 500 mM potassium acetate and 0.01% (v/v) NP-40 and eluted with 500 µL CTD peptide (YSPSTS8) at 1 mg·mL⁻¹ in buffer C. The fraction containing pure RNAPII was dialysed against buffer C and used for the transcription experiments. RNAPII holoenzyme was prepared from TAP-SpMed7 (a gift from C. Gustafsson, Karolinska Institute, Novum, Sweden) cell extracts. Extracts were prepared as described above from TAP-SpMed7 and dialysed against 20 mM Hepes pH 7.5, 200 mM potassium acetate, 10% (v/v) glycerol, 1 mM dithiothreitol, 0.1% (v/v) NP-40 and 0.5 mM phenylmethylsulfonyl fluoride. After dialysis, 2 mL of extract was incubated with 500 µL IgG beads (Amersham Biosciences) for 1 h at 4 °C. The IgG beads were loaded into a column and washed with dialysis buffer. After washing with 10 mL tobacco etch virus (TEV) protease buffer, the beads were resuspended in 1 mL TEV protease buffer and incubated at 30 °C for 1 h with 300 U TEV protease. Eluates were collected and dialysed against dialysis buffer. TEV protease was eliminated from the eluates for passage onto a Ni-NTA–agarose column. The RNAPII holoenzyme was further purified on a heparin–Sepharose column to separate the RNAPII holoenzyme from TRAP240 complex.

Purification of the TFIIID complex

We used two methods to purify the TFIIID complex, which consists of TBP and the TAFs. First, 500 µL of protein A–agarose containing crosslinked anti-TAF110 Igs were mixed with 2 mL cell extract in buffer containing 20 mM Hepes pH 7.5, 100 mM potassium acetate, 10% (v/v) glycerol, 1 mM dithiothreitol, 0.1% (v/v) NP-40, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM EDTA and incubated at 4 °C for 1 h. The resin was then washed with the same Hepes buffer, and protein was eluted with 500 µL 6 M urea, 20 mM Hepes pH 7.5, 0.1 mM EDTA, 1 mM dithiothreitol. The eluate was dialysed against incubation buffer and concentrated fivefold with a Centricon. The native TFIIID complex was also purified from whole-cell extracts using conventional chromatographic methods (hydroxyapatite, phosphocellulose, DE52, Mono S, Mono Q, Phenyl-Superose and ACA22 gel filtration columns). We purified the TAF-containing complex until we obtained the same set of polypeptides that were present in the complex that was affinity purified with anti-TAF110. Both, the affinity and chromatographic purified TAF-containing complex (TFIID) were free of RNAPII, Mediator and GTFs.

Purification of the Gal4 DNA binding domain, Gal4-AP2, Gal4-VP16 and GAL4-CTF

The gene that encodes the Gal4-AP2 transcriptional activator, which contains the Gal4 DNA binding domain and an activation domain from the AP2 transcription factor, was obtained from a Sc. pombe expression vector (a gift from J. E. Remacle, Department of Cell Growth, University of Leuven, Belgium) and cloned into pET15b (Novagen) by PCR. Gal4-AP2 was then expressed in bacteria and purified under native conditions on Ni-NTA–agarose. Gal4-CTF and Gal4-Sp1 were purified by the same method. Purified Gal4 and Gal4-VP16 were a gift from D. Reinberg.

Preparation of antibodies to TFIIIFβ, Srb4, Tfb1, TAF110, and TAF72

Antibodies were prepared by injecting rabbits with 500 µg of either purified recombinant TFIIIFβ, Srb4 and Tfb1, or TAF110 and TAF72, each mixed in Complete Freund’s adjuvant according to NIH guidelines. Fifty days after the first injection, another injection was given in Incomplete Freund’s adjuvant. After 3 weeks, blood was obtained from each rabbit and serum was prepared. The antibodies were purified from the serum by protein A–agarose chromatography and used for Western blots.

