The Med proteins of yeast and their function through the RNA polymerase II carboxy-terminal domain

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Mediator was resolved from yeast as a multiprotein complex on the basis of its requirement for transcriptional activation in a fully defined system. Three groups of mediator polypeptides could be distinguished: the products of five SRB genes, identified as suppressors of carboxy-terminal domain (CTD)-truncation mutants; products of four genes identified as global repressors; and six members of a new protein family, termed Med, thought to be primarily responsible for transcriptional activation. Notably absent from the purified mediator were Srbs 8, 9, 10, and 11, as well as members of the SWI/SNF complex. The CTD was required for function of mediator in vitro, in keeping with previous indications of involvement of the CTD in transcriptional activation in vivo. Evidence for human homologs of several mediator proteins, including Med7, points to similar mechanisms in higher cells.

[Key Words: RNA polymerase II holoenzyme; transcription; CTD; Saccharomyces cerevisiae]

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Mediator was discovered as an activity in a crude fraction that relieved activator inhibition of transcription (squelching) in yeast nuclear extract (Kelleher et al. 1990). The same crude fraction was subsequently shown to be required for enhancement of transcription in a partially fractionated yeast system (Flanagan et al. 1991). Other, initially unrelated studies identified SRB genes from a screen for RNA polymerase II carboxy-terminal domain (CTD)-interacting proteins and showed the association of these proteins with the polymerase in a large complex (Koleske and Young 1994). These two lines of work converged with the resolution of mediator to homogeneity as a complex of ∼20 proteins, including several SRB gene products (Kim et al. 1994). The only discordant finding was that Srb proteins were present at a level ∼6% that of RNA polymerase subunits in yeast extracts, whereas about half the RNA polymerase purified from the extracts was associated with mediator. This apparent conflict was resolved by evidence for the recycling of mediator to initiation complexes and the absence of mediator from elongating polymerases, which constitute the majority of the enzyme in a cell (Svejstrup et al. 1997).

Purified mediator was found to possess three functional activities (Kim et al. 1994): It stimulated basal transcription in a system reconstituted from essentially homogeneous yeast proteins ∼10-fold; it enabled a response of the reconstituted system to activator proteins by as much as 30-fold, for an overall effect on transcription as great as 300-fold; and it stimulated phosphorylation of the CTD by TFIIH kinase by 30- to 50-fold. Major objectives of further work have been to identify the components of mediator responsible for these activities, to determine the mechanisms, and to establish the relevance for transcriptional regulation in vivo. To this end, characterization with antisera against various yeast proteins enabled the provisional identification of Gal11, Sug1, Srb2, Srb4, Srb5, and Srb6 among the set of mediator polypeptides (Kim et al. 1994). Subsequent peptide sequence analysis confirmed the presence of Gal11 and identified three further mediator polypeptides as products of the previously described SIN4, RGR1, and ROX3 genes (Li et al. 1995; Gustafsson et al. 1997). The significance of this work was several fold: It united the Gal11, Sin4, and Rgr1 proteins, products of disparate genetic screens, in a common biochemical entity; it revealed the occurrence in mediator of proteins implicated by genetic studies in transcriptional repression in vivo, thus showing a role for mediator in negative, as well as positive, regulation of transcription; and it pointed to a modular organization of the mediator, with Gal11, Sin4, Rgr1, and a 50-kD polypeptide forming a separable subassembly of the larger complex. This work, however, did not address the questions of physiologic relevance or mecha-
nism. It remained to make any connection between functions in vitro and phenotypes in vivo.

Here, we present a nearly complete molecular description of mediator. In addition to the products of known genes, we identify a novel family of mediator polypeptides required for transcriptional activation. We provide direct evidence for mediator function through the CTD in vitro, in keeping with the phenotypes of CTD truncation mutants in vivo. The occurrence of human homologs of a number of mediator proteins points to the possible existence of a corresponding mechanism in higher cells.

