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

Lawrence C. Myers,^{1,3} Claes M. Gustafsson,^{1,3} David A. Bushnell,¹ Mary Lui,² Hediye Erdjument-Bromage,² Paul Tempst,² and Roger D. Kornberg^{1,4}

¹Department of Structural Biology, Stanford University School of Medicine, Stanford, California 94305-5400 USA;

²Molecular Biology Program, Memorial Sloan-Kettering Cancer Center, New York, New York 10021 USA

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*]

Received August 26, 1997; revised version accepted October 27, 1997.

Mediator was discovered as an activity in a crude fraction that relieved activator inhibition of transcription (squenching) 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 TFIIF 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-

³These authors contributed equally to this work.

⁴Corresponding author.

E-MAIL kornberg@stanford.edu; FAX (415) 723-8464.

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 *Med2* at ~30% (Fig. 2).

Figure 1. Separation of free mediator and RNA polymerase II holoenzyme by chromatography on Mono Q. Hydroxyapatite fractions containing both mediator and polymerase II were applied to Mono Q and eluted with a linear gradient of potassium acetate. Fractions were analyzed by immunoblotting with antibodies against the mediator subunit *Srb4* and against the polymerase II subunits *Rpb1* and *Rpb3*. Mediator, in the free form, peaked at 600 mM potassium acetate and, in the holoenzyme form, peaked at 800 mM.

Figure 2. Polypeptide composition of free mediator. Peak fractions from Bio-Sil SEC 400 were pooled and analyzed by SDS-PAGE in an 11% gel. Proteins were revealed by staining with Coomassie blue. *Srb6* was present, but was too small to be detected in the gel system used. Positions of molecular mass markers are indicated on the right.

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 TFIIF 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

Figure 3. Functional characterization of free mediator. (A) Activities of pure mediator (heparin fraction) in transcription. The final gel filtration fraction used to assess the stoichiometry of mediator polypeptides (see text) was functionally indistinguishable from the heparin fraction used here and of comparable purity (data not shown). Transcription was performed with templates containing binding sites for Gal4 (GAL4:G-) and Gcn4 (GCN4:G-) upstream of the *S. cerevisiae* *CYC1* promoter fused to a G-less cassette (Kim et al. 1994). Reaction mixtures contained 2 μ g of RNA polymerase II, 0.5 μ g of mediator (lanes 2,3), and 10 ng of Gal4-VP16 (lane 3), in addition to basal transcription factors as described (Kim et al. 1994). Enhancement of basal transcription (lane 2) was 20.5-fold and could vary between 5- and 60-fold depending on reaction conditions. Activation of transcription by Gal4-VP16 (lane 3) was 18.4-fold, and could vary between 10- and 20-fold depending on reaction conditions. (B) Free mediator stimulates CTD phosphorylation by TFIIF. RNA polymerase II (Core Pol II, 100 ng) was phosphorylated by holoTFIIF (20 ng) in the presence of increasing amounts of a free mediator (heparin fraction). Stimulation of CTD kinase activity was 17.3-fold (lane 3) and 36.3-fold (lane 4).

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 mod-

ule, 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 Δ CTD) was prepared by proteolysis as described (Li and Kornberg 1994). Pol II Δ CTD was indistinguishable from wild-type enzyme in a basal transcription reaction (Fig. 7). Media-

Table 1. Mediator subunits

Gene	Deletion phenotype	Human homolog	Protein mass (kD)	Apparent mass (kD) ^a	pI	Reference ^b (yeast ORF)
<i>GAL11</i>	conditional		120	130	10.0	(1)
<i>RGR1</i>	inviable	+	123	115	9.0	(1)
<i>SIN4</i>	conditional		111	100	8.8	(1)
<i>SRB4</i>	inviable		78	98	5.1	(2)
<i>MED1</i>	conditional		64	70	4.7	(3)
<i>MED2</i>	conditional		48	62	4.4	this study (YDL005c)
<i>PGD1</i>^c	conditional		47	48	9.4	this study
<i>MED4</i>	inviable		32	38^d	4.5	this study (YOR174w)
<i>MED6</i>	inviable	+	32	36 ^d	5.2	(4)
<i>SRB5</i>	conditional		34	35	4.7	(2)
<i>MED7</i>	inviable	+	32	31	6.7	this study (YOL135c)
<i>MED8</i>	inviable		25	30	5.3	this study (YBR193c)
<i>ROX3</i>	inviable		25	30	6.6	(5)
<i>SRB2</i>	conditional		23	27	5.2	(2)
<i>SRB7</i>	inviable	+	16	19	4.8	(6)
<i>SRB6</i>	inviable		14	15	4.6	(2)

Boldface type indicates the five new mediator subunits reported in this paper.

