Mediator Influences *Schizosaccharomyces pombe* RNA Polymerase II-dependent Transcription *in Vitro*

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The fission yeast *Schizosaccharomyces pombe* has proved an important model system for cross-species comparative studies of many fundamental processes in the eukaryotic cell, such as cell cycle control and DNA replication. The RNA polymerase II transcription machinery is, however, still relatively poorly understood in *S. pombe*, partially due to the absence of a reconstituted *in vitro* transcription system. We have now purified *S. pombe* RNA polymerase II and its general initiation factors TFIIB, TFIIF, TFIIE, and TFIIH to near homogeneity. These factors enable RNA polymerase II to initiate transcription from the TATA box promoter, whereas Mediator lacking these subunits has a stimulatory effect on transcription. Our findings thus demonstrate that the spSrb8/spTrap240/spSrb10/spSrb11 subcomplex governs the ability of Mediator to stimulate or repress basal transcription *in vitro*.

The minimal set of general transcription factors required for basal transcription *in vitro* include, RNA polymerase II (pol II), the TATA-binding protein (TBP), and transcription factors (TFs) IIB, IIE, IIF, and IIF. These factors are both necessary and sufficient for proper initiation of transcription in transcription systems reconstituted from *Saccharomyces cerevisiae* and mammalian cells (1). The structure and function of *Schizosaccharomyces pombe* pol II has been investigated in great detail. The enzyme is composed of 12 subunits, Rpb1 to Rpb12, similar to what has been described for the budding yeast *S. cerevisiae* and mammalian cells (2, 3). Biochemical analysis of the enzyme has revealed the presence of several distinct subcomplexes and has proved important in the analysis of the recently published x-ray crystallography structure of *S. cerevisiae* pol II (4, 5). Previous characterization of *S. pombe* TFIIH has only concerned a specific subcomplex of this transcription factor, containing the Mcs6, Mcs2, and Pmh1 gene products (6). These proteins correspond to the cyclin-dependent kinase 7 (Cdk7), cyclin H, and Mat1, in mammalian TFIIH. Cdk7 phosphorylates the C-terminal domain (CTD) of the largest subunit of pol II upon initiation of transcription (7–9). Interestingly, Cdk7 is also the mammalian cdk-activating kinase (Cak): the kinase needed for the activating phosphorylation of other cyclin-dependent kinases (10). A similar situation also appears to exist in *S. pombe*, where the trimeric Mcs6-Mcs2-Pmh1 complex also possesses CAK activity (6).

We have reported previously on the purification and characterization of the *S. pombe* Mediator (11–13). The Mediator complex is essential for basal and regulated expression of nearly all pol II-dependent genes in *S. cerevisiae* (14), and deletion of human Mediator from nuclear extracts abolishes transcription by pol II (15). The *S. pombe* Mediator complex exists in at least two specific forms (13): one smaller core Mediator in complex with pol II and one larger form of Mediator, devoid of pol II, but containing the spSrb8, spTrap240, spSrb10, and spSrb11 proteins. Homologues to these four proteins are found in certain *S. cerevisiae* and human Mediator preparations and they are collectively referred to as the Srb–11 module (13, 16). The Srb11 and Srb10 genes encode cyclin C and the cyclin C-dependent kinase, respectively (17), and genetic analysis indicates that the Srb8–11 module is involved in the negative regulation of a small subset of genes during exponential growth (13, 18). Why Mediator exists in two different forms and what differential roles these complexes have in transcriptional regulation are still unclear.

**MATERIALS AND METHODS**

*Genetic Manipulations—* The *S. pombe* strains used in this work are: L972 (h+), Mp12 (h− ade6-M210) and Mp13 (h− ade6-M216), TP33 (h+ sptrap240:G418), TP46 (h− ade6-M216 smed7−:TAP), and TP68 (h+ ade6-M216 sptrap240−:TAP). The TAP-Pmh1 strain (h+ pmh1−:pmh1-TAP kanR ade6−210 ura4−D18 leu1−32 his3−D1) was constructed as described previously (19) with the following oligonucleotides: 5′-GTCATTTGCGATGCTTATATGAGGCTTTAGTGGCATTG-3′ and 5′-GTTAAATACGACTCACTATAGGG-3′.