Results

Isolation of free mediator complex

In past work, mediator was isolated either as a complex with 12-subunit core RNA polymerase II, or following displacement from this holoenzyme complex by anti-CTD antibodies (Kim et al. 1994). For the isolation of mediator for peptide sequence analysis, we turned to commercial yeast (Fleischmann’s), and noticed the resolution of mediator polypeptides in two peaks in the fourth step of the fractionation procedure, chromatography on Mono Q. The first peak was devoid of polymerase subunits and apparently represented free mediator, whereas the second peak contained the previously described holoenzyme (Fig. 1).

The free mediator was further purified by chromatography on TSK-Heparin-5-PW and Mono S, followed by gel filtration through Bio-Sil SEC 400. Peak fractions from gel filtration were pooled and analyzed by SDS-PAGE and Coomassie Blue staining (Fig. 2). As many as 18 polypeptides were resolved in the molecular weight range 14–130 kD, 16 of which are described here. In addition to the products of known genes, there were seven products of novel genes, which we have termed MED, for mediator. Densitometry of the SDS–gel indicated that all mediator polypeptides were present in roughly equal amounts, except for Srb5, at ~40% of the level of the other proteins, members of the Gal11 module (Gal11, Sin4, Rgr1, and p50, here termed Med3), at variable levels between 50% and 75%, and Med 2 at ~30% (Fig. 2). The amount of Med2 may be underestimated, because it formed a diffuse band in the gel. Functional assays of the homogeneous free mediator showed all the activities found previously for the protein derived from RNA polymerase holoenzyme: The free mediator stimulated basal transcription in a fully reconstituted system 20.5-fold, enabled a further increase in transcription in the presence of Gal4–VP16 activator protein of 18.4-fold (Fig. 3A), and stimulated phosphorylation of the CTD by TFIIH as much as 40-fold (Fig. 3B).

Identification of five new mediator subunits

A highly purified mediator fraction was resolved by SDS-PAGE, transferred to a polyvinylidene difluoride (PVDF) membrane, and stained with Ponceau S. Bands denoted Med2, Med3, Med4, Med5, Med7, and Med8 (see Fig. 2) were excised and digested with trypsin, and the resulting peptides were fractionated by reverse-phase high-pressure liquid chromatography (HPLC) and sequenced. Perfect matches were found to sequences in the Saccharomyces cerevisiae genome database (Table 1), 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. Med4 and Med5 yielded peptide sequences from the same uncharacterized open reading frame, and thus derived from a single protein migrating as a doublet, hereafter referred to as Med4. The final member of the Gal11 module, the p50/Med3 protein, proved to be the product
of the previously identified PGD1 (HRS1) gene (Bröhl et al. 1994). Med7 derived from an uncharacterized open reading frame. The Med8 band gave rise to peptide sequences from two open reading frames, one corresponding to the previously identified holoenzyme component Rox3 (Gustafsson et al. 1997), and a second, equally abundant protein, from an open reading frame partially characterized in the yeast chromosome II sequencing effort (Démolis et al. 1993), for which we retain the designation Med8.

Although none of the MED sequences show enzymatic motifs or other strong homologies clearly related to mediator function in transcription, there are some features of note in the newly defined sequences. The existence of a putative leucine zipper motif in MED8 at amino acids 69–90 has been recognized previously (Démolis et al. 1993). MED2 contains an asparagine-rich region at the carboxy terminus of >100 amino acids. A polyglutamine stretch, common to many transcription proteins, including Gal11, has been noted in PGD1 (HRS1) (Santos-Rosa et al. 1996). Along with other members of the Gal11 module, PGD1 (HRS1) is basic, whereas Med7 and Rox3 are neutral, and the rest of the mediator subunits are acidic (Table 1).