^aAs judged by SDS-PAGE analysis with respect to molecular weight standards.

^b(1) Li et al. (1995); (2) Thompson et al. (1993); (3) Bjorklund et al. (unpubl.); (4) Lee et al. (1997); (5) Gustafsson et al. (1997); (6) Hengartner et al. (1995).

^cThis gene is also known as *HRS1*.

^dMed4 and Med6 are often observed as doublets.

tor, however, failed to stimulate basal transcription by Pol II Δ CTD, nor did it elicit a response of Pol II Δ CTD 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

Figure 4. Med proteins comigrate during hydroxyapatite chromatography and gel filtration. (A) The peak of RNA polymerase II holopolymerase from DEAE-Sephacel was applied to a 20 ml hydroxyapatite column and eluted with a 200 ml gradient of 0.01–0.2 mM potassium phosphate. Fractions were analyzed by SDS-PAGE in a 10% gel and immunoblotted with antibodies directed against the proteins indicated. (B) The mediator peak from Mono Q was subjected to gel filtration through Bio-Sil SEC 400. Fractions were analyzed by SDS-PAGE in a 10% gel and immunoblotted with antibodies directed against the proteins indicated.

Med proteins function through the CTD

Figure 5. Co-immunoprecipitation of Med proteins with RNA polymerase II holoenzyme. (A) Immunoprecipitation by anti-Med2 antibody coupled to protein A-Sepharose. An RNA polymerase II holopolymerase fraction from Mono Q (Load, 5 μ g) was incubated with anti-Med2 antibody beads. The supernatant (Sup) was removed, the beads were washed, and immunoprecipitated protein was eluted (Pel). Equal amounts of the load, supernatant, and eluted proteins were analyzed by immunoblotting with antibodies against the proteins indicated. (B) Immunoprecipitation with anti-Med4 antibody coupled to protein A-Sepharose, as in A.

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

Figure 6. Mediator binds directly to the CTD. Free mediator was applied to GST-CTD and GST (control) resins and eluted with glutathione (Elution 1) and SDS (Elution 2). Proteins were analyzed by immunoblotting with antibodies directed against Med4, Med8, and the CTD (to reveal the presence of GST-CTD).

Figure 7. CTD requirement for mediator function in vitro. Transcription was performed as described with wild type polymerase (1 μ g) or Pol II Δ CTD (1 μ g) in the presence of mediator (500 ng), Gal4-VP16 (10 ng), and GCN4 (10 ng) as indicated.

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 *med6^{ts}* 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-

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 Geitz 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 tetrads (>15) were dissected on YPD agar and scored for nutritional auxotrophies and growth at various temperatures.

Dissection of each tetrad from the *MED4/med4::TRP1* (MG101), *MED7/med7::HIS3* (MG102), and *MED8/med8::LEU2* (MG103) diploids resulted in two or fewer viable spores. All viable spores were auxotrophs of the appropriate marker, indicating that *MED4*, *MED7*, and *MED8* were essential. A PCR product containing ~300 bp upstream and ~200 bp downstream of each essential *MED* gene was cloned into the *Bam*HI-*Xho*I 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 *MED2/med2::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 *Bam*HI-*Xho*I 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-Sephacel (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) 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, 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 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 A-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 multiprotein 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-

