Purification and Characterization of RNA Polymerase II Holoenzyme from Schizosaccharomyces pombe*

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We have purified the RNA polymerase II holoenzyme from Schizosaccharomyces pombe to near homogeneity. The Mediator complex is considerably smaller than its counterpart in Saccharomyces cerevisiae, containing only nine polypeptides larger than 19 kDa. Five of these Mediator subunits have been identified as the S. pombe homologs to Rgr1, Srb4, Med7, and Nut2 found in S. cerevisiae and the gene product of a previously uncharacterized open reading frame, PMC2, with no clear homologies to any described protein. The presence of Mediator in a S. pombe RNA polymerase II holoenzyme stimulated phosphorylation of the C-terminal domain by TFIIFII purified from S. pombe. This stimulation was species-specific, because S. pombe Mediator could not stimulate TFIIFII purified from S. cerevisiae. We suggest that the overall structure and mechanism of the Mediator is evolutionarily conserved. The subunit composition, however, has evolved to respond properly to physiological signals.

An in vitro system reconstituted with pure general transcription factors and RNA polymerase II from Saccharomyces cerevisiae can support basal transcription, but it is not responsive to transcriptional activators. This observation prompted a search for co-activators, which could support activated transcription in vitro. Such an activity was purified to homogeneity and demonstrated to be a holoenzyme form of RNA polymerase II made up of core polymerase and a Mediator complex (1). Mediator was subsequently purified as a discrete entity (2). In addition to its function as a co-activator, Mediator can stimulate both basal transcription and phosphorylation of the C-terminal domain (CTD) of the polymerase (1, 2).

The twenty different polypeptides that have been identified as members of Mediator in S. cerevisiae can be divided into three subgroups. The first group contains the SRB gene products, which were identified in a screen for RNA polymerase II CTD-interacting proteins (3). The Srb proteins were subsequently isolated in a complex with RNA polymerase II giving the first indication of the existence of a RNA polymerase II holoenzyme (4). A second group contains the products of the GAL11, RGR1, SIN4, PGD1/HRS1, NUT1, NUT2, and ROX3 genes, which were all discovered in genetic screens for mutations affecting transcription in positive as well as negative ways (5, 6). The third subgroup comprises the MED genes, which were all previously uncharacterized, and whose products were identified through peptide sequencing (2, 7, 8).

The critical importance of Mediator in transcriptional regulation has been demonstrated using genome-wide expression analysis. Loss of Srb4 function essentially eliminates transcription from all RNA polymerase II-dependent promoters (9). For some other Mediator components the effects are less dramatic, e.g. Med6 is needed for expression of approximately 10% of all genes in the yeast genome (9). In another study, deletion of the Med2 subunit abolished activation by Gal4-VP16 both in vitro and in a pure in vitro system for transcription (10). The effect of this deletion was specific, because activation with another activator, Gcn4, as well as stimulation of basal transcription and enhancement of the TFIIFII-associated CTD kinase activity were unaffected.

Coactivator complexes containing homologs to Mediator subunits have now been identified in mouse and human cells (11–15). The human SRB/MED-containing cofactor complex (SMCC) can act synergistically with the coactivator PC4 and support the function of the p53 activator domain (13). Importantly, SMCC also contained components (TRAP220, TRAP170/hRgr1, and TRAP100), which are involved in thyroid hormone receptor function (16). Another complex, CRSP, was isolated on the basis of its ability to support Sp1-dependent activation in vitro (14). CRSP shares many subunits with SMCC, and the two complexes might even be identical. These data demonstrate the presence of Mediator in higher cells and strongly indicate that this complex plays an essential role for a wide spectrum of transcriptional activators.

Transcription in Schizosaccharomyces pombe differs from S. cerevisiae in several ways. Glutamine-rich activation domains act similarly in S. pombe and mammalian cells but are unable to stimulate transcription in S. cerevisiae (17). In mammalian cells and S. pombe, initiation of transcription occurs ~30 bp downstream of the TATA box, whereas in S. cerevisiae this distance can vary between 40 and 120 bp (18). A description of the molecular basis for these differences would be of importance for our understanding of transcription mechanisms in higher cells.

