Identification of New Mediator Subunits in the RNA Polymerase II Holoenzyme from Saccharomyces cerevisiae*

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Mediator was isolated from yeast on the basis of its requirement for transcriptional activation in a fully defined system. We have now identified three new members of mediator in the low molecular mass range by peptide sequence determination. These are the products of the NUT2, CSE2, and MED11 genes. The product of the NUT1 gene is evidently a component of mediator as well. NUT1 and NUT2 were earlier identified as negative regulators of the HO promoter, whereas mutations in CSE2 affect chromosome segregation. MED11 is a previously uncharacterized gene. The existence of these proteins in the mediator complex was verified by copurification and co-immunoprecipitation with RNA polymerase II holoenzyme.

An in vitro system reconstituted with pure general transcription factors and RNA polymerase II from yeast can support basal transcription but is not responsive to transcriptional activators (1). This observation prompted a search for coactivators, 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 that not only enables transcriptional activation but also stimulates both basal transcription and phosphorylation of the C-terminal domain of the polymerase (1, 2).

Earlier studies identified 16 different subunits in the mediator (1–5). These can be divided into three subgroups. The SRB genes products were identified in a screen for RNA polymerase II C-terminal domain-interacting proteins and were subsequently isolated in a complex with RNA polymerase II, giving the first indication of the existence of the RNA polymerase II holoenzyme (6). The mediator subunits encoded by GAL11, RGR1, SIN4, PGD1/HRS1, and ROX3 were all found by earlier genetic studies to affect both the positive and negative regulation of transcription (7). Biochemical evidence indicates that at least Gal11, Rgr1, Sin4, and Pgd1/Hrs1 belong to a physically distinct mediator subassembly (3). The third subgroup is comprised of the MED genes, which were all previously uncharacterized, and whose products were identified through peptide sequencing. Genetic and biochemical evidence indicates that several members of this group of genes are essential for the function of individual transcriptional activators, such as Gal4 and Gcn4, both in vivo and in vitro (5).

The widespread effect of the mediator on transcription is reflected in genetic studies that have characterized its subunits in a diverse array of cellular activities (7). Here we report that the products of the NUT2, CSE2, and MED11 genes are all components of the mediator. We present evidence for the occurrence of the NUT1 gene product in mediator as well. Med11 is the product of a previously uncharacterized open reading frame. The characterization of NUT1 and NUT2, identified as negative regulators of the HO gene (9) and Cse2, whose mutations affect chromosome segregation (10), adds to the list of different functions regulated through specific mediator subunits.

EXPERIMENTAL PROCEDURES

Protein Sequencing—Peptides were generated from bands corresponding to proteins of 12 and 21 kDa, bound to polyvinylidene difluoride membrane by tryptic digestion in situ (11, 12), and fractionated by reverse-phase HPLC (13) with the use of a 1-mm Reliasil C18 column. Selected peak fractions were analyzed by a combination of automated Edman chemical degradation (14) and matrix-assisted laser-desorption ionization time-of-flight mass spectrometry. Peptide sequences were compared with entries in the Saccharomyces Genome Data Base (Stanford Genomic Resources, Stanford University) with the use of the National Center for Biotechnology Information BLAST program. Experimental masses of these and additional peptides were compared with the theoretical average isotopic masses of fragments expected to result from tryptic digestion of the identified proteins (with the use of PeptideSearch software; Dr. Matthias Mann, European Molecular Biology Laboratory, Heidelberg, Germany).

Antibodies and Immunoblot Analyses—Recombinant Cse2 and Med11 proteins fused to glutathione S-transferase 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. The purified glutathione S-transferase fusion proteins and a synthetic polypeptide corresponding to the 15 N-terminal amino acids of Nut2 were used to immunize rabbits. The antisera used in this study were taken 10 days after the second booster injection (Antibody AB, Sweden).

Protein Purification—Purification of the RNA polymerase II holoenzyme was as described (15) with the following modifications. After fractionation on hydroxyapatite, the mediator/holopolymerase peak was loaded on a Mono Q 10/10 column (Amersham Pharmacia Biotech). After washing with 40 ml of buffer Q-0.15 and 40 ml of buffer Q-0.5, the column was developed with a linear gradient (112 ml) of buffer Q-0.5 to Q-0.0.

