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Head Module Control of Mediator Interactions

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Summary

Yeast Mediator proteins interacting with Med17(Srb4) have been expressed at a high level with the use of recombinant baculoviruses and recovered in homogeneous form as a seven subunit, 223 kDa complex. Electron microscopy and single-particle analysis identify this complex as the Mediator head module. The recombinant head module complements "headless" Mediator for the initiation of transcription in vitro. The module interacts with an RNA polymerase II-TFIIF complex, but not with the polymerase or TFIIF alone. This interaction is lost in the presence of a DNA template and associated RNA transcript, recapitulating the release of Mediator that occurs upon the initiation of transcription. Disruption of the head module in a temperature-sensitive mutant in vivo leads to the release of middle and tail modules from a transcriptionally active promoter. The head module evidently controls Mediator-RNA polymerase II and Mediator-promoter interactions.

Introduction

Most, if not all, RNA polymerase II promoters are transcribed by a common set of proteins, comprising the multisubunit RNA polymerase, five general transcription factors, and the Mediator of transcriptional regulation. RNA polymerase II alone is capable of unwinding, transcribing, and rewinding duplex DNA. The general transcription factors are responsible for promoter recognition and are crucial for formation of a transcription bubble and for the initiation of RNA synthesis (Conaway and Conaway, 1993). Mediator provides the interface with activator and repressor proteins. It transduces regulatory information from enhancers and other DNA elements to promoters in all eukaryotes (Kornberg, 2005).

Twenty-two of the 25 proteins so far identified with yeast Mediator have homologs in higher cells (Bourbon et al., 2004). Nearly half of the yeast Mediator proteins were revealed by genetic screens for mutations affecting the regulation of transcription (Gustafsson et al., 1997; Li et al., 1995). One screen, for suppressors of truncation mutants of the RNA polymerase II C-terminal domain (CTD), led to the isolation of so-called *SRB* genes, nine of which encode Mediator subunits (Kim et al., 1994), and five of which are essential for yeast cell growth (Nonet and Young, 1989; Thompson et al., 1993). A temperature-sensitive mutation of one of these genes, now designated *MED17*(*SRB4*), has proven particularly informative about the role of Mediator in vivo (Thompson and Young, 1995) and in vitro (Takagi and Kornberg, 2006). At a restrictive temperature, transcription ceases in the mutant cells (Holstege et al., 1998) and cannot be reconstituted in a cell-free system (Takagi and Kornberg, 2006). Mediator is evidently required not only for regulated transcription but for any transcription at all. Indeed, Mediator stimulates "basal" (unregulated) transcription in vitro (Kim et al., 1994). Mediator also stimulates phosphorylation of the polymerase II CTD by general transcription factor TFIIH, and binding of Mediator to the CTD has been thought to underlie Mediator-polymerase II interaction (Kim et al., 1994).

The earliest evidence of Mediator-polymerase II interaction came from copurification and coimmunoprecipitation (Kim et al., 1994; Koleske et al., 1992; Koleske and Young, 1994; Thompson et al., 1993). Structural analysis by electron microscopy (EM) and image processing revealed Mediator alone as a globular entity, unfolding to a crescent shape that largely envelops the polymerase in the complex formed between them (Figure 1A) (Asturias et al., 1999; Davis et al., 2002). Beyond its presumed interaction with the CTD, Mediator makes extensive contact with the polymerase along the "back" side of the molecule (the "upstream" end in a transcribing complex) (Asturias, 2004; Davis et al., 2002). Mediator appears, even at the low resolution of the analysis, to be divided into three modules that have been termed head, middle, and tail (Figure 1A). The tail can be deleted without loss of yeast cell viability (Li et al., 1995) or of the remaining structure in the electron microscope (Dotson et al., 2000), supporting its existence as a discrete module. There are indications that Med17(Srb4) and other Srb proteins are prominent in the head module, while proteins important for negative regulation may be found in the middle module (Guglielmi et al., 2004; Lee and Kim, 1998).

The intimate nature of Mediator-polymerase interaction poses fundamental questions about the Mediator mechanism: how is regulatory information transmitted through the Mediator-polymerase interface? How are polymerase-general transcription factor interactions accommodated as well? How is the extensive interface disrupted following the initiation of transcription, recycling Mediator to new initiation complexes and freeing polymerase to traverse the body of a gene?

The size (>1 MDa) and complexity of Mediator pose formidable challenges for biochemical analysis. We report here on an approach to the problem one module

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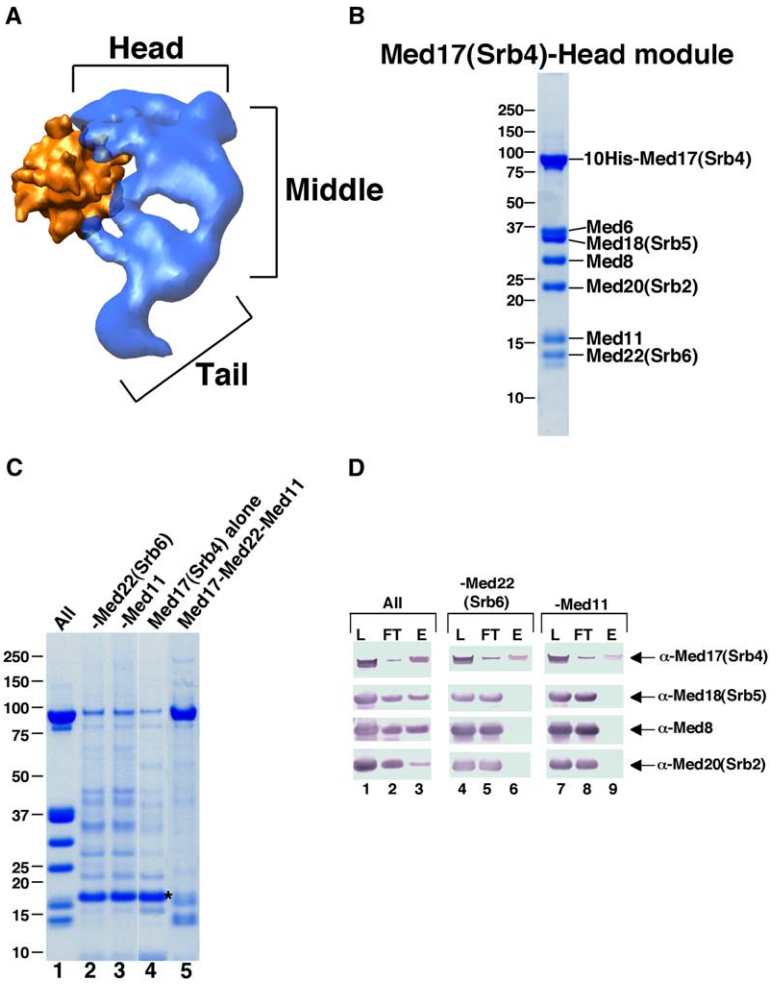

Figure 1. Recombinant Med17(Srb4)-Head Module

(A) RNA polymerase II-Mediator structure determined by EM and single-particle analysis (Davis et al., 2002). Head, middle, and tail modules are indicated.

