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Preponderance of Free Mediator in the Yeast *Saccharomyces cerevisiae**

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Biochemical evidence suggesting that the predominant form of Mediator in the yeast *Saccharomyces cerevisiae* might be one in which the complex is associated with RNA polymerase II to form a holoenzyme has led to the proposition of a holoenzyme-based model for transcription initiation. We report that polymerase-free Mediator, isolated early on during a whole-cell extract fractionation protocol, is in fact the most abundant form of the Mediator complex. The existence of free Mediator would make possible independent recruitment of Mediator and RNA polymerase II to the preinitiation complex. This is in agreement with reports from *in vivo* studies of time and spatial independence of Mediator and RNA polymerase II promoter interaction, with current models of preinitiation complex structure in which promoter DNA upstream of the transcription start site is positioned between Mediator and polymerase, and with the proposed role of Mediator as the major component of the Scaffold complex involved in transcription reinitiation.

Mediator is a global regulator of transcription, first identified in the yeast *Saccharomyces cerevisiae* (1, 2), which acts as an interface between gene-specific regulator proteins and the general transcription machinery (3, 4). Mediator complexes have been identified in all eukaryotic organisms examined on the basis of moderate sequence homology of their component subunits to corresponding subunits in the yeast complex (5) and appear to play a universal role in integrating information for control of gene expression. The limited sequence homology among Mediator subunits of different eukaryotes points to a high degree of evolutionary divergence. However, it has been suggested that sequence conservation could be highest in protein segments involved in inter-subunit interactions, suggesting that the overall organization of Mediator, and perhaps its mechanism, could be conserved (6). In agreement with this scenario, structural studies have revealed a limited degree of structural similarities among Mediator complexes from yeast to man (7–9).

Mediator was initially identified in a crude yeast fraction as an activity required to relieve inhibition and enable activator response in a partially purified reconstituted RNA polymerase II transcription system (1, 2). The purified activity, dubbed Mediator, was shown to possess three biochemical functions: stimulation of basal transcription, support of activated transcription, and stimulation of CTD[1] phosphorylation by the kinase activity of TFIIH (3). Several Mediator complex subunits are products of genes previously linked to transcription, such as the SRB genes identified by genetic screens for mutations that compensate for CTD truncations (10), the SIN4 and RGR1 genes previously identified as participants in activation and repression (11), and a number of proteins that are products of novel MED genes (4). Research in the last few years has resulted in biochemical characterization of Mediator homologues in higher organisms and identification of their component subunits (5, 6, 12).

A significant impediment to studying the biochemical properties and structural organization of yeast Mediator stems from the inherent difficulty in obtaining the complex in pure form, free from the other components of the transcription machinery. Although protocols for isolation of free Mediator have been published (4, 13), it has proven extremely challenging to obtain reproducible results,[2] and as a consequence, the bulk of biochemical studies published to date have relied on partially purified holoenzyme (Mediator-RNA polymerase II (RNAPII)) fractions (14–17). The same type of preparation was also used for initial structural characterization of the Mediator-RNAPII interaction (18). Immunoprecipitation has been used to obtain an enriched Mediator fraction from these holoenzyme preparations and the purity of the resulting material is sufficient to identify individual Mediator subunits bands by SDS-PAGE analysis (14, 15). However, more precise structural and biochemical characterizations of Mediator have suffered from the lack of suitable free Mediator preparations.

The existence of a free subspecies of Mediator, not associated with RNAPII, has important implications for the gene regulatory properties of the complex and for the possible assembly mechanism of the preinitiation complex (19). A longstanding model of the transcription initiation process based on biochemical studies proposed that initiation would start with sequential assembly of the components of the basal transcription machinery (RNAPII plus at least five general transcription factors: TFIIB, TFIIE, TFIID (TBP), TFIIF, and TFIIH) (20–22). Reports suggesting the existence of a stable Mediator-

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[1] The abbreviations used are: CTD, C-terminal domain of Rpb1; RNAPII, RNA polymerase II; TFII, general transcription factor for RNA polymerase II; TBP, TATA-binding protein; WCE, yeast whole-cell extract; HA, hemagglutinin; PEI, polyethylenimine; PI, protease inhibitor; HAP, hydroxyapatite resin; EM, electron microscopy; βME, β-mercaptoethanol; TAP, tandem affinity purification; NTA, nitrilotriacetic acid.

[2] Y. Tagaki and F. J. Asturias, unpublished results.

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RNAPII holoenzyme have called into question this "ordered recruitment" model. Depending on the details of the purification protocol, the holoenzyme complex was reported to include not only Mediator and RNAPII but also TBP and other TFIID components, various general transcription factors such as TFIIE and TFIIH, and even such large complexes as the SWI-SNI chromatin remodeling complex (23–25). Observation of these large complexes led to the proposition of a "holoenzyme-based" model for transcription initiation in which one large complex containing all required components of the machinery would be recruited to the promoter by an activator. Although the holoenzyme-based model for initiation is largely based on indirect evidence from studies in yeast, it has influenced the interpretation of observations in other systems, with evidence for recruitment of an individual factor interpreted as indication of recruitment of an entire holoenzyme complex (17, 26, 27), and has sparked an effort to identify a holoenzyme complex in human cells (28). Several recent observations have questioned the validity of the holoenzyme-based model for transcription initiation. Chromatin immunoprecipitation analysis of *HO* and *Gal* promoters and other *SBF*-regulated promoters clearly points to independent recruitment of Mediator and RNAPII *in vivo* (29–31), and in contrast to what has been observed in yeast, all mammalian Mediator complexes isolated to date have been obtained in free form, not in complex with RNAPII (32–35).