We now present the purification and a partial characterization of RNA polymerase II holoenzyme from S. pombe. This multiprotein complex contains homologs to Rgr1, Srb4, Med7,
and Nut2 proteins, representing each of the three subgroups identified in Mediator purified from *S. cerevisiae*. We also identify the gene product of a previously uncharacterized open reading frame, PCM2, as a member of the *S. pombe* RNA polymerase II holoenzyme.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Immunoblot Analyses**—Recombinant spMed7 (aa 178–376) and Pmc2 (aa 295–454) proteins fused to glutathione S-transferase (GST) were overproduced in *Escherichia coli* BL21(DE3)pLysS cells and subsequently purified using glutathione-Sepharose 4B (Amersham Pharmacia Biotech) according to the supplier’s recommendations. Recombinant spSrb4 (aa 307–542) fused to a 6xHis tag was purified using nickel-nitrilotriacetic acid superflow (Qiagen’s recommendations). Recombinant spSrb4 (aa 307–542) fused to a 6xHis tag was purified using nickel-nitrilotriacetic acid superflow (Qiagen’s recommendations). Recombinant spMed7, GST-Pmc2, and 6xHis-spSrb4 proteins were used to immunize rabbits. Rabbits were also immunized with a synthetic polypeptide corresponding to the 15 N-terminal amino acids of spNut2. The antiserum used in this study was taken 10 days after the second booster injection (Antibody AB, Lund, Sweden).

**Protein Purification**—A wild type *S. pombe* strain (972h-) was grown to an *A*<sub>600</sub> value of 3 in 96 liters of YPD. Cells were harvested by centrifugation and resuspended in double distilled H<sub>2</sub>O and centrifuged again. The yeast (900 g) was suspended in 3 × lysis buffer (containing 0.45 M Hepes-KOH (pH 7.6), 30% glycerol, 3 mM EDTA, 0.15 M KAc, 3 mM DTT, and protease inhibitors) and the cells were disrupted in a Mini BeadBeater (20 cycles, 30 s activity, 90 s rest). The supernatant was collected after centrifugation (Beckman JA-10, 9000 rpm, 25 min, 4 °C) and 1/9 volume of 5 M potassium acetate was added followed by stirring for 15 min. Polyethyleneimine was added to a final concentration of 0.2% (w/v), and stirring was continued for another 30 min. The lysate was centrifuged in a Beckman JA-20 rotor at 15,000 rpm for 90 min. The supernatant (15 g of total protein) was diluted with buffer A-0 (containing 25 mM Hepes-KOH (pH 7.6), 10% glycerol, 1 mM EDTA, 1 mM DTT, protease inhibitors, and the molarity of potassium acetate indicated after the hyphen) to the conductivity of buffer A-0.15 and loaded on a 400-ml Bio-Rex 70 column at 2 column volumes/h. The column was washed with 1 column volume buffer A-0.15 and 2 column volumes of buffer A-0.3 and eluted with buffer A-0.6. The protein peak (1.24 g of total protein) was diluted with buffer B-0 (containing 20 mM Tris acetate (pH 7.8), 10% glycerol, 1 mM EDTA, 1 mM DTT, protease inhibitors, and the molarity of potassium acetate indicated after the hyphen) to the conductivity of buffer B-0.1 and fractionated on a 40-ml DEAE-Sepharyl Fast Flow column (Amersham Pharmacia Biotech) column. The column was loaded at 8 column volumes/h, washed with 80 ml of buffer B-0.1 and 120 ml of buffer B-0.2, and eluted with buffer B-0.55. The protein peak (184 mg of total protein) was applied to a hydroxyapatite column, which was developed as described earlier (19). The holoenzyme peak (15.5 mg of total protein) was diluted with one volume buffer Q-0 (containing 20 mM Tris-Ac (pH 7.8), 10% glycerol, 1 mM EDTA, 1 mM DTT, protease inhibitors, and the molarity of potassium acetate indicated after the hyphen) and loaded at 0.5 ml/min on a Mono Q 5/5 column (Amersham Pharmacia Biotech). After washing with 10 ml of buffer Q-0.12 and 10 ml of Q-0.4, the column was developed with a linear gradient (15 ml) of buffer Q-0.4 to Q-1.2. The peak of holoenzyme (2.73 mg of total protein) was pooled and dialyzed against H-0.1 (containing 20 mM potassium phosphate (pH 7.6), 10% glycerol, 0.2 mM EDTA, 1 mM DTT, protease inhibitors, and the molarity of potassium acetate indicated after the hyphen) at room temperature for 4 h.