Specific transcription reactions

The conditions for the transcription reactions were based on those reported by Lue et al. [9]. The DNA template contained the adenovirus major late promoter (Ad-MLP) promoter fused to the 377 bp G-minus cassette of Sawadogo and Roeder [10]. The template contained five Gal4 binding sites located 30 bp upstream from the TATA box. Reactions mixtures (30 µL) contained proteins and template as indicated in the legend of each figure. Transcription reactions were performed in 50 mM Hepes pH 7.8, 50 mM potassium glutamate, 15 mM magnesium acetate, 2.5 mM dithiothreitol, 1 U ribonuclease inhibitor (Promega), 10% (v/v) glycerol, 2% (w/v) polyethylene glycol, 0.4 mM ATP, 0.4 mM CTP, 0.5 µCi [³²P]dUTP [²P], 4 mM phosphoenolpyruvate. After 30 min at 25 °C, the transcription reaction was stopped by the addition of 100 µL stop buffer (10 mM Tris/HCl pH 5.7, 300 mM NaCl, 5 mM EDTA, 10 U RNAaseTI) and incubated at 37 °C for 15 min. The reaction was treated with 10 µL 10% SDS and 100 µg proteinase K for 15 min at 37 °C. The transcripts were precipitated with 3 vols ethanol and analysed by electrophoresis in a 6% polyacrylamide/7 M urea gel in Tris/borate/EDTA buffer. The gels were dried and analysed by autoradiography.

Purification of DNA templates

Supercoiled DNA templates were purified with Wizard columns (Promega) followed by polyethylene glycol precipitation. Relaxed DNA templates were purified on Qiagen columns, and the preparation contained only 5% supercoiled templates. However, using the Wizard columns we obtained ≥95% supercoiled templates. Linear templates were obtained by cutting the template with a restriction enzyme (EcoRI).
Results

Identification and purification of TFIIE, TFIIF, TFIIB, TBP and the Tfb1 subunit from Sc. pombe

Using the NCBI BLASTP program, we identified Sc. pombe homologs of TFIIE, TFIIF, TFIIB and Tfb1 by querying with the amino acid sequences of the homologous human and S. cerevisiae factors. The genes that encode the various factors where then cloned from Sc. pombe and expressed and purified as described in Materials and methods. After purification, the factors were at least 90% pure as judged by SDS/PAGE followed by Coomassie blue staining (Fig. 1A–D). Sc. pombe TFIIE is composed of two subunits, α (CAC32853) and β (CAC20446). The Sc. pombe α subunit contains 434 amino acids and shares 26% amino acid sequence identity with its S. cerevisiae homolog and 21% amino acid sequence identity with human TFIIEα. Sc. pombe TFIIEβ (CAA20446) contains 285 amino acids and shares 38% amino acid sequence identity with its S. cerevisiae homolog and 30% amino acid sequence identity with its human homolog. Sc. pombe TFIIF is also composed of two subunits, α (CAA22493) and β (NP595082). Sc. pombe TFIIFα contains 490 amino acids and shares 33% amino acid sequence identity with its S. cerevisiae homolog. However, no homology with human TFIIFα was detected. Sc. pombe TFIIFβ has 301 amino acids and displays 27% amino acid sequence identity with human TFIIFβ and 37% identity with S. cerevisiae TFIIFβ. The Sc. pombe Tfb1 subunit of TFIIFH has 457 amino acids and shares 28% amino acid sequence identity with the human p62 subunit of TFIIFH. Tfb1 also displays 29% identity with its S. cerevisiae homolog.