Med2, Med4, Med7, and Med8 were expressed in recombinant form, and polyclonal antibodies were obtained. The association of these proteins with RNA polymerase II holoenzyme was shown by copurification and coimmunoprecipitation. RNA polymerase II holoenzyme was purified from strain BJ926 by chromatography on Bio-Rex 70, DEAE–Sephacel, hydroxyapatite, Mono Q, and Bio-Sil SEC 400. Immunoblot analysis showed coelution from hydroxyapatite and Bio-Sil SEC 400 of Med2, Pgd1 (Hrs1), Med4, Med7, Med8, the polymerase subunits Rpb1 and Rpb3, and Srb4 (Fig. 4).

Immunoprecipitation was performed with anti-Med2 antibodies coupled to protein A–Sepharose. Holopolymerase was incubated with the antibody–Sepharose, followed by washing under stringent conditions. Immunoblot analyses revealed that Med2, Pgd1 (Hrs1), Med4, Med7, Med8, Rpb3, and Srb4 were almost entirely bound by the antibody–Sepharose (Fig. 5A). Immunoprecipitation with anti-Med4 antibodies gave the same result (Fig. 5B). We conclude that Med2, Pgd1 (Hrs1), Med4, Med7, and Med8 in the holopolymerase preparation were entirely associated with the holoenzyme.

MED gene requirement for cell viability

Yeast strains were constructed in which each of the previously uncharacterized open reading frames encoding Med proteins was deleted. Following sporulation and tetrad dissection, viability segregated 2:2 for the med4, med7, and med8 deletions. Tetrad dissection of the med2 deletion strain yielded four viable spores, but the two spores containing the disrupted gene were significantly smaller and unable to grow on galactose as the sole carbon source (data not shown).

Mediator binds directly to a CTD peptide

A GST fusion protein that included all 26 repeats of the yeast CTD and 40 amino acids of linker sequence was loaded on glutathione–Sepharose. Pure mediator was completely bound by the resulting GST–CTD Sepharose, whereas none was bound by a GST–Sepharose control (Fig. 6). What little mediator was not bound by the GST–CTD Sepharose could most likely be attributed to a small amount of GST–CTD that bled off the column. Approximately half of the bound mediator was eluted with glutathione, which also displaced a majority of the GST–CTD. The remaining mediator and GST–CTD were eluted under denaturing conditions. There was no mediator in eluates from the GST–Sepharose control.

CTD required for mediator function in vitro

RNA polymerase II lacking a CTD (Pol II ΔCTD) was prepared by proteolysis as described (Li and Kornberg 1994). Pol II ΔCTD was indistinguishable from wild-type enzyme in a basal transcription reaction (Fig. 7). Medi-
tor, however, failed to stimulate basal transcription by Pol II DCTD, nor did it elicit a response of Pol II DCTD to Gal4–VP16 or GCN4, in marked contrast to its effect on the wild-type polymerase (Fig. 3A; Kim et al. 1994).

Human homolog of yeast Med7

A cDNA encoding a human homolog of the Med7 protein (hMed7) was identified in a search of the NCBI database of expressed sequence tags (Fig. 8A). The region of homology between the deduced amino acid sequences of the two proteins encompasses almost all of the yeast protein and 211 of 235 residues of the human protein (Fig. 8B). The sequences exhibited 31.8% identity and 59.2% similarity in this extended region, essentially the same as the degree of homology between yeast and human general transcription factors, and between yeast and human Med6 (Lee et al. 1997) and Srb7 (Chao et al. 1996). Full-length hMed7 failed to complement deletion of the yeast homolog (data not shown), as also found for Med6 and Srb7.