(B) SDS-PAGE of recombinant Med17(Srb4)-head module. Approximately six micrograms of a peak of Ni column fraction was electrophoresed in a 4%–12% NuPAGE, followed by staining with Coomassie blue.

(C) Med22 and Med11 are required for assembly of Med17(Srb4)-head module. All seven subunits of Med17(Srb4)-head module (lane 1), all subunits except Med22(Srb6) (lane 2), all subunits except Med11 (lane 3), Med17(Srb4) alone (lane 4), and Med17(Srb4), Med22, and Med11 (lane 5) were expressed in insect cells and purified by means of a 10 histidine tag on the Med17(Srb4) subunit. Approximately six micrograms of Ni column fraction was electrophoresed in a 4%–12% NuPAGE, followed by staining with Coomassie blue. Molecular weight markers are indicated on the left. Asterisk indicates proteolytic fragment of Med17(Srb4).

(D) Expression levels of Med17(Srb4), Med18, Med8, and Med20 subunits in the presence (lanes 1–3) or absence of Med22 (lanes 4–6) or the absence of Med11 (lanes 7–9). Clarified cell lysate (load) was applied to a Ni column. Ten microliters of load (L), flowthrough (FT), and eluate (E) of Ni column was resolved by 4%–12% NuPAGE, with detection by immunoblotting with anti-Med17, anti-Med18, anti-Med8, and anti-Med20 antibodies.

at a time. Production of the head module in recombinant form has opened the way to structural and functional studies. The first such studies have given unexpected insight into the architecture of the module, its role in transcription, and the dynamics of the transcription machinery.

Results

Recombinant Med17(Srb4) Module

Previous studies have shown the possibility of expressing Mediator modules with the use of recombinant baculovirus in insect cells (Kang et al., 2001; Koh et al., 1998). The amounts of material obtained were small, however, requiring detection by immunoblotting or silver staining, and insufficient for detailed biochemical and structural analysis. We sought to overcome this limitation by screening recombinant baculoviruses for high levels of expression of Mediator subunits. Repeated rounds of screening resulted in a set of baculoviruses for Mediator proteins, including all those previously associated with Med17(Srb4) at a low expression level: Med6, Med18(Srb5), Med8, Med20(Srb2), Med11, Med22(Srb6), and Med19(Rox3). Insect cell cultures (100 ml) were infected with various combinations of these viruses and with viruses for expression of Med21(Srb7), Med7, Med1, Med2, Med4, Med9, and Med10 as well. Proteins associated with Med17(Srb4), which bore a decahistidine tag, were recovered by cell breakage, ammonium sulfate precipitation, and affinity purification on Ni resin and were revealed by SDS-PAGE and staining with Coomassie blue (Figure 1B). A stoichiometric complex of seven proteins was obtained, comprising those previously found to be associated with Med17(Srb4), with the exception of Med19(Rox3), which was expressed but failed to bind the Ni resin and could be omitted without effect on the assembly of the rest of the complex (see Figure S1 in the Supplemental Data available with this article online). Following further purification on a HiTrap Q column, approximately 0.3 mg of the seven subunit complex was obtained from 100 ml of insect cell culture in homogeneous form.

Med17(Srb4) Module Organization

Omission of either of the smallest subunits, Med11 or Med22(Srb6), resulted in virtually no Med17(Srb4) module at all (Figure 1C, lanes 2 and 3). This was not due to an effect on expression, as shown by immunoblotting with antibodies against Med8, Med17(Srb4), Med18(Srb5), and Med20(Srb2) (Figure 1D). Rather, Med11 and Med22(Srb6) were evidently required for recovery of Med17(Srb4), since expression of the three proteins resulted in

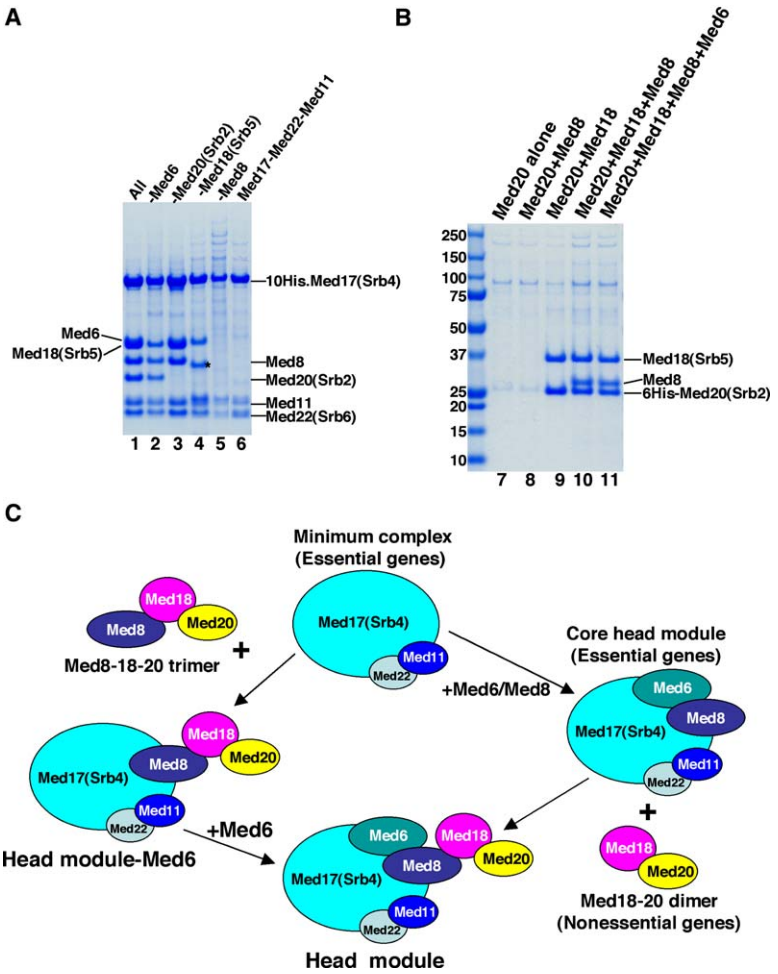

Figure 2. Molecular Architecture of Med17(Srb4)-Head Module

(A) All subunits (lane 1) or all subunits with the omission of Med6 (lane 2), Med20 (lane 3), Med18 (lane 4), or Med8 (lane 5) were expressed in insect cells and purified by means of a ten histidine tag on Med17(Srb4). Protein complexes were resolved by 4%–12% NuPAGE and stained with Coomassie blue. Asterisk indicates apparent slight proteolysis of Med8 in the absence of Med18(Srb5).