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Q-1.2, and 1.2-ml fractions were collected. Immunoblotting revealed a peak of free mediator around Q-0.6 and a peak of holopolymerase eluting around Q-0.8. Purification of free mediator was as described earlier (2).

**Immunoprecipitation**—Anti-Med2 and anti-Cse2 antibodies were coupled to protein A-Sepharose beads and used to immunoprecipitate purified RNA polymerase II holoenzyme as described (3) with the following modifications. RNA polymerase II holopolymerase Mono Q fraction 89 (10 μl) was diluted with 30 μl of buffer Q, 0.5 (25 mM Tris acetate, pH 7.5, 10% glycerol, 1 mM EDTA, 0.5 mM potassium acetate) and sedimented for 5 min at 13,000 rpm prior to incubation with the beads. Following incubation with the diluted fraction for 10 h at 4 °C, the beads were washed three times with 100 μl of IP-400 buffer (20 mM Tris-Cl, pH 7.5, 10% glycerol, 0.1 mM EDTA, 0.1% Nonidet P-40, 0.4 mM potassium acetate) and eluted twice for 10 min at room temperature with 50 μl of 5 M urea. To the combined eluates was added 20 μl of 2× SDS loading buffer (20% glycerol, 10% 2-mercaptoethanol, 4.6% SDS, 125 mM Tris-Cl, pH 6.8, 0.1% (w/v) bromphenol blue) for SDS-PAGE and immunoblotting.

**RESULTS**

In previous work, free mediator complex was purified and 16 polypeptides in the size range between 14 and 130 kDa were described. Analysis of mediator by SDS-PAGE and Coomassie Blue staining indicated the presence of additional subunits in the molecular mass range below 21 kDa. To investigate further, mediator was analyzed by SDS-PAGE in a 15% gel containing 2% glycerol and staining with silver, leading to the identification of five bands, ranging from 10 to 19 kDa (Fig. 1.). Two of these corresponded to the previously reported mediator components, Srb6 and Srb7. In addition there were two protein bands of about 19 kDa that we termed Med9 and Med10 and a protein band about 14 kDa that we termed Med11.

Purified mediator was separated by 12% SDS-PAGE and transferred electrophoretically to a polyvinylidene difluoride membrane. The Med9, Med10, and Med11 bands were cut out and subjected to tryptic digestion. Because the Med9 and Med10 bands migrated so closely together, these two bands were cut out together and analyzed simultaneously. The resulting peptides were fractionated by reverse-phase HPLC and sequenced. Perfect matches were found to sequences in the *Saccharomyces cerevisiae* genome data base (Table I), and the masses of peptides determined by mass spectrometry were in good agreement with the theoretical masses of the expected tryptic peptides for the individual gene products. The Med9 and Med10 bands proved to be the products of two previously characterized genes, *NUT2* and *CSE2*. The Med11 band yielded peptide sequences from an uncharacterized open reading frame, denoted *MED11*. The Cse2 protein contained an earlier noted putative basic region leucine zipper motif (10), but otherwise we could not find any significant sequence features related to transcription for any of the these proteins.

Cse2 and Med11 were expressed in recombinant form, and polyclonal antibodies were obtained. Polyclonal antibodies were also obtained against a 15-amino-acid polypeptide corresponding to the N-terminal end of Nut2. The association of these proteins with RNA polymerase II holoenzyme was shown by copurification and co-immunoprecipitation. RNA polymerase II holoenzyme was purified from strain BJR26 by chromatography on Bio-Rex 70, DEAE-Sephacel, hydroxyapatite, and Mono Q. Immunoblot analysis showed coelution from Mono Q of Med4, Nut2, Cse2, Med11, and the polymerase subunit Rpb1 (Fig. 2).