Discussion

Fractionation of RNA polymerase II transcription systems has revealed two multiprotein complexes that confer responsiveness to activators, the TAF (TATA binding protein-associated factor) complex (Pugh and Tjian 1990; Burley and Roeder 1996) and mediator (Kelleher et al. 1990; Kim et al. 1994). Subsequent studies were directed...
toward the molecular characterization of these complexes, as a prelude to genetic and mechanistic analysis. A major goal has been to test the relevance of transcriptional activation supported by the complexes in vitro to gene regulation in vivo. Eight RNA polymerase II TAFs were identified, and genes for five of the proteins were mutated in yeast. These mutations had few effects on transcription, pointing to a role for TAFs at only a small number of yeast promoters (Moqtaderi et al. 1996; Walker et al. 1996).

In contrast, mediator appears to play a more general role. The five new mediator genes reported here bring to sixteen the number so far identified, with the possibility of another two remaining. Three groups of mediator genes may be distinguished: a subset of the SRB genes, which were originally isolated from a genetic screen for CTD-interacting proteins, and whose products may be involved in CTD binding; GAL11 and related members, whose roles in vivo extend to transcriptional repression; and the MED genes, analyzed here. A temperature-sensitive mutation in the SRB4 gene was shown to abolish transcription from all promoters tested at the restrictive temperature, indicating a general requirement of mediator for transcription (Thompson and Young 1995). The consequences of MED gene mutation establish a role in transcriptional activation. A med6ts strain (Lee et al. 1997) is defective in GAL gene induction in vivo, and the isolated med6 ts holoenzyme exhibits no response to transcriptional activators in vitro.

The correlation between genetic and biochemical studies of transcription control is extended by evidence for a CTD requirement. Transcription from some promoters is reduced by CTD truncation in vivo (Scafe et al. 1990) and in nuclear extracts (Liao et al. 1991). Here we report that the response to activator proteins in the presence of mediator is abolished by removal of the CTD from RNA polymerase II in a pure transcription system. Consistent with these findings, proteins from yeast whole cell extract including Srb proteins are retained by CTD polypeptide immobilized on a solid support (Thompson et al. 1993), and we show that pure mediator, reduced to a minimal set of polypeptides required for function in vitro, and devoid of other proteins, is bound by such a CTD column as well.

An important difference between the mediator de-
scribed here and that reported previously (Kim et al. 1994) is our isolation of the complex in a free form, not associated with RNA polymerase II in a holoenzyme. The relative abundance of free and polymerase-bound mediator was somewhat variable from one preparation to another, possibly dependent, in part, on the yeast strain used. The occurrence of free mediator is in keeping with the mediator cycle proposed elsewhere (Svejstrup et al. 1997). Isolation as a free, stoichiometric species underlines the integrity of the complex, further distinguishing it from a collection of proteins interacting independently with the polymerase CTD.

Proteins absent from the mediator isolated here are also noteworthy. In particular, TFIIF was separated from the mediator during fractionation, as noted elsewhere (Svejstrup et al. 1997). Other suppressors of this phenotype include genes encoding Srb2 (Piruat and Aguilar 1996), Rpb2, and TFIIB (Fan et al. 1996). The deletion of HRS1 was shown to greatly reduce the frequency at which sequences between direct repeats are deleted (Santos-Rosa et al. 1996). This result was obtained not only for hpr1Δ cells, but also for wild-type and Rad52 cells. It seems likely that the connection between HRS1 and recombination is indirect and involves transcription, because the stability of direct repeats in yeast depends on their level of transcription (Thomas and Rothstein 1989), and mediator mutations may affect global gene expression. A direct role for mediator in the control of genetic stability, however, is not excluded.