(B) A subcomplex of Med8, Med18(Srb5), and Med20(Srb2) was revealed by expressing 6His-Med20 alone (lane 7); Med20 and Med8 (lane 8); Med20 and Med18 (lane 9); Med20, Med18, and Med8 (lane 10); and Med20, Med18, Med8, and Med6 (lane 11). Protein complexes were purified by means of a six histidine tag on Med20(Srb2) and were resolved by 4%–12% NuPAGE and stained with Coomassie blue. Molecular weight markers are at the left.

(C) Schematic representation of Med17(Srb4)-head module and subcomplexes based on (A) and (B).

a stoichiometric complex (Figure 1C, lane 5). In the absence of the two small proteins, Med17(Srb4) may have been degraded, and the 10 histidine tag associated only with a small fragment (Figure 1C, lanes 2–4).

Other subcomplexes of the Med17(Srb4) module could be formed as well. Thus, omission of either Med6 or Med20(Srb2) resulted in complexes of the remaining six proteins (Figure 2A, lanes 2 and 3). Omission of Med18(Srb5) led to a complex also lacking Med20(Srb2) (Figure 2A, lane 4). Consistent with this, expression of only Med18(Srb5) and Med20(Srb2) yielded a complex of these two proteins alone (Figure 2B, lane 9). Finally, omission of Med8 resulted in the loss of all of the three proteins Med6, Med18(Srb5), and Med20(Srb2) from the complex with Med17(Srb4), Med11, and Med22(Srb6) (Figure 2A, lane 5). Conversely, coexpression of Med8 with Med6, Med18(Srb5), and Med20(Srb2) yielded a ternary Med8-Med18(Srb5)-Med20(Srb2) complex, though without Med6 (Figure 2B, lanes 10 and 11).

These results lead to a picture of Mediator Med17(Srb4) module architecture based on a minimal complex of Med17(Srb4), Med11, and Med22(Srb6) (Figure 2C). Interaction with Med6 and Med8 (which also interact with one another) results in a "core" module made up entirely of subunits encoded by essential genes. The core complex binds Med6 (essential) and a Med18(Srb5)-Med20(Srb2) dimer, both through Med8, to form the complete module. This picture is consistent with results from pairwise two-hybrid analysis in yeast (Guglielmi et al., 2004).

Identification of Med17(Srb4) Module with the Mediator Head

The surface topography of the recombinant complex was revealed by EM and image processing of negatively stained specimens. Images of 358 particles were classified according to direction of view and averaged (Figure 3A). These averages corresponded with views of the head region of an RNA polymerase II-Mediator complex (Figures 3B and 3C) and were clearly distinguishable from views of the middle and tail regions (data not shown). On this basis, we identify the seven subunit Med17(Srb4) module with the Mediator head.

Functional Activity of Recombinant Head Module

The recombinant module could be assayed for activity with extract from med17(srb4)ts cells, which is incapable of transcription at 30°C (Takagi and Kornberg, 2006). Head module proteins dissociate from the mutant complex (Takagi and Kornberg, 2006) (C.M. Gustafsson, personal communication), resulting in a "headless" Mediator at 30°C (C.M. Gustafsson, personal communication). Addition of recombinant head module to the mutant cell extract restored activity to about the level

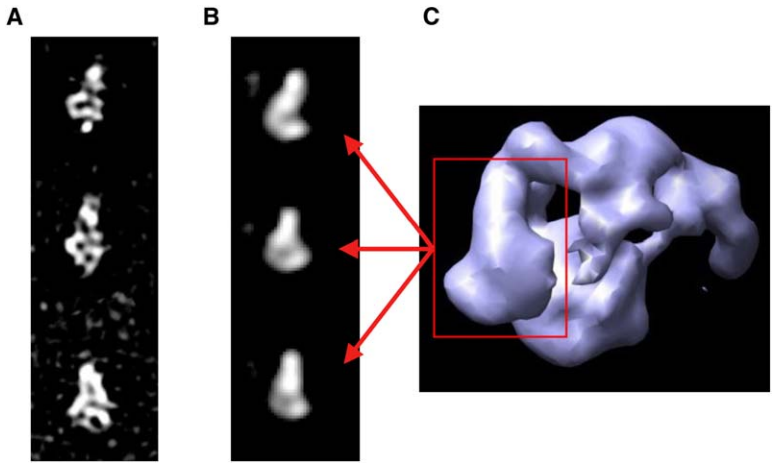

Figure 3. EM and Single-Particle Analysis of the Recombinant Head Module, and Comparison with the Head Portion of the Mediator Structure

(A) Class averages obtained after alignment and classification of 358 images of recombinant head module particles preserved in stain.

(B) Selected 2D projections of the head portion of the Mediator structure (boxed in [C]) (Davis et al., 2002).

(C) 3D reconstruction of yeast Mediator-RNA polymerase II complex, calculated from images of particles preserved in stain.

observed with complete wild-type (wt) Mediator (Figure 4A), whereas addition of recombinant Med17(Srb4) protein alone was without effect (data not shown). In a fully reconstituted system with purified RNA polymerase II and general transcription factors, the head module had little effect (Figure 4B) and failed to support transcriptional activation or to stimulate CTD kinase activity (data not shown). Evidently, these functions require additional Mediator modules, and the restoration of transcriptional activity in med17(srb4)ts cell extract was due to complementation between the head module and headless Mediator.