**Protein Identification**—Holoenzyme fractions from the Heparin stage were separated on SDS-PAGE (12%) and revealed by staining with.
Coomassie Brilliant Blue R250. Each protein band from the Coomassie Brilliant Blue-stained SDS-gel was cut out, placed in separate siliconized tubes, and gently macerated with a hand-hold mixer. The Coomassie Brilliant Blue dye was removed by adding 80 μl of ammonium bicarbonate (25 mM) in 50% acetonitrile, and the tube was vortexed for 10–15 min before the supernatant was removed. The washing procedure was repeated three times. The destained gel pieces were dried for 30 min in a speed-Vac before 10–15 μl (depending on the size of the gel) of trypsin in ammonium bicarbonate (25 mM, pH 8) was added until the gel pieces were covered with solution. The tubes were incubated overnight at 37 °C. The protein was extracted from the gel with 10–15 μl of 75% acetonitrile/5% trifluoroacetic acid. The tubes were vortexed for 20 min and centrifuged for 2 min at 5000 × g. The matrix-assisted laser desorption ionization spectra were collected on a Micromass time of flight-Spec E (Micromass, Manchester, United Kingdom) with delayed extraction in reflectron mode. First, 0.5 μl of matrix solution (containing 10 mg of α-cyano-4-hydroxy-cinnamic acid (Sigma) in 1 ml of acetonitrile/water 1:1) was added to the target. Subsequently, 0.5 μl of the supernatant of the sample was mixed with the matrix solution directly on the target and left to dry in air. The peptide monoisotopic masses from the matrix-assisted laser desorption ionization spectra were then matched against theoretical tryptic peptides in a protein data base for identification (20). MS-Fit was used for this purpose.

**RESULTS**

To investigate the occurrence of a *S. pombe* RNA polymerase II holoenzyme, we used all the previously identified subunits of the *S. cerevisiae* Mediator in a BLAST similarity search of the *S. pombe* data base. Three open reading frames encoding possible *S. pombe* homologs to the Srb4, Med7, and Nut2 proteins were identified and denoted *spSRB4*, *spMED7* (Fig. 1A and B), and *spNUT2* (21). Polyclonal antibodies were generated against the three putative *S. pombe* Mediator subunits and used, in concert with the 8WG16 antibody directed toward CTD (22), to monitor the fractionation of a *S. pombe* extract.

Total cell extract was prepared from *S. pombe* 972h- cells and processed over Bio-Rex 70, DEAE-Sepharcl FF, and hydroxyapatite. At this stage fractions were analyzed with immunoblotting. One distinct peak of RNA polymerase II around 80 mM KPO₄ coincided with a peak of the spNut2 protein, indicating the possible existence of a RNA polymerase II holoenzyme (data not shown). The peak fractions from the hydroxyapatite step were further fractionated over Mono Q and heparin columns. At this stage, an immunoblot analysis demonstrated that Rpb1, spSrnb4, spMed7, and spNut2 all comigrated exactly with each other (Fig. 2A). The peak of possible RNA polymerase II holoenzyme subunits coincided with a peak of several polypeptides revealed by SDS-PAGE (Fig. 2B). A peak fraction (Fig. 2B, fraction 20) from the heparin stage was subjected to gel filtration through Superose 12 at high ionic strength. The analysis revealed RNA polymerase II holoenzyme as a distinct complex of about 15 polypeptides above 19 kDa (Fig. 3A and B). Six of these polypeptides corresponded in size to previously identified components of core RNA polymerase II. The bands corresponding to spSrnb4, spMed7, and spNut2 were confirmed with immunoblotting (data not shown). Six additional bands were unaccounted for and denoted Pmc1 to Pmc6 for pombe Mediator complex.