Figure 8. Human homolog of yeast Med7 protein. (A) Human Med7 homolog sequence (GenBank accession no. AF031383). (B) Human and yeast Med7 alignment. Colons indicate identity and dots similarity by the program FASTA (Pearson and Lipman 1988). FASTA aligned the sequences using the BLOSUM50 matrix resulting in a Smith–Waterman score of 291, and a 31.8% identity and 59.2% similarity in a 211-amino-acid region of overlap.

such as Gal11, Sin4, Rgr1, and Med3. The similar behavior of these four proteins is in keeping with three additional lines of evidence. First, the four proteins are basic, in contrast with the neutral or acidic nature of all other mediator polypeptides (Table 1). Second, Rgr1 truncation causes the loss of Gal11, Sin4, and p50/Med3 from mediator (Li et al. 1995). Third, mutations in Gal11, Sin4, and Rgr1 confer similar phenotypes (Jiang and Stillman 1995; Jiang et al. 1995). We now identify p50/Med3 as the product of the PGD1 (HRS1) gene. Others have noted that Hrs1 mutations have similar consequences to those of Gal11, Sin4, and Rgr1 deficiencies and have suggested its identification with p50/Med3 (Piruat et al. 1997). HRS1 was found in a genetic screen for mutations that could suppress a hyper-recombinant phenotype of hpr1Δ cells (Santos-Rosa et al. 1995). Other suppressors of this phenotype include genes encoding Srb2 (Piruat and Aguilar 1996), Rpb2, and TFIIB (Fan et al. 1996). The deletion of HRS1 was shown to greatly reduce the frequency at which sequences between direct repeats are deleted (Santos-Rosa et al. 1996). This result was obtained not only for hpr1Δ cells, but also for wild-type and Rad52 cells. It seems likely that the connection between HRS1 and recombination is indirect and involves transcription, because the stability of direct repeats in yeast depends on their level of transcription (Thomas and Rothstein 1989), and mediator mutations may affect global gene expression. A direct role for mediator in the control of genetic stability, however, is not excluded.
Finally, we note the likely relevance of the activation mechanism in yeast to that in higher cells. Conservation of MED7 from yeast to man is shown here, and similar conservation of MED6 is reported elsewhere (Lee et al. 1997). In all likelihood, an entire Med protein subcomplex, important for activation in yeast, plays a similar role in higher organisms. Homologs of SRB7 (Chao et al. 1996) and RGR1 (Y.W. Jiang and R.D. Kornberg) also occur in man, pointing to conservation of the Srb and Gal11 subcomplexes as well.

Materials and methods

Genetic manipulations

Yeast transformations employed lithium acetate (Schiestel and Getz 1989). Plasmid shuffle techniques were performed as described (Boeke et al. 1987) with the use of 5-fluoro-orotic acid (5-FOA) as a selective agent against URA3 plasmids.

Deletions of the entire coding regions of the MED2, MED4, MED7, and MED8 genes were performed by a single-step gene disruption technique utilizing PCR amplification of selectable markers (Lorenz et al. 1995). A set of bifunctional PCR primers was constructed with a region of 50 bp at the 5' end homologous to the flanking sequence of the MED genes. The 3' end contained 18 bases of homology to the flanking sequence of the marker genes in the pRS303-306 series of vectors (Sikorski and Hieter 1989). PCR knockout products marked MED7 with HIS3, MED8 with LEU2, and MED2 and MED4 with TRP1. These PCR products were transformed into the S. cerevisiae strain CRY3 (Kean et al. 1993) and plated on the appropriate selective media. Yeast genomic DNA was isolated and PCR analysis was performed to confirm that the marker gene had recombined correctly into the target MED gene (see Table 2). The diploids were sporulated and tetrad (>15) were dissected on YPD agar and scored for nutritional auxotrophies and growth at 30°C.

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Dissection of each tetrad from the MED4::med4::TRP1 (MG101), MED7::med7::HIS3 (MG102), and MED8::med8::HIS3 (MG103) diploids resulted in two or fewer viable spores. A PCR product containing ~300 bp upstream and ~200 bp downstream of each essential MED gene was cloned into the BamHI–XhoI site of pRS316 (URA3), resulting in pGM23 (URA3 MED4), pGM24 (URA3 MED7), and pGM25 (URA3 MED8). To confirm that these genes were essential, pGM23, pGM24, and pGM25 were transformed into the strains GM101, GM102, and GM103 and plated on synthetic medium lacking uracil. These diploid transformants were sporulated and the tetrads (>10) were dissected. Viable spores, which were Ura+, and Trp+ (MED4), His+ (MED7), or Leu+ (MED8), were streaked on 5-FOA-containing medium. The inability to grow on 5-FOA confirmed that MED4, MED7, and MED8 were essential.