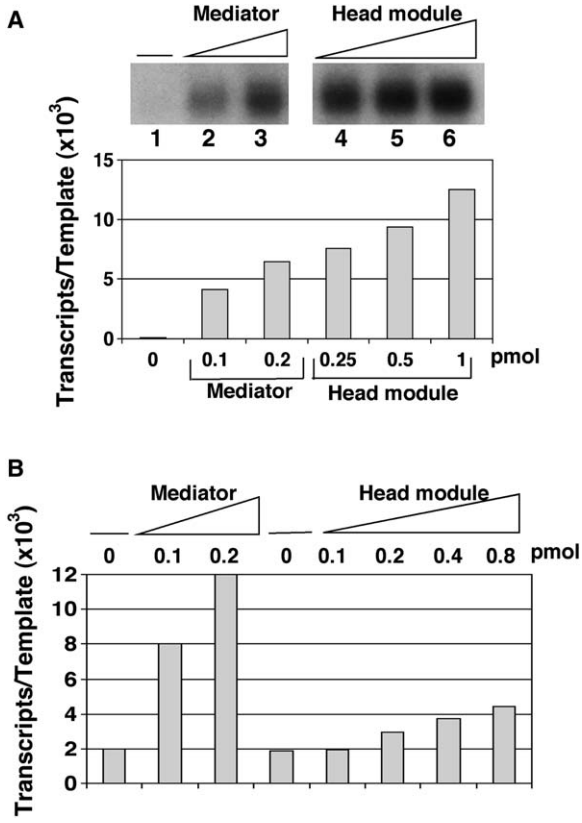

Figure 4. Transcription Activity of the Head Module

(A) Transcription was performed with *srb4ts* mutant extract at 30°C as described (Takagi and Kornberg, 2006), with addition of purified Mediator (0, 0.1, and 0.2 pmol in lanes 1–3) or of recombinant head module (0.25, 0.5, and 1 pmol in lanes 4 and 5). Transcripts (~360 bp) separated by 6% denaturing PAGE and revealed by autoradiography are shown (top) and quantified by PhosphorImager analysis (bottom).

(B) Transcription was performed with purified proteins at 24°C as described (Takagi and Kornberg, 2006), with 0, 0.1, and 0.2 pmol of purified Mediator or with 0, 0.1, 0.2, 0.4, and 0.8 pmol of reombinant head module. Transcripts were quantified by PhosphorImager analysis.

Head Module Interactions

The head module has been reported to form complexes with the RNA polymerase II CTD and with transcriptional activators (Kang et al., 2001; Koh et al., 1998). Interaction with the CTD was anticipated from the original isolation of the *SRB* genes, based on suppression of a CTD truncation mutation (Nonet and Young, 1989). We investigated head module-CTD interaction with mixtures of decahistidine-tagged head module and GST-CTD. There was no retention of GST-CTD on a Ni column nor any retention of head module on a glutathione column (Figure S2A) and thus no evidence of stable complex formation. In all likelihood, genetic suppression was due to an indirect effect and not to Srb protein-CTD interaction. Recombinant head module also failed to form stable complexes with TBP (Figure S2B), TFIIB (Figure S2C), or transcriptional activation domains (Figure S3) or DNA (Figure S4).

Despite the apparent contact of the head module with the polymerase in the electron microscope structure of the Mediator-polymerase II complex (Figure 1A), recombinant head module failed to bind stably to the polymerase (Figure S5A). The head module did, however, bind stably to a polymerase-TFIIF complex, giving rise to a stoichiometric assemblage of 22 protein molecules (Figures 5A and 5B). This result, together with the lack of binding to the polymerase or TFIIF alone (Figure S5B), suggests a simultaneous interaction of the head module with both polymerase and TFIIF.

Release of Head Module from a Transcribing Complex

Chromatin immunoprecipitation (ChIP) has revealed a close proximity of Mediator to upstream activating sequences (UASs) and promoters, but not to open reading

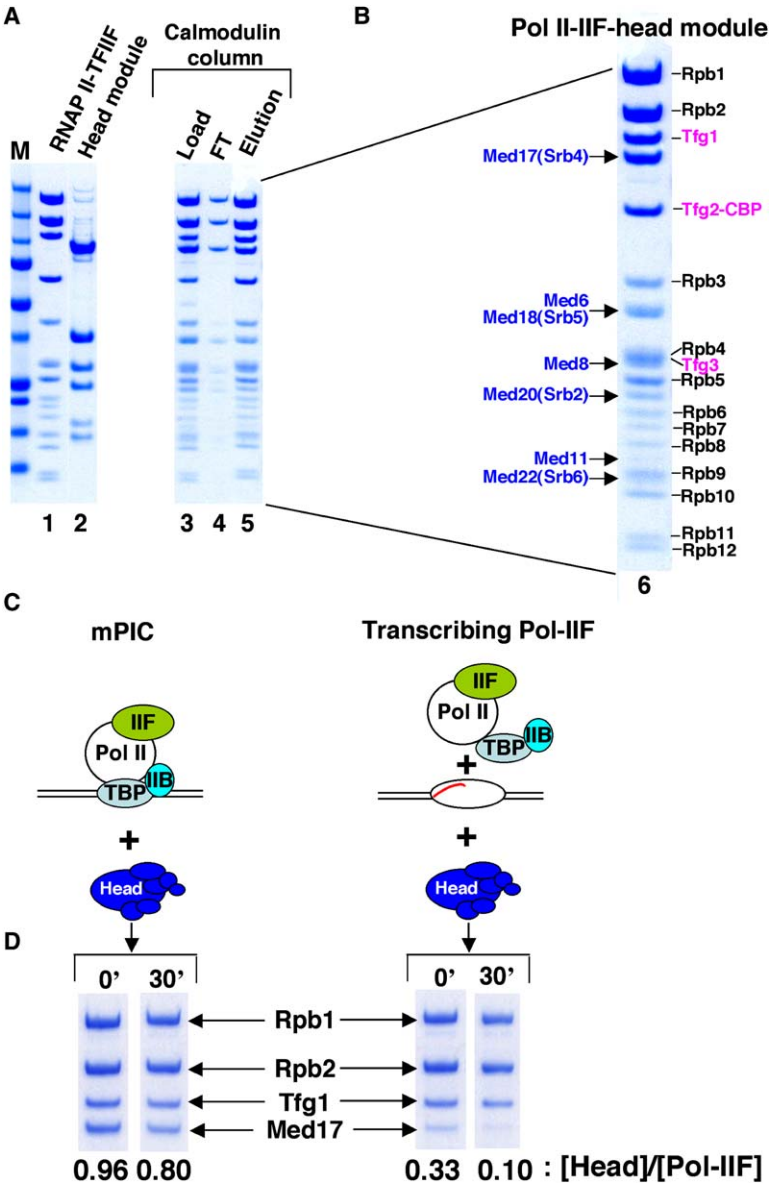

Figure 5. The Head Module Interacts with an RNA Polymerase II-TFIIF Complex before, Not after, Transcription Initiation

(A) RNA polymerase-TFIIF complex (lane 1) and head module (lane 2) were mixed, kept on ice for 30 min (load, lane 3), and purified on a calmodulin column by means of a calmodulin binding peptide (CBP) on the C terminus of the Tfg2 subunit of TFIIF. Six to ten micrograms of column fractions were subjected to 4%–12% NuPAGE and stained with Coomassie blue. The gel profile of the eluate (lane 5) is enlarged in (B) (lane 6). Subunits of the head module are indicated in blue, subunits of RNA polymerase II in black, and subunits of TFIIF in pink. Head module is released from a transcribing complex. (C) Components diagrammed schematically were mixed, adsorbed to a calmodulin column, and washed with either 5 column vol (time 0) or 200 column vol (over a 30 min period) of buffer B(125). (D) Elution was performed with buffer containing EGTA, and ~1 μg of the eluates was subjected to 4%–12% NuPAGE and staining with Coomassie blue. The gel was scanned, and ratios of [Head] to [Pol-IIF] were determined, as indicated below the gels.