Purification of RNAPII and RNAPII holoenzyme from Sc. pombe

RNAPII and the RNAPII holoenzyme were purified from Sc. pombe cell extracts as described in Materials and methods. RNAPII was purified by affinity chromatography with antibodies against the CTD. The RNAPII holoenzyme was purified by subjecting cell extracts made with the TAP-SpMed7 Sc. pombe strain to affinity chromatography on IgG beads. The purity of both preparations was evaluated by SDS/PAGE followed by silver staining. As shown in Fig. 2A, all of the RNAPII subunits were present in the RNAPII holoenzyme preparation, and the holoenzyme preparation also contained additional polypeptides, some of which were identified by N-terminal amino acid microsequencing. Furthermore, Srb4 and the smallest subunit of TFIIF were present in the RNAPII holoenzyme as detected by Western blot analysis (Fig. 2B). The smallest subunit of TFIIF and Srb4 were not detected in the affinity purified RNAPII. We did not detect TBP, TFIIB, TFIIE, TFIIFH,

Fig. 1. Purification of recombinant factors. The various factors were amplified by PCR and cloned in the vector pET-15b. The plasmids containing the cDNA encoding the various factors were transformed into BL21-(DE3) cells and the expression of the protein was induced with isopropyl thio-β-D-galactoside. The various factors were purified as described Materials and methods. Five micrograms of each factor were analysed by SDS/PAGE (8% acrylamide) followed by staining with Coomassie blue. (A) TFIIE; (B) TFIIF; (C) TFIIB; (D) pTBP. Migration positions of molecular size standards are indicated to the left of the panels.
TAF72 or TAF110 in preparations of the *Sc. pombe* RNAPII holoenzyme, as measured by Western blot analysis (data not shown). We estimated that affinity-purified RNAPII preparation contained twofold more RNAPII than the affinity-purified RNAPII holoenzyme preparation, as measured by Western blotting with antibodies to the CTD (Fig. 2C). TFIIH was purified from cell extracts as described in Materials and methods, and its elution from the ACA22 column was followed by Western blotting with antibodies to Srb4, TFIIF and the CTD (Fig. 3B). A very precise coelution of Tbf1 and transcription activity can be seen in Fig. 3A. The TFIIH preparation appeared to contain only a few polypeptides and some of them were identified by N-terminal amino acid microsequencing (Fig. 3B).

**Reconstitution of transcription in vitro using purified recombinant factors from *Sc. pombe***

To reconstitute transcription in vitro, we used purified recombinant *Sc. pombe* TBP, TFIIF, TFIIB, TFIIH, affinity-purified RNAPII, and TFIIH. We used the G-minus template described above, which contained the Ad-MLP promoter and five Gal4 binding sites upstream from the TATA box. The preparation of DNA template was a relaxed plasmid. This promoter has been used by Kornberg’s laboratory to study transcription in *Sc. pombe* [6].

As shown in Fig. 4A, strong transcription from the Ad-MLP was detected when all the factors were present in the reaction (lanes 1 and 8). However, the omission of TBP (lane 2), TFIIH (lane 3), TFIIF (lane 4), TFIIE (lane 5), TFIIIB (lane 6) or RNAPII (lane 7) resulted in no detectable transcription from the Ad-MLP promoter. The transcripts were produced by RNAPII, as their production was sensitive to 5 μg·mL⁻¹ and 10 μg·mL⁻¹ α-amanitin (lanes 9 and 10). Furthermore, the assay is completely dependent on the addition of *Sc. pombe* RNAPII (lane 7). These results indicate that RNAPII and the GTFs are necessary and sufficient to reconstitute *Sc. pombe* transcription in vitro. Furthermore, transcription was initiated at the same position as it was in whole-cell extracts as measured by primer extension (data not shown). We also used the *Sc. pombe* adh promoter in the in vitro transcription assay. The adh promoter displayed essentially the same requirements for transcription as did the Ad-MLP promoter, although transcription levels were lower with the adh promoter than with the Ad-MLP promoter (Fig. 4B, compare lanes 1 and 2 with lanes 3 and 4).