To further analyze the subunit composition of the RNA polymerase II holoenzyme, large amounts of protein were separated on a SDS-PAGE and stained with Coomassie blue. Bands were cut out and identified through trypsin cleavage and mass fingerprinting using matrix-assisted laser desorption ionization-time of flight. In this way, the presence of all known subunits of core RNA polymerase II, as well as spSrnb4 and spMed7 could be confirmed with high accuracy (Table I). Two of the Pmcns, Pmc1 and Pmc2, generated mass fingerprinting data compatible with hypothetical open reading frames in the *S. pombe* data base (Table I). The Pmc3, Pmc4, Pmc5, and Pmc6 proteins apparently correspond to genes not yet sequenced in the *S. pombe* genome project, because no definitive matches could be found despite good quality mass fingerprinting data.

The amino acid sequence of Pmc1 and Pmc2 were used for a BLAST similarity search of the GenBank™ or EST data bases. Here, Pmc1 revealed a short stretch of homology to hRgr1/TRAP170/CRSP150 (data not shown). On the basis of this homology Pmc1 was compared with Rgr1, hRgr1/TRAP170/CRSP150, and a Rgr1 homolog from *C. elegans* using the Best Fit software (Fig. 4A). This comparison revealed a short stretch of homology between each of these proteins and the region spanning amino acids 46 and 118 in Pmc1. The homologous regions were then used for a multiple sequence alignment using CLUSTAL (Fig. 4B). The weak sequence homologies in concert with the biochemical identification in Mediator led us to conclude that Pmc1 is the *S. pombe* homolog to Rgr1.

No significant homologies could be found for Pmc2 with any entries in the GenBank™ or EST data bases. This protein therefore falls into the family of Mediator subunits with no
known homologies in other eukaryotic cells. To confirm its presence in the Mediator, Pmc2 was expressed in recombinant form, and polyclonal antibodies were obtained. Immunoblot analysis of heparin fractions demonstrated coelution of Pmc2 with spSrb4 and Rpb1 (Fig. 5).

Because no fully defined S. pombe transcription system has been established yet, we have not been able to obtain in vitro evidence for the ability of the Mediator to support activated transcription. Another hallmark activity of S. cerevisiae Mediator, however, is stimulation of the TFIIH-associated CTD kinase (1, 2). To test if our Mediator was active in this respect, S. cerevisiae TFIIH was incubated with equimolar amounts of core RNA polymerase II or RNA polymerase II holoenzyme from S. pombe (Fig. 6A). In this experiment no specific stimulation of CTD kinase activity could be detected. However, when the experiment was repeated with TFIIH purified from S. pombe a 4.7-fold stimulatory effect was observed (Fig. 6B). We conclude that S. pombe Mediator can stimulate the TFIIH-

![Table I](image)

| Gene   | MOWSE scorea | S. cerevisiae homolog | Human homolog | Protein mass | Apparent massb | S. pombe ORFc |
|--------|--------------|-----------------------|---------------|--------------|----------------|----------------|
| RPB1   | $5.22 \times 10^{12}$ | +                     | +             | 194          | 190            |                 |
| RPB2   | $4.8 \times 10^9$     | +                     | +             | 138          | 120            |                 |
| PMC1   | $6.01 \times 10^6$    | +                     | +             | 77.3         | 90             | SPBC1A4.10c     |
| SRB4   | $2.15 \times 10^7$    | +                     | +             | 62.5         | 62             | SPBC31F10.04c   |
| MED7   | $7.51 \times 10^7$    | +                     | +             | 43.5         | 51             | SPBC14F.08      |
| PMC2   | $3.02 \times 10^7$    | +                     | +             | 51.4         | 49             | SPAC2F7.04      |
| RPB3   | $9.08 \times 10^6$    | +                     | +             | 33.5         | 43             |                 |
| PMC4   | 38            |                       |               |              |                |                 |
| PMC5   | 33            |                       |               |              |                |                 |
| PMC6   | 30            |                       |               |              |                |                 |
| RPB5   | $6.25 \times 10^4$   | +                     | +             | 23.9         | 30             |                 |
| RPB6   |  +            |                       |               | 15.7         | 25             |                 |
| RPB7   | $1.05 \times 10^7$   | +                     | +             | 19.1         | 22             |                 |
| NUT2d  |              |                       |               | 15           | 20             | SPBC31F10.09c   |

a Ref. 28.
b As judged by SDS-PAGE analysis with respect to molecular weight standards.
c ORF, open reading frame.
d The Nut2 band was not analyzed using matrix-assisted laser desorption ionization-time of flight spectrometry.