frames (ORFs) of yeast genes in vivo (Kuras et al., 2003; Pokholok et al., 2002) From this and other evidence (Svejstrup et al., 1997), it appears that Mediator is discharged from its association with RNA polymerase II following the initiation of transcription in vivo. We therefore investigated the affinity of the head module for a transcribing complex in vitro. Whereas the module bound stably to an RNA polymerase II-TFIIF-TFIIB-TBP-promoter DNA complex (minimal preinitiation complex, mPIC), binding to a complex containing a DNA bubble and nine residue complementary RNA (transcribing complex) was weaker and short-lived (Figure 5C). From the rate of head module dissociation, we estimate an order-of-magnitude difference in affinity of the head module for preinitiation and transcribing complexes.

Head Module Control of Mediator-Promoter Interaction

The discrete nature of the head module and the capacity of headless Mediator to complement head module in transcription raised the question of whether the head module is required for association of middle and tail modules with promoters in vivo. We addressed the question by ChIP analysis, with the use of *med17(srb4)ts* strains bearing TAP tags on subunits representative of the head (Med22[Srb6]), middle (Med14[Rgr1]), and tail (Med15[Gal11]) modules. Cells were grown in the presence of galactose for activation of *GAL* promoters and maintained at 30°C (permissive temperature) or raised to 37°C (restrictive temperature). At the permissive temperature, all three tagged proteins were associated with the *GAL10* UAS (Figure 6), but not with a negative control sequence (located on chromosome V and lacking any known genes) or with the *GAL10* ORF (data not shown), consistent with previous reports on Med20(Srb2) (Bhaumik et al., 2004) and Med14(Rgr1) (Bhaumik et al., 2004; Kuras and Struhl, 1999). Upon shift to the restrictive temperature, the association of all three tagged proteins was diminished to a greater extent in the mutant strain than in a wt control (Figure 6). We

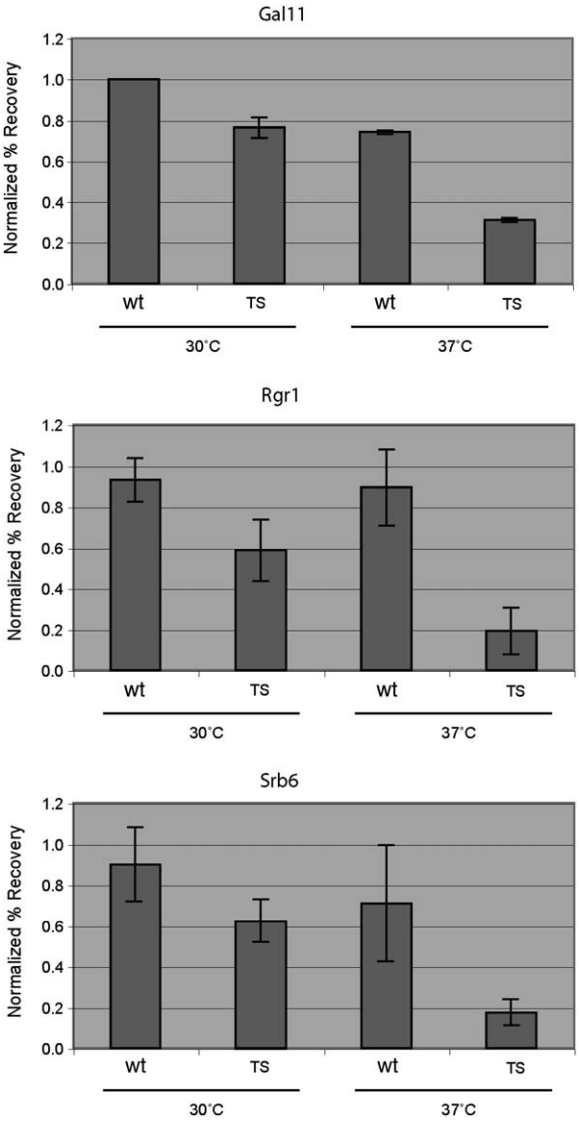

Figure 6. Loss of All Three Mediator Modules from a Transcriptionally Active Promoter in med17(srb4)ts Strains at a Restrictive Temperature, as Revealed by ChIP

Percent recoveries of immunoprecipitated GAL1-10 promoter DNA crosslinked to one of the three TAP-tagged Mediator subunits indicated were normalized by division by the largest value obtained. Standard deviations of three measurements for each culture for Rgr1 and Srb6, and of two measurements for each culture for Gal11, are shown.

conclude that disruption of the Mediator head module leads to the release not only of head module (Bhaumik et al., 2004), but also of middle and tail modules from a promoter in vivo.

Discussion

Our development of a high-level expression system for the Mediator head module has opened the way to definitive biochemical and structural analysis. While confirming the findings of others in a general way (Guglielmi et al., 2004; Kang et al., 2001; Koh et al., 1998; Lee and Kim, 1998), our results differ in a number of regards and extend the previous findings. Besides correcting the composition of the Med17(Srb4) module (by the omission of Med19[Rox3]), we demonstrate its equivalence with the head region of Mediator seen in the electron microscope. Our findings support previous inferences regarding the association of Med17(Srb4) with the head region and the modular nature of this region (Dotson et al., 2000). They bear out the notion of rigid, discrete modules as the underlying basis for Mediator structure.

We could detect no interaction of recombinant head module with the RNA polymerase II CTD or with transcriptional activators. Rather, we demonstrate strong, specific binding to an RNA polymerase II-TFIIF complex, which requires both polymerase and TFIIF, and which is reversed by the initiation of transcription. Simultaneous interaction of the head module with both polymerase and TFIIF is consistent with electron microscope structures, showing a close proximity of the head- and TFIIF-interacting regions on the polymerase surface (Figure 7B) (Chung et al., 2003). The loss of the head module from a transcribing complex can also be rationalized in terms of the available structural information. Promoter DNA is believed to pass above or alongside the head- and TFIIF-interacting regions in a preinitiation complex (Figure 7A). Following DNA melting, the template strand descends into the polymerase cleft, and RNA-DNA hybrid emerges near the head- and TFIIF-interacting regions of the transcribing complex (Figure 7C) (Craighead et al., 2002). The DNA moves a distance of 20 Å or more and changes its direction of curvature across the polymerase surface in the process. Movement or conformational change of TFIIF may accompany the movement of DNA, depriving the head module of an essential contact and leading to its release.