It has been reported that transcription driven by the adh promoter is partially dependent on TFIIH [11]. To address this discrepancy between the previous data and the data reported here we tested, in reconstituted in vitro transcription assays, both supercoiled and linear purified DNA templates that carried the Ad-MLP (Fig. 4C). The supercoiled Ad-MLP was transcribed in the absence of TFIIH at levels similar to those obtained in the presence of TFIIH (Fig. 4D, compare lanes 1 and 2). However, the transcription of linear Ad-MLP templates required TFIIH (compare lanes 3 and 4). We also tested the requirements of the *Sc. pombe* adh promoter in reconstituted in vitro transcription assays. Similar to the results of Sphar et al. [11], we found that the supercoiled templates with the adh promoter can be transcribed in the absence of TFIIH, although transcription was less efficient under these conditions than it was with the Ad-MLP (the minus-TFIIH assay yielded 50% of the amount of transcripts produced in the presence of TFIIH; Fig. 4E).
The RNAPII holoenzyme stimulates basal and activated transcription in a reconstituted transcription system

Next, we tested whether the RNAPII holoenzyme, which contains the Mediator complex, is able to stimulate basal transcription and support activated transcription in our reconstituted system. As shown in Fig. 5A, no detectable levels of basal transcription were observed in the presence of the RNAPII holoenzyme if TBP (lane 1), TFIIB (lane 2), TFIIH (lane 3) or TFIIIE (lane 4) was omitted from the transcription assay. And, as expected, omitting the RNAPII holoenzyme from the reaction completely eliminated basal transcription (lane 5). When TFIIIF was omitted, detectable levels of transcription were observed (lane 6), indicating that the RNAPII holoenzyme preparation contains TFIIF (as detected by Western blotting; Fig. 2B). The addition of TFIIF did not augment transcription, which indicates that TFIIF is in saturating amounts in the RNAPII holoenzyme preparation (lane 7).

The RNAPII holoenzyme was able to carry out basal transcription at a level that was twofold greater than that observed with affinity-purified RNAPII (Fig. 2B, compare lanes 6 and 7 with lanes 8 and 9). In this series of assays, we used the same amount (in ng of protein) of the largest subunit of RNAPII.

The RNAPII holoenzyme was also able to support transcriptional activation (at least fivefold, as measured with a Phosphoimager) by the mammalian Gal4-AP2 transcriptional activator protein (lane 11), indicating that the Mediator is involved in activated transcription. Neither the inclusion of human recombinant TFIIA nor the Gal4 DNA binding domain had an effect on activated transcription (lanes 12 and 10, respectively). We have not tested Sc. pombe TFIIA in our reconstituted assay.

The levels of transcriptional activation observed in the RNAPII holoenzyme assay were lower than those observed with in whole-cell extracts (Fig. 5B). A 20-fold activation of transcription was observed with whole-cell extracts plus VP16 (lane 4), AP2 (lane 3) and CTF (lane 6) transcriptional activator proteins. In the reconstituted assay with the RNAPII holoenzyme, only a 10-, six-, and sixfold activation of transcription was obtained with VP16 (lane 7), AP2 (lane 8), and CTF (lane 9), respectively. The same levels of transcription were obtained in the whole-cell extract without (lane 1) or with (lane 2) Gal4 DNA binding domain and in the reconstituted assay with Gal4 binding domain (lane 3). We also tested the ability of the acidic activator, Gal4-VP16, to activate transcription in the presence of the RNAPII holoenzyme in the reconstituted assay, and we obtained essentially the same results as those observed with Gal4-AP2 (Fig. 5C). However, the activation achieved with Gal4-VP16 was 10-fold, which indicates that the VP16 transcriptional activation domain is more potent than the AP2 domain.