Table 2. Yeast strains

Strain	Genotype
CRY3	<i>MATa/α ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1</i>
BJ926	<i>MATa/MATα trp1/TRP1 Prc1-126/Prc1-126 pep4-3/pep4/3 prp1-1122/prp1-1122 can1/can1</i>
MG101	<i>MATa/α ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 med4Δ1::TRP1/MED3</i>
MG102	<i>MATa/α ade2-1 can1-100 his3-11 15leu2-3,112 trp1-1 ura3-1 med7Δ1::HIS3/MED7</i>
MG103	<i>MATa/α ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 med8Δ1::LEU2/MED8</i>
MG104	<i>MATa/α ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 med2Δ1::TRP1/MED2</i>

jument-Bromage et al. 1994; Lui et al. 1996) and were fractionated by reversed-phase HPLC (Elicone et al. 1994) with the use of a 1-mm Reliasil C₁₈ column. Selected peak fractions were analyzed by a combination of automated Edman chemical degradation (Tempst et al. 1994) and matrix-assisted laser-desorption ionization time-of-flight mass spectrometry. Peptide sequences were compared with entries in the Saccharomyces Genome Database (SGD, Stanford Genomic Resources, Stanford University) with the use of the National Center for Biotechnology Information (NCBI) 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 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 A. Aguilera (Universidad de Sevilla, Spain). Anti-RPB3 antibodies were a gift from R. Burgess and N. Thompson (McArdle Laboratory for Cancer Research, Madison, WI).

Immunoprecipitation

Anti-Med2 and anti-Med4 antibodies were coupled to protein A-Sepharose beads and were used to immunoprecipitate a purified holopolymerase as described (Li et al. 1995), with the following modifications. Prior to incubation with the holopolymerase fraction, 50 μ l of the anti-Med6 beads were washed with 100 μ l of 50 mM glycine (pH 2.3), 150 mM NaCl, followed by several washes with 100 μ l of IP-200 wash buffer (20 mM HEPES at pH 7.6, 10% glycerol, 12.5 mM MgCl₂, 0.1 mM EDTA, 0.2% NP-40, 0.1 mM DTT, 200 mM potassium acetate). A Mono Q fraction of holopolymerase (10 μ l) was diluted with 40 μ l of buffer Q-0.5 (no dithiothreitol or protease inhibitors), centrifuged for 5 min at 13,000 rpm, and incubated with the beads for 4 hr at 4°C. The beads were then washed three times with 200 μ l of buffer IP-200 and eluted twice with 17.5 μ l of 5 M urea (10 min at room temperature). The eluates combined with 20 μ l of 2 $\times$ SDS gel-loading buffer were loaded on gels for immunoblotting.

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 TFIIF 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*

Plasmid	Description
pGM23	pRS316 with the <i>Bam</i> HI- <i>Xho</i> I fragment removed and a PCR product, using the oligonucleotides 5'-dCGCGGGATCCAGAATGCGAATGCACAG-3' and 5'-dCGCGCTCGAGTTTCCAGTTCCTGTGG-3' containing the full <i>MED4</i> coding sequence inserted.
pGM24	pRS316 with the <i>Bam</i> HI- <i>Xho</i> I fragment removed and a PCR product (PCR with oligonucleotides 5'-dCGCGGGATCCCATCAACATCCATCCTG-3' and 5'-dCGCGCTCGAGAATCTCGGAAATACGTC-3') containing the full <i>MED7</i> coding sequence inserted.
pGM25	pRS316 with the <i>Bam</i> HI- <i>Xho</i> I fragment removed and a PCR product (PCR with the oligonucleotides 5'-dCGCGGGATCCAGTTATTACTTGGGTCC-3' and 5'-dCGCGCTCGAGAACCTACTAAGGTTTCC-3') containing the full <i>MED8</i> coding sequence inserted.
pGM26	pRS316 with the <i>Xba</i> I- <i>Xho</i> I fragment removed and a PCR product (PCR with oligonucleotides 5'-dGCGCTCTAGAGCAAGGCCAGAGCTTGTCAT-3' and 5'-dCGCGCTCGAGGCATGCATCTCTCATGACG-3') containing the full <i>MED2</i> coding sequence inserted.
pGM27	pGEX-3X with the <i>Bam</i> HI- <i>Eco</i> RI fragment removed and a PCR product (PCR with oligonucleotides 5'-dGCGCGCGGATCCCCATGTCTGTCCAAGATACTAAAG-3' and 5'-dCCGGCCGAATTCTTAGAAGTCGTCCGGATCAAACA-3') containing the full <i>MED4</i> coding sequence inserted.
pGM28	pGEX-3X with the <i>Bam</i> HI- <i>Eco</i> RI fragment removed and a PCR product (PCR with oligonucleotides 5'-dGCGCGCGGATCCCCATGTCCAATGATCCTGGTAAT-3' and 5'-dCCGGCCGAATTCTTATTGGGACGACGATGGAGGAG-3') containing the full <i>MED7</i> coding sequence inserted.
pGM29	pGEX-3X with the <i>Bam</i> HI- <i>Eco</i> RI fragment removed and a PCR product (PCR with oligonucleotides 5'-dGCGCGCGGATCCCCATGTACAATCTACTGCATCA-3' and 5'-dCCGGCCGAATTCTTATTACTAGATGATGTTGAAGTA-3') containing the full <i>MED8</i> coding sequence inserted.
pGM30	pGEX-3X with the <i>Bam</i> HI- <i>Eco</i> RI fragment removed and a PCR product (PCR with oligonucleotides 5'-dGCGCGCGGATCCCCATGGTAGTACAAAATAGCCC-3' and 5'-dCCGGCCGAATTCTTATCGTTGAACATTACATCCGA-3' containing the first 25 kD of the <i>MED2</i> coding sequence inserted.