ChIP analyses have previously demonstrated Mediator-UAS and Mediator-core promoter associations in vivo (Bhoite et al., 2001; Bryant and Ptashne, 2003; Cosma et al., 2001; Kuras et al., 2003). We now find that disruption of the head module discharges all Mediator modules from the UAS. The question arises whether release of the head module from RNA polymerase II upon transcription initiation leads to the release of all modules as well. While the middle module appears to contact RNA polymerase II directly (Figures 1A and 7B), persistent binding of the middle module may require a ternary head-middle-polymerase interaction. Disruption of the head module would then lead to release of the middle module. Indeed, we have not isolated any Mediator proteins in association with RNA polymerase II from *med17(srb4)ts* cells (Y.T. and R.D.K., unpublished data); instability of the head module apparently diminishes such interactions in these cells. Middle and tail modules may also contact the polymerase CTD (Myers et al., 1998), in an unphosphorylated state (Svejstrup et al., 1997), but activity in transcription likely depends on the head module.

Experimental Procedures

Expression and Purification of Mediator Head Module and Subcomplexes

ORFs of genes encoding Med6, Med18(Srb5), Med8, Med19(Rox3), Med20(Srb2), Med22(Srb6), and Med11 were amplified from yeast

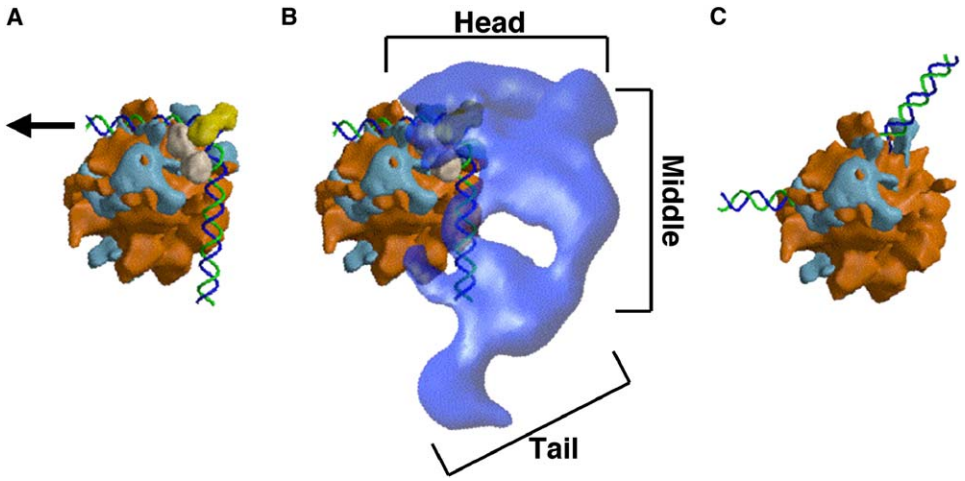

Figure 7. Models of the mPIC, mPIC-Mediator Complex, and RNA Polymerase II-TFIIF Transcribing Complex

(A) Model for mPIC was derived from EM study of an RNA polymerase II-TFIIF complex (Chung et al., 2003) and from the X-ray structure of a TBP-IIB-DNA complex (Tsai and Sigler, 2000). RNA polymerase II, TFIIF, TFIIB, and TBP are brown, light blue, yellow, and white, respectively. Arrow indicates direction of transcription.

(B) Model for mPIC-Mediator complex was produced by superimposing an EM structure of Mediator-RNA polymerase II complex (Davis et al., 2002) on the mPIC from (A). Mediator is blue. Head, middle, and tail modules are indicated.

(C) Model for an RNA polymerase II-TFIIF transcribing complex was derived from EM study of an RNA polymerase II-TFIIF complex (Chung et al., 2003) and the X-ray structure of an RNA polymerase II transcribing complex (Westover et al., 2004).

genomic DNA by polymerase chain reaction (PCR) and cloned in the pBacPAK9 baculovirus expression vector (Clontech). A 10 histidine-tagged Med17(Srb4) ORF was amplified by PCR from the vector pCT127(10His-Srb4), as described (Takagi and Kornberg, 2006). Recombinant viruses were produced in monolayers of Sf21 cells, as described (Takagi et al., 2003).

For recombinant protein expression, 100 ml of Hi5 cells (~1.5 × 10^6 cells/ml) in a 500 ml flask was infected with various combinations of cloned viruses at a multiplicity of infection of 2–10. After 72 hr, the cells were harvested by centrifugation and stored as a pellet at −80°C until use. Cells were lysed by resuspension in 20 ml of lysis buffer (50 mM HEPES-KOH [pH 7.6], 10% glycerol, and 5 mM β-mercaptoethanol) containing 400 mM potassium chloride, 0.01% NP-40 (Calbiochem), and protease inhibitor mix (final concentrations of 6 mM leupeptin, 20 mM pepstatin A, 20 mM benzamidine, and 10 mM PMSF). The cell lysate was stirred at 4°C for 30 min and clarified by centrifugation in a Beckman JA20 rotor at 15,000 rpm for 20 min and then in a Beckman Ti70 rotor at 50,000 rpm for 60 min. Ammonium sulfate was added to 35% of saturation, followed by centrifugation in a Beckman Ti70 rotor at 40,000 rpm for 20 min. The pellet was resuspended in 6 ml of buffer A (50 mM HEPES-KOH [pH 7.6], 10% glycerol, 5 mM β-mercaptoethanol) containing 10 mM imidazole (pH 8.0) and protease inhibitor mix, clarified by centrifugation in a Beckman Ti80 rotor at 40,000 rpm for 20 min, and loaded on a 0.5 ml column of Ni resin (HIS-Select, Sigma-Aldrich) in lysis buffer containing 10 mM imidazole (pH 8.0). After washing with 5 ml of buffer A containing 1.2 M potassium acetate and 0.01% NP-40 and with 5 ml of buffer A containing 150 mM potassium acetate, proteins were eluted with buffer A containing 150 mM potassium acetate and 300 mM imidazole (pH 8.0). Peak fractions (3–10 ml) were analyzed by SDS-PAGE in 4%–12% gradient gels and by staining with Bradford solution (Bio-Rad). When necessary, head module from the Ni column was further fractionated on a 1 ml HiTrap Q column in buffer A, developed with a linear gradient of 0.1–1 M potassium acetate. Head module eluted at about 600 mM potassium acetate.