We also investigated whether affinity-purified RNAPII can support activated transcription. As shown in Fig. 5D, affinity-purified Sc. pombe RNAPII was unable to support activated transcription (lanes 3–5). The addition of human TFIIA (lane 6) had no effect on basal transcription carried out by the affinity-purified RNAPII, in fact, human TFIIA inhibited basal transcription with the RNAPII, indicating that its presence may be involved in squelching of the function of transcription factors necessary for transcription. RNAPII holoenzyme is at least twofold more potent in directing basal transcription (compare lanes 1 and 2). Also, we tested the effect of the transcriptional activation domains of CTF and Sp1 in the reconstituted assay using the RNAPII holoenzyme and found that Gal4-CTF is able to activate basal transcription (Fig. 5E, compare lanes 1, 2, and 3) whereas Gal4-SP1 is not (compare lanes 1, 4, and 5).

Antibodies against Srb4 inhibit activated but not basal transcription

We next tested whether the ability of the RNAPII holoenzyme to support activated transcription was the
result of the presence of the Mediator. Antibodies to Srb4, a component of the Mediator complex, were introduced into the *in vitro* transcription reactions with the RNAPII holoenzyme. As shown in Fig. 6A, preimmune serum or antibodies to TAF110 did not inhibit the activation of basal transcription by Gal4-VP16 from the Ad-MLP promoter (Fig. 6, compare lane 2 with lanes 3 and 4). However, antibodies to Srb4 inhibited activated transcription (with the RNAPII holoenzyme and Gal4-VP16) (lanes 5 and 6) but not basal transcription (with the RNAPII holoenzyme) (compare lanes 2, 3 and 4 with lanes 5 and 6). Anti-Srb4 also was not able to inhibit basal transcription when affinity-purified RNAPII was used (lanes 7 and 8). Anti-CTD Igs completely inhibited transcription.
The TAF-containing complex does not have an effect on activated transcription

To test the effect of TAFs on activated transcription, we purified the TAF complex by two methods and tested the effect TAFs on activated transcription using the reconstituted in vitro transcription assay with the RNAPII holoenzyme, the activator Gal4-VP16, and the Ad-MLP. We used affinity chromatography with antibodies to TAF110 as well as conventional chromatography to isolate the TAF complex. The TAF complex isolated by conventional chromatography was similar in its properties to the complex purified by affinity chromatography. A precise coelution from the ACA22 column was observed for TAF110, TAF72 and TBP, as detected by Western blotting of the column fractions with specific antibodies (Fig. 7A). Also, when the affinity-purified and conventionally purified preparations were subjected to gradient SDS/PAGE, the same set of polypeptides was observed in both preparations (Fig. 7B). Also, we observed that the same set of polypeptides can be immunoprecipitated with antibodies against TAF72 and TBP from a preparation that was first immunoprecipitated with anti-TAF110 (lanes 2–4). Furthermore, the complex purified by conventional
chromatography shows the same set of polypeptides as the complex purified by affinity chromatography (lane 5) and the same polypeptides are immunoprecipitated by anti-TAF110 from the ACA22 column (lane 6).

Next, we investigated the role of the TAFs in activated transcription. As seen in Fig. 7C, the addition of affinity purified TAFs to in vitro transcription reactions containing the RNAPII holoenzyme and Gal4-VP16 did not stimulate activated transcription from the Ad-MLP, as the same activation fold is seen in the presence and absence of TAFs (compare lanes 1, 3, 4 and 5). We know that the TAF-containing complex contains TBP, because it can replace TBP, which is a required component, in the transcription assay (lane 2).

Because we used harsh conditions to elute the TAF-containing complex from the anti-TAF110 column, which might have affected TAF coactivator function, we purified the TAF-containing complex under native conditions by conventional chromatography. As shown in Fig. 7D, this TAF-containing complex did not stimulate basal transcription directed by the RNAPII holoenzyme (compare lanes 1 and 2) either in the absence (lane 2) or presence of TFIIA (lane 3). Likewise, TAFs were not able to stimulate basal transcription directed by purified RNAPII (lanes 4–7) either in the absence or presence of TFIIA (lane 5). TAFs were also unable to stimulate activated transcription (compare lanes 9, 10 and 11) even in the presence of TFIIA (lane 11). TBP is contained in the conventionally purified TAF-containing complex, because it can replace TBP in the reconstituted assay (lane 12), which is dependent on the presence of TBP (lane 13).