Immunoprecipitation was performed with anti-Cse2 antibodies coupled to protein A-Sepharose. Holopolymerase was incubated with the antibody Sepharose, followed by washing under stringent conditions. Immunoblot analyses revealed that Med2, Med4, Nut2, Cse2, and Med11 were almost entirely bound by the antibody Sepharose (Fig. 3). Immunoprecipitation with anti-Med2 gave the same result (data not shown). Antibodies generated against Nut2 and Med11 were not useful for immunoprecipitation. We conclude that Nut2, Cse2, and Med11 in the holopolymerase preparation were entirely associated with the holoenzyme.

Reinvestigation of of the 130-kDa region of the purified mediator indicated that Nut1, genetically closely related to Nut2 might be a mediator component as well. Peptide mass fingerprinting revealed many tryptic fragments with amino acid sequences corresponding to Nut1 (data not shown). The genetic studies (9) and presence of Nut1 in a highly purified mediator fraction make a strong case for Nut1 being a mediator subunit, although its tight association with the complex and stoichiometry remain to be shown.

A BLAST similarity search of the GenBank™ data base revealed homologs of Nut2 in both *Schizosaccharomyces pombe* (spNut2) and *Caenorhabditis elegans* (ceNut2) (Fig. 4). The region of homology between the deduced amino acid sequences of Nut2 and spNut2 encompasses almost all of the *S. pombe* protein and 144 of 157 residues of the *S. cerevisiae* protein. The ceNut2 shared homology with Nut2 over a 132-amino-acid region. In addition, several putative mouse and human homologs of the Nut2 protein were identified in a search of the National Center for Biotechnology Information data base of expressed sequence tags (data not shown).

**Table I**

| Gene   | Deletion phenotype | Human homolog | Protein mass (kDa) | Apparent mass (kDa) | pI | Reference (yeast open reading frame) |
|--------|--------------------|---------------|--------------------|---------------------|----|-------------------------------------|
| NUT2   | In viable          | +             | 18                 | 21                  | 4.7| 9                                   |
| CSE2   | Conditional        | +             | 17                 | 21                  | 6.2| 10                                  |
| SRB7   | In viable          | +             | 16                 | 19                  | 4.8| 16                                  |
| SRB6   | In viable          | +             | 14                 | 14                  | 4.6| 17                                  |
| MED11  | ND                 |               | 15                 | 12                  | 5.1| This study (YMR112c)                 |

* *As judged by SDS-PAGE analysis with respect to molecular mass standards.*
DISCUSSION

Our results demonstrate the presence of Cse2, Nut2, and Med11 in mediator and RNA polymerase II holoenzyme. Immunoprecipitation experiments and copurification data establish that these three proteins are true components of the holoenzyme preparations. In addition, peptide mass fingerprinting indicated that Nut1 is likely to be a subunit of mediator as well.

The CSE2 gene was originally identified in a search for mutations affecting chromosome segregation in S. cerevisiae. Disruption of CSE2 results in an increase in chromosome missegregation, slower growth, and both a cold- and temperature-sensitive phenotype. It seems likely that the connection between CSE2 and chromosome segregation is indirect and involves transcription. CSE2 might, for example, be necessary for cell cycle-dependent activation of certain genes required for proper chromosome segregation. A high copy number suppressor of a CSE2 mutation has been isolated and identified as SCM2, a gene encoding a yeast tryptophan permease. SCM2 suppresses the cold-sensitive phenotype of the cse2 mutation but fails to suppress temperature sensitivity and chromosome missegregation. The function-specific suppression of the CSE2 disruption phenotype may result from the participation of this mediator subunit in the regulation of several different sets of genes. The 17-kDa Cse2 protein contains a putative basic region leucine zipper motif, indicating that it may possess a DNA binding activity. It will be of interest to learn whether mutations in this DNA binding domain give rise to a specific phenotype. Leucine zipper motifs have also been observed in the Med7 and Med8 subunits of the mediator, indicating that these motifs might serve a role in the organization of the mediator.

HO transcription is dependent on Swi4p and Swi6p for relief of repression by the URS2 region upstream of the HO promoter (8). NUT1 and NUT2 were recently isolated in a screen for mutants that would suppress the Swi4p/Swi6p dependence of a...
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3 H. Spåhr and C. M. Gustafsson, unpublished observations.

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