EM and Image Analysis of Recombinant Head Module

Recombinant head module and RNA polymerase II-TFIIF fractions (5 mg/ml and 1 mg/ml) were mixed in a 3:1 molar ratio, kept for 60 min at 4°C, and diluted to concentrations of 8.5 μg/ml and 17 μg/ml with 25 mM KCl, 25 mM Tris-HCl (pH 7.8), and 5 mM DTT. Immediately after dilution, 3 μl was applied to carbon-coated, 400 mesh Cu/Rh EM grid that had been glow discharged in the presence of amylamine. After about 30 s, excess buffer was blotted and 3 μl of a 1% uranyl acetate solution was applied. Blotting and uranyl acetate application were repeated twice more. Approximately 1 min after the last application, the grid was submerged in uranyl acetate and covered with a second layer of carbon, creating a carbon layer sandwich (Davis et al., 2002). Samples were imaged under low-dose conditions at a magnification of ×66,000 using a CM200 TEM (Philips/FEI) fitted with a field emission electron source and operating at an accelerating voltage of 120 kV. Micrographs were digitized on an SCAI scanner (ZI/Carl Zeiss) with 7 μm sampling. Scanned images were decimated three times, resulting in a final sampling of 3.18 Å/pixel.

Image processing was carried out with the Spider software package (Frank et al., 1996). A total of 994 particle images were hand-selected from four micrographs and windowed in a 120 × 120 pixel frame. Judging from the size and appearance of the particles, the image set appeared to be a mixture of free recombinant head module, RNA polymerase II-TFIIF, and RNA polymerase II-TFIIF-head module particles. Based on the size of the head module in the RNA polymerase II-Mediator complex (Davis et al., 2002), 358 particles within the range expected for the head module (±30 Å) were selected, filtered to 20 Å, and reference-free aligned. Multivariate statistical analysis and k-means classification were performed on the aligned particles. Class averages were then calculated, yielding 2D projection maps of the head module.

GST Fusion Protein Expression and Analysis

The yeast *GCN4* ORF was amplified by PCR and cloned between BamHI and XhoI sites of pGEX6P-1 (Amersham Biosciences), yielding the expression vector pTK041 (GST-GCN4). The C-terminal region (amino acids 1535–1731) of the yeast *RPB1* ORF was amplified by PCR with a Flag tag sequence added to the N terminus and was cloned between BamHI and XhoI sites of pGEX6P-1, yielding the expression vector pTK065 (GST-Flag-CTD). The Gal4-VP16 ORF was amplified by PCR and cloned between BamHI and NotI sites of pGEX6P-1, yielding the expression vector pTK035 (GST-Gal4.VP16). Expression and purification of GST-Flag-CTD was performed essentially as described (Takagi et al., 2003).

GST fusion proteins (~20 μg) were immobilized on 0.1 ml of GST-agarose resin in buffer A containing 50 mM potassium acetate. The resin was mixed with head module (~50 μg) and kept for 1 hr at 4°C. Flowthrough, wash (3 ml buffer A containing 50 mM potassium

acetate), and eluates (0.2 ml of wash buffer containing 20 mM reduced glutathione) were collected, and 7–10 μg of each fraction was analyzed by 4%–12% NuPAGE (Invitrogen).

Immunoblot Analysis

Load, flowthrough, and eluates of Ni columns were subjected to 4%–12% NuPAGE (Invitrogen), transferred to Protran membranes (Schleicher & Schuell), and probed with anti-Med17(Srb4), anti-Med8, anti-Med17(Srb5), and anti-Med20(Srb2) antibodies as described (Takagi et al., 2003; Takagi and Kornberg, 2006). Detection was with alkaline phosphatase-conjugated anti-rabbit antibodies (Bio-Rad) followed by color development by the BCIP/NBT liquid substrate system (Sigma-Aldrich).

Transcription

General transcription factors TBP, TFIIB, TFIIE, and TFIIH and RNA polymerase II were purified from yeast as described (Myers et al., 1997). RNA polymerase II-TFIIF complex was purified from yeast with the use of a TAP tag on the Tfg2 subunit and with IgG affinity chromatography as described (Chung et al., 2003). TFIIF was purified in the same way, except that RNA polymerase II was removed by washing the IgG column with buffer containing 500 mM ammonium sulfate.

Transcription assays with whole-cell extract of the *srb4ts* mutant strain (*srb4-138*) were performed as described (Takagi and Kornberg, 2006). Transcription reconstituted with purified proteins was performed as described (Takagi and Kornberg, 2006). Free Mediator was purified as described (Takagi et al., 2005). Quantitation was performed with a PhosphorImager and ImageQuant software.

Head Module Interactions

Peak fractions from the HiTrap Q column were concentrated to 5 mg/ml with the use of a Vivaspin Centrifugal Concentrator (30,000 Da cutoff). The concentrated head module (200 μg) was mixed with GST-CTD (140 μg) and dialyzed against 50 mM HEPES-KOH (pH 7.6), 50 mM potassium acetate, 5% glycerol, and 5 mM β-mercaptoethanol containing 10 mM imidazole (pH 8.0) for 2 hr at 4°C. The dialysate was adjusted to 200 μl with dialysis buffer, divided in two, and mixed with either 0.1 ml of Ni resin (HIS-select, Sigma) or 0.1 ml of GST-agarose (Sigma) in dialysis buffer. After 30 min at 4°C, flowthrough and wash (3 ml of the same buffer) fractions were collected. The Ni resin was eluted with the same buffer containing 300 mM imidazole (pH 8.0), while the GST-agarose was eluted with the same buffer containing 20 mM reduced glutathione. Load, flowthrough, and eluate (7–10 μg) were analyzed by 4%–12% NuPAGE (Invitrogen).

Binding of head module (100 μg) to TBP (35 μg), TFIIB (40 μg), and RNA polymerase II (100 μg)-TFIIF (50 μg) complex was analyzed with the use of Ni resin in similar fashion. Alternatively, for production of head module-RNA polymerase II-TFIIF complex, RNA polymerase II-TFIIF (prepared with the use of a TAP tag, as described above, and so bearing a calmodulin binding peptide on the Tfg2 subunit) was incubated with a 1.5-fold molar excess of head module at room temperature for 20 min in buffer B(50) (50 mM KCl, 25 mM Tris [pH 7.5], and 5 mM DTT, where the quantity in parentheses indicates the KCl concentration). The mixture was applied to a calmodulin-Sepharose (Stratagene) column, washed with 20 column vol of buffer B(125) to remove excess head module, and eluted with buffer B(25) containing 2.5 mM EGTA. Load, wash, and eluate were analyzed by 4%–12% NuPAGE (Invitrogen).

Affinity of the Head Module for the Minimal Preinitiation Complex and Transcribing RNA Polymerase II-TFIIF Complex

DNA oligonucleotides (from IDTDNA) were 53 residues in length, with a TATA box 30 residues upstream of the transcription initiation site and a seven residue TFIIB recognition element (BRE) as described (Tsai and Sigler, 2000). The template strand of the transcribing complex included 12 residues noncomplementary to the nontemplate strand. A nine residue RNA oligonucleotide complementary to the template strand was added to form DNA-RNA hybrid as described (Westover et al., 2004).