Segregants of each tetrad from the MED4::med4::TRP1 (MG104) diploid scored 2:2 for tryptophan prototrophy and all tryptophan prototrophs exhibited weak temperature-sensitive, Gal+, and small colony phenotypes, indicating that MED2 deletion strains are conditionally viable. A PCR product containing ~300 bp upstream and ~200 bp downstream of the MED2 gene was cloned into the BamHI–XhoI site of pRS316 (URA3), yielding pGM26 (URA3 MED2). The haploid med2::TRP1 strain (MG105) was transformed with pGM26 and plated on synthetic medium lacking uracil. This transformed strain complemented all phenotypes of the deletion strain, confirming MED2 as the source of these phenotypes.

Protein purification

Fractionation of whole cell extract by chromatography on Bio-Rex 70 (Bio-Rad), DEAE–Sephadex (Pharmacia), Bio-Gel HTP hydroxyapatite (Bio-Rad), and Mono Q was as described (Li et al. 1996) with the following modifications. Active dry yeast (2.6 kg) was suspended in lysis buffer and the cells were disrupted as described (Kim et al. 1994). After fractionation on hydroxyapatite, the mediator/holopolymerase peak, detected by immunoblotting, was loaded on a Mono Q 10/10 column (Pharmacia). After washing with 40 ml of buffer Q-0.15 and 40 ml of Q-0.5, the column was developed with a linear gradient (112 ml) of buffer Q-0.5 to Q-1.2. Immunoblotting revealed a peak of free mediator eluting around Q-0.6 and a peak of holopolymerase eluting around Q-0.8. The peak of free mediator 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 for 4 hr. The dialysate was diluted with H-0 to the conductivity of H-0.1 and applied to a TSK–Heparin–5-PW HPLC column (3.3 ml, 75 × 7.5 mm, Supelco) equilibrated in H-0.1. After fractionation on hydroxyapatite, the mediator/holopolymerase peak was collected before drying (Fleischmann's, Oakland, CA) was suspended in lysis buffer and the cells were disrupted as described (Kim et al. 1994). After fractionation on hydroxyapatite, the mediator/holopolymerase peak was detected by immunoblotting, was loaded on a Mono Q 10/10 column (Pharmacia). After washing with 40 ml of buffer Q-0.15 and 40 ml of Q-0.5, the column was developed with a linear gradient (112 ml) of buffer Q-0.5 to Q-1.2. Immunoblotting revealed a peak of free mediator eluting around Q-0.6 and a peak of holopolymerase eluting around Q-0.8. The peak of free mediator was pooled and dialyzed against H-0.1 containing 20 mM potassium phosphate (pH 7.6), 10% glycerol, 1 mM EDTA, 1 mM DTT, protease inhibitors, and the molarity of potassium acetate indicated after the hyphen, adjusted to a conductivity of 0.1, and applied to a TSK–Heparin–5-PW HPLC column (3.3 ml, 75 × 7.5 mm, Supelco) equilibrated in H-0.1. After washing with two column volumes of H-0.1 and 4 column volumes of H-0.25, the column was developed with a linear gradient (33 ml) of H-0.25–H-0.75 at a flow rate of 0.5 ml/min, and fractions of 0.75 ml were collected. The peak of mediator was at ~H-0.4.