![Fig. 4](image)

A, Pmc1 homologous to Rgr1, hRgr1/TRAP170/CRSP150, and C. elegans Rgr1. A, results from a Best Fit alignment of Pmc1 and Rgr1 homologs. B, a multiple sequence alignment of the sequences found in A using CLUSTAL. Identical and related amino acids are shaded.

![Fig. 5](image)

Fig. 5. Pmc2 comigrates with Rpb1 and spSrb4 during heparin chromatography. The peak of RNA polymerase II holopolymerase from Mono Q was applied to a 1 ml of heparin HiTrap column and eluted with a 12 ml gradient of 0.1-1.0 M potassium acetate. Fractions were separated on a 12% SDS-PAGE and immunoblotted with antibodies directed against the proteins indicated.
FIG. 6. RNA polymerase II holoenzyme specifically stimulates CTD phosphorylation by S. pombe TFIIH. Equimolar amounts of RNA polymerase II (Core) and RNA polymerase II holoenzyme were phosphorylated by TFIIH purified from S. cerevisiae (A). No stimulatory effect of Mediator could be detected. B, the same as in A but with TFIIH purified from S. pombe. CTD kinase activity was 4.7-fold higher in the presence of Mediator. C, Western blot analysis of CTD.

associated CTD kinase activity and that this stimulation is species-specific.

DISCUSSION

Some fundamental aspects of transcription differ between S. pombe and S. cerevisiae. In S. cerevisiae the distance between the TATA box and the start site for transcription is between 40 and 120 bp, whereas in S. pombe and human cells the distance is about 30 bp. To investigate the molecular basis for this difference Li et al. (18) employed an in vitro transcription system derived from S. cerevisiae and replaced combinations of general transcription factors with their S. pombe counterparts. This analysis demonstrated that RNA polymerase II and TFIIIB together are responsible for the distance from the TATA box to the start site for transcription. The ability of different activator domains to elicit transcriptional activation also differ significantly between S. pombe and S. cerevisiae (17). For example, mammalian activator proteins containing acidic-rich and/or proline-rich activation domains have been shown to stimulate transcription in both S. cerevisiae and S. pombe. In contrast, the glutamine-rich activation domains of Oct1, Oct2, and Sp1 can stimulate transcription only in S. pombe. It therefore appears that the function of glutamine-rich domains in transcription is conserved in S. pombe and human cells but not in S. cerevisiae. The molecular basis for the observed differences in activator function is unclear, but it is evident that an in vitro comparison of RNA polymerase II holoenzyme purified from S. cerevisiae and S. pombe would provide insight into Mediator-mediated transcriptional activation in higher cells.

In this paper we have developed a method to purify RNA polymerase II holoenzyme from S. pombe to near homogeneity. SDS-PAGE analysis of the purified holoenzyme revealed about 15 different subunits of a molecular mass greater than 19 kDa (Fig. 3). Six of these subunits corresponded to previously identified components of core RNA polymerase II (Table I). Three subunits, spSr84 (Fig. 1A), spMed7 (Fig. 1B), and spNut2 (21), were homologs to proteins present in the S. cerevisiae Mediator. The Mediator part of the holoenzyme also contained six additional subunits, which were denoted Pmc1–6 for pombe Mediator complex. Pmc1 and Pmc2 could be identified through peptide mass fingerprinting as the products of two previously uncharacterized open reading frames. No open reading frames could be identified for the Pmc3–6 proteins, despite good quality mass fingerprinting data. We therefore assume that these proteins are products of genes not yet sequenced in the S. pombe genome sequencing project.

Mutations in SRB4 essentially eliminate transcription of all RNA polymerase II-dependent promoters in S. cerevisiae (9, 23). Despite this general importance, no homologs have been found in higher cells. In fact, S. pombe is the first additional organism to have a homolog to Srb4. Apparently factors of broad and essential importance for transcriptional regulation can be conserved between S. pombe and S. cerevisiae but absent from human cells.