TFIIB, TBP, a TBP-TFIIB-DNA complex, and an RNA polymerase II-TFIIF complex were prepared as described (Myers et al., 1997). The minimal preinitiation complex (mPIC) was formed by mixing the TBP-TFIIB-DNA complex RNA polymerase II-TFIIF and was purified by affinity chromatography on calmodulin-Sepharose (see above). The transcribing complex was formed by incubation of DNA-RNA hybrid with RNA polymerase II-TFIIF (5:1 molar ratio) for 30 min at room temperature in the presence of 2 mM $MgCl_2$, followed by affinity chromatography on calmodulin-Sepharose for removal of excess DNA-RNA hybrid.

The recombinant head module was incubated in 1.5-fold molar excess with the mPIC or with the transcribing complex in buffer B(50) for 30 min at room temperature, followed by adsorption of half the mixture on each of two calmodulin-Sepharose columns. Stability was tested by washing one column with 5 vol (time 0) and the other column with 200 vol (over a 30 min period) of buffer B(125), followed by elution with a buffer containing EGTA (see above). The eluates were subjected to 4%–12% NuPAGE followed by staining with Coomassie blue. The protein bands were quantified with ImageQuant software (Molecular Dynamics), and the dissociation rate k_d was determined from the following equation:

$$k_d = -\frac{1}{30} \ln \frac{[\text{polII} - \text{IIF} - \text{head}]_{30\text{min}}}{[\text{polII} - \text{IIF} - \text{head}]_{0\text{min}}}$$

The half-life $t_{1/2} = \frac{\ln 2}{k_d}$ was 111 min for the mPIC-head module complex and 17.7 min for the transcribing RNA polymerase II-TFIIF complex.

Construction of Tagged Yeast Strains

The PvuII-SpeI fragment from the vector pUG6 (De Antoni and Gallwitz, 2000), containing the *loxP-KanMX-loxP* gene, was blunted and subcloned between the blunted ApaI and HindIII sites of pBS1479 (Rigaut et al., 1999), yielding the *KAN* marker-containing vector pYT4(A). TAP tags were introduced at the C termini of Srb6 and Gal11 in both wt and *srb4* mutant yeast strains by PCR from pYT4(A) with primer sets targeting the *SRB6* and *GAL11* genomic loci, as described (Rigaut et al., 1999). The PCR products were used to transform yeast strain Z572 (*MATa his3^200 leu2-3, 112 ura3-52 srb4^2::HIS3 [CEN, URA3, SRB4], Srb6::Srb6-TAP-Kan*) and Z572 (*MATa his3^200 leu2-3, 112 ura3-52 srb4^2::HIS3 [CEN, URA3, SRB4], Gal11::Gal11-TAP-Kan*), yielding yeast strains YT140 and YT141. Finally, pCT127 (*SRB4*) was transformed into YT140 by plasmid shuffling, yielding the yeast strain YT142 (*SRB4*, *SRB6-TAP*). And pCT127 (*SRB4*) and pCT181 (*srb4ts*) were transformed into YT141 by plasmid shuffling, yielding the yeast strains YT144 (*GAL11-TAP*, wt), and YT145 (*GAL11-TAP, srb4ts*), respectively.

PCR was performed with pYT1(A) (Takagi and Kornberg, 2006) as template and with primer sets targeting *RPB3*, *SRB6*, and *RGR1* genomic loci. The PCR products were used to transform yeast strain Z579 (*MATa his3^200 leu2-3, 112 ura3-52 srb4^2::HIS3 pCT127 [LEU2, SRB4+]*) and Z628 (*MATa his3^200 leu2-3, 112 ura3-52 srb4^2::HIS3 pCT181 [LEU2, srb4-138]*), yielding the yeast strains YT025 (*RPB3-mTAP*, wt), YT016 (*RPB3-mTAP, srb4ts*), YT061 (*Srb6-mTAP, srb4ts*), YT075 (*RGR1-mTAP*, wt), and YT076 (*RGR1-mTAP*, srb4ts).

ChIP Analysis

Two 200 ml cultures of strains used for ChIP were grown to midlog phase in YP + 2% galactose. For heat treatment, an equal volume of medium, either at 44°C or 30°C (control), was added, and cultures were incubated for 1 hr at either 37°C (heat treatment) or 30°C (control). Crosslinking was performed at 30°C by adding 100 ml of 20 mM HEPES (pH 7.4) at either 4°C (heat treatment) or 30°C (control), followed immediately by formaldehyde to a final concentration of 1%. After 15 min, residual formaldehyde was quenched for 5 min with 375 mM glycine. Cells were centrifuged, washed with ice-cold TBS, and lysed by bead beating five times for 30 s. The insoluble fraction was collected by centrifugation and washed twice with 300 mM NaCl, 50 mM HEPES (pH 7.5), 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, and protease inhibitors (FA buffer). Chromatin was sheared to an average size of 500 bp by sonication for a total of 300 s with a Branson 450 Sonifier. TAP-tagged proteins crosslinked to sonicated and solubilized chromatin were immunoprecipitated overnight with rabbit IgG-agarose (Sigma) and washed twice in FA buffer; once in FA buffer containing 500 mM NaCl; once in 10 mM Tris (pH 8.0), 250 mM LiCl, 1 mM EDTA, 0.5%

NP-40, and 0.5% Na-deoxycholate; and once in 10 mM Tris (pH 8.0) and 1 mM EDTA. Immune complexes and corresponding input fractions were eluted, and crosslinks were reversed in 50 mM Tris (pH 8.0), 10 mM EDTA, 1% SDS, and 0.5 μg Proteinase K (Sigma) by heating for 1 hr at 37°C and for 6 hr at 65°C. DNA was purified by phenol/chloroform extraction, followed by ethanol precipitation. PCR reactions were performed in a Roche LightCycler according to the manufacturer's protocol, with the following primer pairs: GAL1-10 UAS, F01052 (5′-GAGCCCCATTATCTTAGCC-3′) and F01053 (5′-TTACTG CCAATTTTTCCTC-3′) (Lei et al., 2001); NO ORF, CK0137 (5′-GGC TGTCAGAATATGGGGCCGTAGTA-3′) and CK0138 (5′-CACCCCG AAGCTGCTTTCACAATAC-3′) (Komarnitsky et al., 2000).

Supplemental Data

Supplemental Data include five figures and can be found with this article online at http://www.molecule.org/cgi/content/full/23/3/355/DC1/.

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