Peak mediator fractions were dialyzed against buffer A–0 containing 20 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, adjusted to a conductivity of 0.1, and applied to a Mono S 5/5 FPLC column (Pharmacia) equilibrated in A–0.1. After washing with five column volumes of A–0.1 and 5 column volumes of A–0.2, the column was developed with a linear gradient (12 ml) of A–0.2–A–1.0 at a flow rate of 0.5 ml/min, and fractions of 0.35 ml were collected. The peak of mediator was at ~A–0.5. To test the homogeneity of the multi-protein complex, a portion (0.25 ml) of the Mono S peak mediator fraction was applied to a Bio-Sil SEC 400 HPLC column (Bio-Rad) equilibrated in A–0.5.

RNA polymerase II holoenzyme was purified from 650 grams of S. cerevisiae strain BJ926 as described in Li et al. (1996).

Protein sequencing

Peptides were generated from Med2, Med3, Med4, Med7, and Med8 proteins bound to PVDF by tryptic digestion in situ (Erd-
Plasmid Description

of a 1-mm Reliasil C18 column. Selected peak fractions were analyzed by reversed-phase HPLC (Elicone et al. 1994) with the use of

PeptideSearch software; Dr. Matthias Mann, European Molecular Biology Laboratory, Heidelberg, Germany).

Antibodies and immunoblot analyses

Recombinant Med2, Med4, Med7, and Med8 proteins fused to glutathione S-transferase (Table 3) were overproduced in Escherichia coli BL21(DE3) pLysS cells and purified from inclusion bodies as described (Cairns et al. 1994). For each of the GST–Med fusions, ∼5 mg of protein was separated by SDS-PAGE and used to immunize rabbits. The antisera used in this study were taken 10 days after the second booster injection (BabCO, Berkeley, CA). Anti-HRS1 antibodies (Santos-Rosa et al. 1996) were a gift from R. Burgess and N. Thompson (McArdle Laboratory for Cancer Research, Madison, WI). Anti-RPB3 antibodies were a gift from A. Aguilera (Universidad de Sevilla, Spain). Anti-RPB3 antibodies as described (Cairns et al. 1994). For each of the GST–Med fusions, ∼5 mg of protein was separated by SDS-PAGE and used to immunize rabbits. The antisera used in this study were taken 10 days after the second booster injection (BabCO, Berkeley, CA). Anti-HRS1 antibodies (Santos-Rosa et al. 1996) were a gift from R. Burgess and N. Thompson (McArdle Laboratory for Cancer Research, Madison, WI).

Transcription and CTD phosphorylation assays

Transcription and CTD phosphorylation assays were performed as described (Kim et al. 1994), with the following modifications. Pure TFIIF (Henry et al. 1992) was added to all transcription reactions, and TFIIH was purified as described by Svejstrup et al. (1994). All transcription reactions were performed at a final potassium acetate concentration of 180 mM.