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purified from a TAP-spMed7 strain in which described in the BacPAK manual (Clontech). volumes used to wash the IgG-Sepharose and calmodulin-Sepharose alternative, less stringent protocol to monitor for additional proteins, brated with Buffer D-0.4. The column was washed with 25 column Bioscience) coupled to antibody at 2 mg/ml), which had been equili-

Toa2). During PCR a His6-tag was introduced at the C terminus of

to basal transcription

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13/Mediator and the pol II holoenzyme

Toa1, sp-

essentially pure TFIIF eluted at about 0.8M potassium acetate concentration in molar units) overnight. The dialyzed material was collected by sedimentation, loaded on a Mono Q 5/5 FPLC column (Amersham Biosciences) which had been equilibrated in buffer Q-0.1. The column was washed with 5 ml of buffer Q-0.1 and eluted by a linear gradient (15 ml) of buffer Q-0.1 to C-1.5. Essentially pure TFIIF eluted at about 900 mKAc.

Purification of TFIIF we co-infected Sf9 cells with recombinant baculoviruses expressing spTfg1, spTfg2, and spTfg3 at a multiplicity of infection of 5 for each virus. Cells were collected 72 h after infection by centrifugation (JA-10, 3,500 rpm 10 min, 4 °C), washed with ice-cold PBS, and centrifuged as before. Cells were dissolved in 10 ml of buffer B-0 (25 mM Tris-HCl (pH 8.0), 10 mM β-mercaptoethanol, and 1× protease inhibitors; the number after the hyphen indicates the potassium acetate concentration in molar units) overnight. The dialyzed material was loaded on a Mono Q 5/5 FPLC column (Amersham Biosciences) which had been equilibrated in buffer Q-0.1.

To analyze Mediator complexes on basal transcription

In Vitro Transcription—Transcription assays were performed as de-
scribed previously (22) with the following modifications. In the G-less cassettes, we used different concentrations of purified proteins, essentially as described previously (12). In Vivo Transcription—Transcription assays were performed as de-
scribed previously (22) with the following modifications. In the G-less cassettes, we used different concentrations of purified proteins, essentially as described previously (12). We wanted to examine the influence of the spSrB8–11 complex on basal transcription in vitro. To this end we needed to purify a defined Mediator complex, lacking the spSrB10 cyclin-dependent kinase. We used a tandem affinity purification

We purified core TFIIH from a strain containing a C-terminal TAP-

we used a tandem affinity purification

For purification of TFIIK we co-infected Sf9 cells with recombinant baculoviruses expressing Toa1 and Toa2 at a multiplicity of infection of 5 for each virus. Cells were collected 72 h after infection by centrifugation (JA-10, 3,500 rpm 10 min, 4 °C), washed with ice-cold PBS, and centrifuged as before. Cells were dissolved in 10 ml of buffer B-0 (25 mM Tris-HCl (pH 8.0), 10 mM β-mercaptoethanol, and 1× protease inhibitors; the number after the hyphen indicates the potassium acetate concentration in molar units) overnight. The dialyzed material was loaded on a Mono Q 5/5 FPLC column (Amersham Biosciences) which had been equilibrated in buffer Q-0.1. The column was washed with 5 ml of buffer Q-0.1 and eluted by a linear gradient (15 ml) of buffer Q-0.1 to C-1.5. Essentially pure TFIIF eluted at about 0.8 mKAc.

Purification of S. cerevisiae TBP was as described previously (21).

The identification of proteins in this report, by MALDI-TOF analysis of in vitro digested proteins was essentially as described previously (12).
(TAP) tag on spMed7 and purified Mediator from a yeast strain carrying a deletion of sptrap240. The sptrap240 Mediator was purified in complex with pol II, forming a holoenzyme (holoenzyme was purified in complex with pol II, forming a holoenzyme assay (Fig. 1, holoenzyme was purified in complex with pol II, forming a holoenzyme complex (Fig. 3 A). The individual subunits were identified with MALDI-TOF mass fingerprinting as Mcs2, Mcs6, and Pmh1 (Table I). A similar subcomplex of TFIIH has also been identified in S. cerevisiae and denoted TFIIK (7). Notably absent in our purified complex were the subunits of core TFIIH.

We next purified S. pombe RNA pol II using an anti-CTD monoclonal antibody column and generated a basically homogenous material (Fig. 2 C). We identified the subunits of pol II using MALDI-TOP mass fingerprinting, and our results were in perfect agreement with the subunit analysis reported previously (data not shown) (2).