Discussion

In this report, we have described a reconstituted in vitro transcription assay that contains components from Sc. pombe: purified recombinant TBP, TFIIF, TFIIIB and TFIIIE, affinity-purified RNAPII, and TFIIH. These are the same components required for basal transcription in in vitro assays with factors from human, Drosophila, rat, and S. cerevisiae. Thus there is notable conservation between the GTFs of Sc. pombe and other species, which indicates that the mechanisms of basal transcription have been conserved throughout evolution.

We also demonstrated that, in the reconstituted assay, the RNAPII holoenzyme was able to stimulate basal transcription and support activated transcription through the associated Mediator complex. TAFs had no effect on in vitro transcription carried out by the RNAPII holoenzyme. Ours is the first reported reconstituted in vitro transcription assay from Sc. pombe that can support activated transcription in the presence of the Mediator. This reconstituted assay could serve as a tool to study the mechanisms of transcription in Sc. pombe, an organism that is widely used to study other biological processes.

We have reconstituted in vitro transcription by Sc. pombe RNAPII using two different promoters, the Ad-MLP and the adh promoter. We found that the two promoters have the same factor requirements, but that the level of basal transcription is lower with adh than Ad-MLP. The lower levels of transcription from the adh promoter is not a result of the absence of adh promoter-specific factors, because the levels of transcription from the adh promoter are the same in whole-cell extracts and in the reconstituted system. We believe that the difference is due to promoter strength, because the Ad-MLP possesses a strong TATA box and Initiator, and the Initiator appears not to be present in the adh promoter. When we use circular relaxed templates in our assay, transcription is absolutely dependent on TFIIH. It has been reported by Sphar et al. [11] that in vitro transcription from the adh promoter is partially independent on TFIIH when supercoiled templates are used. We investigated this issue using supercoiled and linear templates with the Ad-MLP and adh promoters. We found that supercoiled templates with either promoter are transcribed in the reconstituted transcription assay without TFIIH, whereas, like the transcription of circular relaxed templates, transcription of linear templates is absolutely dependent on TFIIH. Because the level of transcription from the adh promoter falls by 50% in the absence of TFIIH, while the amount of transcription from the Ad-MLP is the same under both conditions, we believe that transcription from the adh promoter is more dependent on the presence of TFIIH. The difference in the requirement of TFIIH for transcription from the adh and Ad-MLP promoters could be explained by the presence of an Initiator in the Ad-MLP promoter, which can nucleate preinitiation complexes more efficiently than the adh promoter.

The Mediator is a multiprotein complex that contains several polypeptides involved in transcriptional activation. Mediator binds to the CTD of RNAPII to produce the RNAPII holoenzyme and has been purified from S. cerevisiae and several mammals [12–20] A Mediator complex also has been identified in Sc. pombe [21–23] and contains 10 essential gene products that are homologs of the Mediator subunits of S. cerevisiae as well as three non-essential gene products that do not have homologs in other organisms [22]. Recently, a Mediator complex that contained the spsr8, sptrap240, spsrb10 and spsrb11 subunits was isolated from Sc. pombe [23] in a free form, devoid of RNAPII. It has been suggested that spsr8 and spsrb9 are the Sc. pombe homologs of mammalian TRAP230/ARC240 and TRAP240/ARC250, respectively.
We isolated the RNAPII holoenzyme from *Sc. pombe* using the TAP-SpMed7 strain [23] and purified the holoenzyme further using heparin Sepharose chromatography to separate small amounts of the TRAP240 complex, which copurifies with the RNAPII holoenzyme. The RNAPII holoenzyme was more active in our *in vitro* basal transcription assay than was RNAPII, in agreement with the results of Sphar *et al.* [11]. Our holoenzyme preparation also supported activated transcription with two types of transcriptional activators – acidic (VP16) and proline-rich (AP2...
and CTF) – but it did not support transcriptional activation by Sp1. These results strongly suggest that the RNA polymerase holoenzyme contains the Mediator subunits required for activated transcription. These results are in agreement with in vivo data showing that the activation domains of VP16, AP2, and CTF can activate transcription in Sc. pombe [24]. However, the in vivo targets of these activators were not identified in this report. Therefore, we extend these previous observations by demonstrating that these activation domains function via the Mediator. Because AP2 and CTF do not activate transcription in S. cerevisiae, our results suggest that the transcriptional activation process in Sc. pombe is more similar to that of humans than to that of S. cerevisiae.