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'-GCGCGCGGATCCCCAATGACGCTATGGC-TGGAGG-3' and 5'-CCGGCCGAATTCTTATCATCTGGA-ATTTTCATTTTCAT-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 molarity 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 mM reduced glutathione, 50 mM 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 *BglIII*-*HindIII* fragment from clone 139190 5' was excised and ligated into the *BglIII*-*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.

Acknowledgments

We thank G. Jensen for purified transcription proteins and B. Cairns for much help in the analysis of the *Δmed2* phenotype. L.C.M. was a recipient of a Cancer Research Fund of the Damon Runyon-Walter Winchell Foundation Fellowship, DRG-1361. C.M.G. was a recipient of a Swedish Cancer Society postdoctoral fellowship. This research was supported by National Institutes of Health grant GM36659 to R.D.K., by National Science Foundation grant DBI-940123 to P.T., and by National Cancer Institutes core grant 5 P30 CA08748 to the Sloan-Kettering Sequencing Laboratory.

The publication costs of this article were defrayed in part by payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 USC section 1734 solely to indicate this fact.

References

Boeke, J., J. Truehart, B. Natsoulis, and G.R. Fink. 1987. 5-Fluoroorotic acid as a selective agent in yeast molecular genetics. *Methods Enzymol.* **154**: 164-175.

Bröhl, S., T. Lisowsky, G. Riemen, and G. Michaelis. 1994. A new nuclear suppressor system for a mitochondrial RNA polymerase mutant identifies an unusual zinc-finger protein and a polyglutamine domain protein in *Saccharomyces cerevisiae*. *Yeast* **10**: 719-731.

Burley, S.K. and R.G. Roeder. 1996. Biochemistry and structural biology of transcription factor IID (TFIID). *Annu. Rev. Biochem.* **65**: 769-799.

Cairns, B.R., Y.J. Kim, M.H. Sayre, B.C. Laurent, and R.D. Kornberg. 1994. A multisubunit complex containing the SWI1-

ADR6, SWI2-SNF2, SWI3, SNF5, and SNF6 gene products isolated from yeast. *Proc. Natl. Acad. Sci.* **91**: 1950-1954.

Chao, D.M., E.L. Gadbois, P.J. Murray, S.F. Anderson, M.S. Sonu, J.D. Parvin, and R.A. Young. 1996. A mammalian SRB protein associated with an RNA polymerase II holoenzyme. *Nature* **380**: 82-85.

Démolis, N., L. Mallet, F. Bussereau, and M. Jacquet. 1993. RIM2, MSI1 and PGI1 are located within an 8 kb segment of *Saccharomyces cerevisiae* chromosome II, which also contains the putative ribosomal gene L21 and a new putative essential gene with a leucine zipper motif. *Yeast* **9**: 645-659.

Elicone, C., M. Lui, S. Geromanos, H. Erdjument-Bromage, and P. Tempst. 1994. Microbore reversed-phase high-performance liquid chromatographic purification of peptides for combined chemical sequencing-laser-desorption mass spectrometric analysis. *J. Chromatogr.* **676**: 121-137.

Erdjument-Bromage, H., M. Lui, D.M. Sabatini, S.H. Snyder, and P. Tempst. 1994. High-sensitivity sequencing of large proteins: Partial structure of the rapamycin-FKBP12 target. *Protein Sci.* **3**: 2435-2446.