In S. cerevisiae and higher eukaryotic cells, TFIIH contains nine subunits (25). To purify S. pombe TFIIH, we introduced a TAP-tag on the C terminus of Pmh1. Purification on IgG-Sepharose and Ca\(^{2+}\)/calmodulin-Sepharose generated a trimeric complex (Fig. 3A). The individual subunits were identified with MALDI-TOP mass fingerprinting as Mcs2, Mcs6, and Pmh1 (Table I). A similar subcomplex of TFIIH has also been identified in S. cerevisiae and denoted TFIIK (7). Notably absent in our purified complex were the subunits of core TFIIH.

In an attempt to isolate the holo-form of TFIIH (core + TFIIK), we used an alternative protocol, in which we decreased the Nonidet P-40 detergent concentrations during the TAP purification (see “Materials and Methods”). In these preparations we could identify substoichiometric amounts of Rad15 the S. pombe homologue to the S. cerevisiae Rad3 helicase (Fig. 3B) (26). No other components of TFIIH was, however, found in the TAP-Pmh1 preparations, suggesting that TFIIH may be a less stable complex in S. pombe than what has been described in S. cerevisiae and human cells (25). To purify the S. pombe core-TFIIH complex, we fused a TAP-tag to the C-terminal part of spTib2. TAP purification generated a five-subunit complex, which was characterized with MALDI-TOP mass fingerprinting as Ere3sp, spTib1, spTib2, spSsl1, and spTib4 (Fig. 3B).
The MALDI-TOF fingerprinting data of the identified TFIIH subunits are summarized in Table I. We tested the ability of TFIIK to phosphorylate the C-terminal domain of pol II in the presence and absence of core TFIIH (Fig. 3C). Phosphorylation was detected in both cases, although the level was slightly lower in the presence of core TFIIH.

We identified the SPAC16E8.16 gene as a putative *S. pombe* TFIIH homologue. The gene product was fused in-frame with a C-terminal His6-tag, expressed in *E. coli*, and purified to near homogeneity (Fig. 4A). TFIIIE contains two subunits in *S. cerevisiae*, which are encoded by the TOA1 and TOA2 genes. We identified two highly conserved homologues to these genes in *S. pombe*, which upon co-expression in insect cells generated dimeric TFIIIE, which could be purified to homogeneity over Ni2+-agarose and DEAE-Sepharose (Fig. 4B).

We next set out to reconstitute transcription *in vitro*. We used negatively supercoiled templates with either the adenovirus major late promoter or the *S. pombe* adh1 promoter (adh1p) followed by a G-less cassette. Addition of all the factors did indeed generate strong, promoter specific transcription from the endogenous *S. pombe* promoter (Fig. 5A). Lower levels of *in vitro* transcription could also be observed from the adenovirus major late promoter construct (data not shown). To test the activity of each individual transcription factor we performed a dropout transcription assay where one factor at the time was left out (Fig. 5A). We found that transcription was completely abolished if TBP, TFIIIB, pol II, or TFIIIE were omitted. In the absence of TFIIIF we could observe a low level of transcription (2% relative the complete reaction), probably due to small amounts of TFIIIF contaminating the purified pol II. Our reactions were less dependent on TFIIH, which had a 3-fold stimulatory effect on basal transcription. In agreement with our observation, TFIIH is not needed for transcription of certain promoters on negatively supercoiled templates in mammalian *in vitro* transcription systems (27–29). In contrast, our *S. pombe* *in vitro* transcription system appears to be more dependent on TFIIIE than the human system (27, 28), since we can observe no transcription in the absence of this factor.

We next tested the effects of holoenzymeSpTrap240 and spSrb8–11/Mediator on basal transcription (Fig. 5B). We found that Mediator devoid of spSrb8–11 had a significant stimulatory effect on basal transcription. In contrast, Mediator containing spSrb8–11 had a strong negative effect, diminishing the basal levels of transcription up to 5-fold. The stimulation of basal transcription observed for holoenzymeSpTrap240 is not merely an effect of adding more pol II, since the addition of free pol II to the same concentrations as found in the holoenzymeSpTrap240 preparation at best had a modest (1.2-fold) stimulatory effect on the transcription reaction (data not shown). Neither do we believe that the inhibitory effect associated with spSrb8–11/Mediator is due to any contaminating factor. When we do repeated elutions of Srb8–11/Mediator at the Ca2+/calmodulin-Sepharose purification step, we find that the levels of inhibition observed in our *in vitro* transcription system correlates well with the relative amounts of Mediator and spSrb10 associated CTD-kinase activity found in the individual eluted fractions (Fig. 5C).