In our experiments, we observed that activated transcription levels were higher in whole-cell extracts than in our reconstituted Sc. pombe assay, which indicates that additional factors are required for more efficient transcription activation in our reconstituted system. These missing factors could be proteins that are not part of the Mediator such as PC4 or it could be that some key subunits of the Mediator are dissociated from the RNAPII holoenzyme during purification. A homolog of human PC4 is present in Sc. pombe [6]. Also, we found that the RNAPII holoenzyme isolated by us contains TFIIH, a transcription factor that was not found in the preparations of the RNAPII holoenzyme isolated by Spahr [11]. We believe that this difference could result from the method used to detect TFIIH: we used immunoblotting and Spahr used MALDI-TOF. An alternative explanation is that TFIIH is merely a contaminant of the RNAPII holoenzyme preparation.

Antibodies to the Mediator component Srb4 were able to inhibit transcriptional activation by the Gal-VP16 activator. However, these antibodies had no effect on basal transcription. This strongly suggests that the Mediator is involved in transcriptional activation in Sc. pombe. The fact that anti-Srb4 did not inhibit basal transcription directed by the RNAPII holoenzyme is surprising. This result can be explained by the fact that Srb4 contacts RNAPII directly [22] and the anti-Srb4 antibodies can dissociate the Mediator from RNAPII, but the enzyme is still able to transcribe. TAFs are another type of transcriptional coactivator [25]. Although TAFs were shown not to be required for the activation of transcription in a human nuclear extract [26], a later report from the same group showed that the TAFs and Mediator can act jointly to achieve activated transcription [4]. In vivo studies in S. cerevisiae suggest that TAFs are not essential for activated transcription; rather, the TAFs participate in transcription by conferring a dependence on core promoter elements [27–29]. We have purified from Sc. pombe a TAF-containing complex that appears to be the homolog of TFIIH in higher eukaryotes, as it possesses TAFI110, which is the homolog of human TAFII190. Also, TAF72 of Sc. pombe is the homolog of human TAFII100 (isoform 1). For these reasons, we believe that the TAF-containing complex that we purified from Sc. pombe is the homolog of human TFIIH. We assessed the role of the Sc. pombe TAFs in our reconstituted in vitro transcription assay; the results strongly suggest that TAFs are not required for basal or activated transcription. It is important to note that Sc. pombe TFIIA was not included in the reactions, and human TFIIA had no effect on the results. However, we do not know whether human TFIIA can functionally replace Sc. pombe TFIIA in in vitro transcription assays. We do know that human TFIIA can stimulate the binding of Sc. pombe TBP to the TATA box. For this reason, and considering that human TFIIA and Sc. pombe TFIIA have a high degree of homology at the amino acid sequence level, we believe that human TFIIA is likely to be able to replace Sc. pombe TFIIA in in vitro transcription assays. The results we obtained with TAFs are in agreement with other reports that indicate that TAFs are not required for transcriptional activation in vitro in S. cerevisiae [27–29]. However, we do not rule out the following two possibilities: (a) for certain promoters in Sc. pombe, TAFs play a role in transcriptional activation; and (b) another Sc. pombe TAF-containing complex that is different from the homolog of human TFIIA can activate transcription in an Sc. pombe in vitro system.

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