Fan, H.-Y., K.K. Cheng, and H.L. Klein. 1996. Mutations in the RNA polymerase II transcription machinery suppress the hyper recombination mutant *hpr1Δ* of *Saccharomyces cerevisiae*. *Genetics* **142**: 749-759.

Flanagan, P.M., R.J. Kelleher III, M.H. Sayre, H. Tschochner, and R.D. Kornberg. 1991. A mediator required for activation of RNA polymerase II transcription in-vitro. *Nature* **350**: 436-438.

Gustafsson, C.M., L.C. Myers, Y. Li, M.J. Redd, M. Lui, H. Erdjument-Bromage, P. Tempst, and R.D. Kornberg. 1997. Identification of Rox3 as a component of mediator and RNA polymerase II holoenzyme. *J. Biol. Chem.* **272**: 48-50.

Hengartner, C.J., C.M. Thompson, J. Zhang, D.M. Chao, S.M. Liao, A.J. Koleske, S. Okamura, and R.A. Young. 1995. Association of an activator with an RNA polymerase II holoenzyme. *Genes & Dev.* **9**: 897-910.

Henry, N.L., M.H. Sayre, and R.D. Kornberg. 1992. Purification and characterization of yeast RNA polymerase II general initiation factor g. *J. Biol. Chem.* **267**: 23388-23392.

Jiang, Y.W. and D.J. Stillman. 1995. Regulation of HIS4 expression by the *Saccharomyces cerevisiae* SIN4 transcriptional regulator. *Genetics* **140**: 103-114.

Jiang, Y.W., P.R. Dohrmann, and D.J. Stillman. 1995. Genetic and physical interactions between yeast RGR1 and SIN4 in chromatin organization and transcriptional regulation. *Genetics* **140**: 47-54.

Kean, L.S., R.S. Fuller, and J.W. Nichols. 1993. Retrograde lipid traffic in yeast: Identification of two distinct pathways for internalization of fluorescent-labeled phosphatidylcholine from the plasma membrane. *J. Cell. Biol.* **123**: 1403-1419.

Kelleher, R.J., III, P.M. Flanagan, and R.D. Kornberg. 1990. A novel mediator between activator proteins and the RNA polymerase II transcription apparatus. *Cell* **61**: 1209-1216.

Kim, Y.J., S. Bjorklund, Y. Li, M.H. Sayre, and R.D. Kornberg. 1994. A multiprotein mediator of transcriptional activation and its interaction with the C-terminal repeat domain of RNA polymerase II. *Cell* **77**: 599-608.

Koleske, A.J. and R.A. Young. 1994. An RNA polymerase II holoenzyme responsive to activators. *Nature* **368**: 466-469.

Lee, Y.C., S. Min, B.S. Gim, and Y.J. Kim. 1997. A transcriptional mediator protein that is required for activation of many RNA polymerase II promoters is conserved from yeast to humans. *Mol. Cell. Biol.* **17**: 4622-4632.

Li, Y. and R.D. Kornberg. 1994. Interplay of positive and negative effectors in function of the C-terminal repeat domain of RNA polymerase II. *Proc. Natl. Acad. Sci.* **91**: 2362-2366.

Myers et al.