The S. pombe Med7 identified here is significantly larger (376 aa) than both its S. cerevisiae (222 aa) and human (233 aa) homologs. A sequence comparison revealed a high degree of conservation between these homologs (Fig. 1B). In spMed7 this homology is split up in two parts by a unique stretch of about 120 amino acids and it seems likely that the N-terminal and C-terminal regions correspond to two specific protein domains. Pmc2 displayed no apparent homologies to any entries in the Swiss-Prot data base. Pmc1 had a short region of homology (about 45–70 aa) to Rgr1 and Rgr1 homologs found in higher eukaryotes (Fig. 4, A and B). Pmc1 is only 673 aa long compared with 1082 aa for Rgr and 1454 aa for hRgr1/TRAP170. Interestingly, Rgr1 and hRgr1/ TRAP170 share a long conserved region near the N terminus (13), which is completely absent in Pmc1. This observation indicates the presence of a domain conserved between S. cerevisiae and mammalian cells but lacking in S. pombe.

Apparently absent from S. pombe Mediator were homologs to the Gal11 and Sin4 proteins (24). In S. cerevisiae, these proteins have a molecular mass of 120 and 111 kDa. It seems unlikely that any homologs will be found among the still unidentified Mediator subunits, because these have apparent molecular masses of 40 kDa or less. In S. cerevisiae, Sin4 is needed to support transcriptional activation by the Gcn4 protein (10). The Gal11 protein is needed to support Gal4-dependent induction of genes involved in galactose metabolism (25). The absence of a Gal11 homolog therefore correlates with the inability of S. pombe to grow on galactose (26).

Our data demonstrate that S. pombe Mediator is clearly distinct from similar complexes found in other eukaryotic cells. Apparently, despite its central role in transcriptional regulation, the subunit composition of the Mediator can vary to a large extent between different eukaryotes. One simple explanation could be that a subset of subunits conserved across species (Pmc1/Rgr1/ TRAP170, Med7, Nut2, and probably also Med6 and Srb7) is needed for interactions with the highly conserved RNA polymerase II and TFIIIB, whereas nonconserved subunits are responsible for interactions with activators and repressors. Such a modular organization of the mediator could be explained from an evolutionary perspective, because regulation of specific genes can be expected to have arisen relatively late during the course of evolution.

Despite divergence on the primary sequence level, low resolution structures of Mediator complexes purified from S. cerevisiae and mouse cells demonstrate striking similarities (27). If the apparent masses of the individual subunits of S. pombe Mediator are added up (Table I), we arrive at a total mass of about 400 kDa, whereas S. cerevisiae Mediator has a total mass of more than 1000 kDa. With the conserved overall structure of Mediator from S. cerevisiae and mouse in mind, we found the comparatively small size of S. pombe Mediator intriguing. One could speculate that a structural comparison of Mediator from S. pombe with the S. cerevisiae or mouse complex could enable the identification of conserved subcomplexes within the Medi-
ator, which is essential for its ability to function as a coactivator.

Further support for the notion of *S. pombe* Mediator being functionally conserved, but structurally distinct from *S. cerevisiae* Mediator came from the species specificity displayed in an activated CTD kinase assay (Fig. 6, A and B). Little is known about the molecular basis for Mediators ability to stimulate CTD phosphorylation by TFIIH. Our observation that *S. pombe* Mediator is unable to stimulate TFIIH derived from *S. cerevisiae* indicates that specific interactions might be formed between Mediator and TFIIH. Future experiments will be aimed at a detailed analysis of the biochemical basis for the observed species specificity. Such an analysis could shed light on the molecular mechanisms of stimulated phosphorylation and be of interest for our understanding of transcriptional activation in eukaryotic cells.

Addendum—We recently found that the mass fingerprinting data generated for Pmc4 is compatible with a hypothetical open reading frame in the GenBank™ data base (SPBC1105.06). This reading frame encodes a protein with high sequence similarity to the *S. cerevisiae* Mediator component Med4.

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