Table 3. Plasmids

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
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<tr>
<td>pGM23</td>
<td>pRS316 with the BamHI–Xhol fragment removed and a PCR product, using the oligonucleotides 5’-dCCGGGATCCAGTTATTACTTGGGTCC-3’ and 5’-dCGCCTCGAGTTTTCCAGTCTCTGTGGG-3’ containing the full MED4 coding sequence inserted.</td>
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<tr>
<td>pGM24</td>
<td>pRS316 with the BamHI–Xhol fragment removed and a PCR product (PCR with oligonucleotides 5’-dCCGGGATCCATCAACATCCATCTCTGTGGG-3’ and 5’-dCGCCTCGAGAATCTCGGAAATACGTCG-3’) containing the full MED7 coding sequence inserted.</td>
</tr>
<tr>
<td>pGM25</td>
<td>pRS316 with the BamHI–Xhol fragment removed and a PCR product (PCR with oligonucleotides 5’-dCCGGGATCCAGTTATTACTTGGGTCC-3’ and 5’-dCGCCTCGAGAATCTCGGAAATACGTCG-3’) containing the full MED8 coding sequence inserted.</td>
</tr>
<tr>
<td>pGM26</td>
<td>pRS316 with the Xbal–Xhol fragment removed and a PCR product (PCR with oligonucleotides 5’-dCCGGGATCCAGTTATTACTTGGGTCC-3’ and 5’-dCGCCTCGAGAATCTCGGAAATACGTCG-3’) containing the full MED2 coding sequence inserted.</td>
</tr>
<tr>
<td>pGM27</td>
<td>pGEX-3X with the BamHI–EcoRI fragment removed and a PCR product (PCR with oligonucleotides 5’-dCCGGGATCCAGTTATTACTTGGGTCC-3’ and 5’-dCGCCTCGAGAATCTCGGAAATACGTCG-3’) containing the full MED4 coding sequence inserted.</td>
</tr>
<tr>
<td>pGM28</td>
<td>pGEX-3X with the BamHI–EcoRI fragment removed and a PCR product (PCR with oligonucleotides 5’-dCCGGGATCCAGTTATTACTTGGGTCC-3’ and 5’-dCGCCTCGAGAATCTCGGAAATACGTCG-3’) containing the full MED7 coding sequence inserted.</td>
</tr>
<tr>
<td>pGM29</td>
<td>pGEX-3X with the BamHI–EcoRI fragment removed and a PCR product (PCR with oligonucleotides 5’-dCCGGGATCCAGTTATTACTTGGGTCC-3’ and 5’-dCGCCTCGAGAATCTCGGAAATACGTCG-3’) containing the full MED8 coding sequence inserted.</td>
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<tr>
<td>pGM30</td>
<td>pGEX-3X with the BamHI–EcoRI fragment removed and a PCR product (PCR with oligonucleotides 5’-dCCGGGATCCAGTTATTACTTGGGTCC-3’ and 5’-dCGCCTCGAGAATCTCGGAAATACGTCG-3’) containing the full MED2 coding sequence inserted.</td>
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Mediator–CTD interaction

To construct a GST–CTD expression construct the carboxy-terminal domain of Rpb1 was amplified by PCR, by use of the primers 5'-GCCGCGCGTACCCCATAGCTAGTGGC- TGAGGG-3' and 5'-GCCGCGGATTCTTCTATCTGGATTCGTTA- ATTTTCATTTCAT-3'. The PCR product was then cleaved with EcoRI and BamHI, and inserted into pGEX-3X (Pharmacia). Pure GST–CTD and GST (30 μg) were bound to 25 μl of Glutathione Sepharose 4B (Pharmacia) following the manufacturer's instructions. The GST–CTD and GST resins were incubated with 1 μg of pure mediator diluted in 100 μl of buffer T-0.3 [buffer A containing 20 mM Tris-Acetate (pH 7.8), 10% glycerol, 0.2 mM EDTA, 0.01% NP-40, 1 mM DTT, and the moleularity of potassium acetate indicated after the hyphen] for 2 hr at 4°C with gentle agitation, followed by three washes with buffer T-0.2 plus 0.1% NP-40. The resins were eluted first with 10 μl reduced glutathione, 50 μM Tris-acetate (pH 7.8) and then by boiling in SDS-PAGE buffer. The load, supernatant, washes, and elutes were analyzed by SDS-PAGE and immunoblotting.

Identification of human MED7 homolog

All of the newly discovered MED genes described here were compared with the human EST database by use of the NCBI BLAST program. Two partially overlapping sequences (GenBank clones 665201 5' and 139190 5') were found with significant homology to yeast MED7 (yMED7). A BglII–HindIII fragment from clone 139190 5' was excised and ligated into the BgIII–HindIII sites in clone 665201 5' to make a construct containing a full-length sequence for hMED7. The construct was sequenced and a start codon assigned based on homology with the yeast protein.

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The Med proteins of yeast and their function through the RNA polymerase II carboxy-terminal domain

Lawrence C. Myers, Claes M. Gustafsson, David A. Bushnell, et al.

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