- Li, Y., S. Bjorklund, Y.W. Jiang, Y.J. Kim, W.S. Lane, D.J. Stillman, and R.D. Kornberg. 1995. Yeast global transcriptional regulators Sin4 and Rgr1 are components of mediator complex-RNA polymerase II holoenzyme. *Proc. Natl. Acad. Sci.* **92**: 10864–10868.
- Li, Y., S. Bjorklund, Y.J. Kim, and R.D. Kornberg. 1996. Yeast RNA polymerase II holoenzyme. *Methods Enzymol.* **273**: 172–175.
- Liao, S.M., I.C.A. Taylor, R.E. Kingston, and R.A. Young. 1991. RNA polymerase II carboxy-terminal domain contributes to the response to multiple acidic activators in vitro. *Genes & Dev.* **5**: 2431–2440.
- Lorenz, M.C., R.S. Muir, E. Lim, L. McElver, S.C. Weber, and J. Heitman. 1995. Gene disruption with PCR products in *Saccharomyces cerevisiae*. *Gene* **158**: 113–117.
- Lui, M., P. Tempst, and H. Erdjument-Bromage. 1996. Methodical analysis of protein-nitrocellulose interactions to design a refined digestion protocol. *Anal. Biochem.* **241**: 156–166.
- Moqtaderi, Z., Y. Bai, D. Poon, P.A. Weil, and K. Struhl. 1996. TBP-associated factors are not generally required for transcriptional activation in yeast. *Nature* **383**: 188–191.
- Pearson, W.R. and D.J. Lipman. 1988. Improved tools for biological sequence comparison. *Proc. Natl. Acad. Sci.* **85**: 2444–2448.
- Piruat, J.I. and A. Aguilera. 1996. Mutations in the yeast SRB2 general transcription factor suppress hpr1-induced recombination and show defects in DNA repair. *Genetics* **143**: 1533–1542.
- Piruat, J.I., S. Chavez, and A. Aguilera. 1997. The yeast HRS1 gene is involved in positive and negative regulation of transcription and shows genetic characteristics similar to Sin4 and GAL11. *Genetics* (in press).
- Pugh, B.F. and R. Tjian. 1990. Mechanism of transcriptional activation by Sp1: Evidence for coactivators. *Cell* **61**: 1187–1198.
- Santos-Rosa, H. and A. Aguilera. 1995. Isolation and genetic analysis of extragenic suppressors of the hyper-deletion phenotype of the *Saccharomyces cerevisiae* hpr1Δ mutation. *Genetics* **139**: 57–66.
- Santos-Rosa, H., B. Clever, W.D. Heyer, and A. Aguilera. 1996. The yeast HRS1 gene encodes a polyglutamine-rich nuclear protein required for spontaneous and hpr1-induced deletions between direct repeats. *Genetics* **142**: 705–716.
- Scafe, C., D. Chao, J. Lopes, J.P. Hirsch, S. Henry, and R.A. Young. 1990. RNA polymerase II C-terminal repeat influences response to transcriptional enhancer signals. *Nature* **347**: 491–494.
- Schiestl, R.H. and R.D. Gietz. 1989. High efficiency transformation of intact yeast cells using single stranded nucleic acids as a carrier. *Curr. Genet.* **16**: 339–346.
- Sikorski, R.S. and P. Hieter. 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics* **122**: 19–28.
- Svejstrup, J.Q., W.J. Feaver, J. Lapointe, and R.D. Kornberg. 1994. RNA polymerase transcription factor IIH holoenzyme from yeast. *J. Biol. Chem.* **269**: 28044–28048.
- Svejstrup, J.Q., Y. Li, J. Fellows, A. Gnatt, S. Bjorklund, and R.D. Kornberg. 1997. Evidence for a mediator cycle at the initiation of transcription. *Proc. Natl. Acad. Sci.* **94**: 6075–6078.
- Tempst, P., S. Geromanos, C. Elicone, and H. Erdjument-Bromage. 1994. Improvements in microsequencer performance for low picomole sequence analysis. *Methods Comp. Meth. Enzymol.* **6**: 248–261.
- Thomas, B.J. and R. Rothstein. 1989. Elevated recombination rates in transcriptionally active DNA. *Cell* **56**: 619–630.
- Thompson, C.M., A.J. Koleske, D.M. Chao, and R.A. Young. 1993. A multisubunit complex associated with the RNA polymerase II CTD and TATA-binding protein in yeast. *Cell* **73**: 1361–1375.
- Thompson, C.M. and R.A. Young. 1995. General requirement for RNA polymerase II holoenzymes in vivo. *Proc. Natl. Acad. Sci.* **92**: 4587–4590.
- Walker, S.S., J.C. Reese, L.M. Apone, and M. Green. 1996. Transcription activation in cells lacking TAF-IIs. *Nature* **383**: 185–188.

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.

Genes Dev. 1998, **12**:

Access the most recent version at doi:[10.1101/gad.12.1.45](https://doi.org/10.1101/gad.12.1.45)

References

This article cites 41 articles, 20 of which can be accessed free at:
<http://genesdev.cshlp.org/content/12/1/45.full.html#ref-list-1>

License

Email Alerting Service

Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article or [click here](#).