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**Fig. 3. Characterization of TFIIH.** A, TFIIK (TAP-Pmh1) was purified according to the high stringency procedure described under “Materials and Methods.” The proteins were separated on a 10% SDS-PAGE gel, revealed by Coomassie Brilliant Blue, and identified with MALDI-TOF mass fingerprinting. Arrows on the left indicate identified proteins, and molecular masses according to standard are indicated on the right. The band below spTfb4 (c) is a contaminant. B, Rad3 associates with TFIIK. TFIIK (TAP-Pmh1) was purified according to the low stringency procedure described under “Materials and Methods.” The eluate was analyzed as described in A. The major contaminants Actin and Ef1a are indicated. C, core TFIIH (TAP-spTfb2) was purified and analyzed as described in the legend to A. D, RNA polymerase II (core) was phosphorylated by TFIIK. The presence of core TFIIH did not significantly influence the CTD kinase activity.

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**Table I**

| Protein | *S. cerevisiae* homologue | Number of peaks | Sequence coverage | Theoretical mass | Gene name (Sanger center) |
|---------|--------------------------|-----------------|------------------|-----------------|--------------------------|
| Pmh1    | Tfb3                     | 13              | 38%              | 37kDa           | SPBC776.18c              |
| Ms6     | Kin28                    | 10              | 33%              | 39kDa           | SPBC19F8.07              |
| Ms2     | Cct1                     | 5               | 14%              | 38kDa           | SPB16F5.02               |
| Rad15   | Rad3                     | 7               | 11%              | 88kDa           | SPAC1D4.12               |
| Erc3sp  | Ssa2                     | 9               | 19%              | 92kDa           | SPAC17A5.06              |
| spTfb1  | Tfb1                     | 11              | 29%              | 55kDa           | SPAC16E8.11c             |
| spTfb2  | Tfb2                     | 13              | 32%              | 51kDa           | SPBC13G1.13              |
| spSs1   | Ss1                      | 6               | 16%              | 45kDa           | SPC16B2.07               |
| spTfb4  | Tfb4                     | 6               | 25%              | 34kDa           | SPBC30B4.07c             |

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We used the in vitro system for regulated pol II transcription in these two yeast species. Here we report on the reconstitution of a highly purified in vitro system for S. pombe pol II transcription. The purified S. pombe TFIIIF contains three subunits, in contrast to metazoan TFIIIF, which only contains two subunits. In S. cerevisiae this additional third subunit, Tfg3/Taf30/Anc1, appears only weakly associated with TFIIIF, since it does not stay associated with the protein complex during gel filtration (24). Interestingly, the Tfg3/Taf30/Anc1 protein has also been identified as a member of the yeast SWI/SNF complex (30) as well as TFIIID (24). The phenotypes of the tfg3Δ S. cerevisiae strain, however, demonstrate that Tfg3 is not essential for many of the tasks associated with these complexes (24). Our finding that spTfg3 is absolutely required for reconstitution of S. pombe TFIIIF in recombinant form could suggest that the protein rather play a role as an assembly factor of multiprotein complexes.

We recently reported that Mediator in S. cerevisiae and S. pombe exists in at least two specific forms (13). The smaller of these forms is the core Mediator, which may interact with pol II and form a holoenzyme. Another larger form, which contains four additional Mediator subunits, spSrb8, spTrap240, spSrb10, spSrb11, is always isolated devoid of pol II. These proteins collectively form the Srb8–11 module, which was recently isolated in free form (31). We here demonstrate that Mediator isolated from a sprapo240Δ deletion strain also lacks spSrb10. Apparently spTrap240 acts as an anchor for the other components of the module. Our isolation of these two well-defined forms of Mediator also allows us to compare their effects on basal transcription in vitro. We find that core Medi-
ator has a stimulatory effect on basal transcription, whereas the spSrb8–11-containing Mediator has an inhibitory effect. This observation could lend support to our previous speculation that the key role of spSrb8–11 may be to repress Mediator function prior to transcriptional activation (13).

Another possible role for the spSrb8–11 submodule is in global down-regulation of transcription upon entry into stationary phase. Specific growth factors and the availability of essential nutrients, control eukaryotic cell proliferation. If either of these signals is lacking, cells may enter into a specialized non-dividing resting state, known as stationary phase or G0, characterized by a considerable down-regulation of transcription upon entry into stationary phase and that the spSrb8–11 module is to down-regulate transcription upon entry into stationary phase and that the in vitro data presented here reconstitute this specific event.

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