To address the discrepancies between the holoenzyme and sequential recruitment models, here we have sought to establish a reliable purification protocol for free Mediator by asking whether free Mediator can be identified and purified from the yeast *S. cerevisiae*. An improved cell lysis procedure and careful reevaluation of the Mediator purification protocol, made possible by detection and tracking of Mediator in crude whole-cell extracts, revealed that free Mediator can be identified and isolated to near homogeneity and also that free Mediator, not holoenzyme, is the predominant form of Mediator. Consistent with the results from the *in vivo* chromatin immunoprecipitation analysis and with the isolation of free Mediator in mammalian cells, these data support a model for initiation in which different components of the transcription machinery are sequentially recruited and Mediator functions as a separate entity. In this scenario, the Mediator-RNAPII holoenzyme is a transient complex formed only during the initiation process.

MATERIALS AND METHODS

Construction of Yeast-tagging Vector—The epitope-tagging vector, pYT006, was created by modifying the vector pU6H3HA (36) using a QuikChange kit (Stratagene); the His_6 tag was disabled by mutating the first four histidine residues to glycines (yielding the vector pYT005), and the sequence of the PreScission protease site (LEVLFQGP) was introduced upstream of three copies of the influenza HA epitope (yielding the vector pYT006).

Construction of Tagged Yeast Strains—The yeast CEN vector carrying wild-type Med17 pCT127, was recovered from the yeast strain Z579 (37). A His_{10} tag was introduced into the N terminus of the Med17 open reading frame using a QuikChange kit (Strategene), yielding the plasmid pCT127 (His_{10}-Med17). Three copies of the HA epitope were introduced into the C terminus of the Med8 subunit using the PCR product of pYT006 as a template, with primer sets targeting Med8 genomic locus as described (36). The PCR product was used to transform the yeast strain Z572 (MAT**a** *his3Δ200 leu2-3*, *112 ura3-52 med17Δ2*::*HIS3* (CEN, URA3, MED17), *Med8*::*Med8-PreScission-3xHA-Kan*) yielding strain YT108. The plasmid, pCT127 (His_{10}-Med17) was then transformed into strain YT108 by plasmid shuffling, yielding strain YT110 (His_{10}-Med17, Med8-PreSci-3xHA).

Analysis of Yeast Genomic DNA—Approximately 50 μl of each sample was mixed with 150 μl of buffer containing 50 mM Tris-HCl (pH 8.0) and 20 mM EDTA and incubated with 120 μg of RNase A for 30 min at 37 °C. 10 μl of 10% SDS and 8 units of protease K (Sigma-Aldrich) were added and incubated for an additional 30 min at 37 °C followed by phenol/chloroform extraction and ethanol precipitation. 20 μg of glycogen was then added as a carrier and one-fifth of the extracted DNA was subjected to 1% agarose gel electrophoresis and detected by ethidium bromide staining.

Purification of Free Mediator—2 kg of yeast strain YT110 was grown in 2× YPD medium as described (38) to an OD_{600} of 8.0 and harvested at 3000 × *g* for 10 min. Cells were washed in ddH_2O and frozen at −80 °C. To lyse yeast cells, 220 g of frozen cells, 100 g of dry ice, and ~300 ml of liquid nitrogen were placed in a 2-liter stainless steel blender jar. This mixture was blended at high speed for 20 min while maintaining the level of liquid nitrogen required to allow the mixture to flow during blending. The whole-cell extract was prepared as described previously (38), except that 600 mM KOAc was used for extraction. The whole-cell extract (WCE) was dialyzed against buffer A (50 mM HEPES-KOH (pH 7.6), 1 mM EDTA, 10% glycerol, 5 mM β-mercaptoethanol (βME), 0.5× protease inhibitor (PI) mix (100× PI mix contains 0.6 mM leupeptin, 2 mM pepstatin A, 2 mM benzamidine, and 1 mM phenylmethylsulfonyl fluoride) and adjusted to the conductivity of buffer A containing 100 mM KOAc (A100). The sample was applied to a 2.5-liter Bio-Rex70 (Bio-Rad) column equilibrated with buffer A100 at 1 column volume/3 h. The resin was then washed with 2 column volumes of buffer A100 followed by 2 column volumes of buffer A400, and the sample was eluted using 2 column volumes of buffer A650.

Fractions containing both Med17 and Med8 were pooled, dialyzed against buffer B (50 mM Tris acetate (pH 7.6), 0.1 mM EDTA, 0.01% Nonidet P-40, 10% glycerol, 5 mM βME, 0.5× PI mix) and adjusted to the conductivity of buffer B containing 100 mM KOAc (B100). The sample was applied to a 220-ml DEAE-Sephacel (Sigma-Aldrich) column pre-equilibrated with buffer B100 at 1 column volume/h. The column was washed with 1 column volume of buffer B100 and eluted with a linear gradient from 100 to 550 mM KOAc over 10 column volumes, with the peak Mediator fractions eluting at ~410 mM KOAc. Fractions containing both Med17 and Med8 but not containing the CTD region of RNAPII were pooled, and $CaCl_2$ was added to a final concentration of 0.2 mM. The sample was applied to a 80-ml hydroxyapatite (Bio-Rad) column pre-equilibrated with buffer H10 (10 mM potassium phosphate (pH 7.8), 100 mM KOAc, 50 μM $CaCl_2$, 10% glycerol, 0.01% Nonidet P-40, 5 mM βME, 0.5× PI mix) at 1 column volume/h. The column was then washed with 1 column volume of buffer H10 and eluted with a linear gradient from 10 to 200 mM potassium phosphate over 10 column volumes. Peak Mediator fractions appeared at ~110 mM potassium phosphate. Mediator-containing fractions were pooled and dialyzed against buffer Q (50 mM Tris acetate (pH 7.6), 10% glycerol, 5 mM βME, 0.5× PI mix) and adjusted to the conductivity of buffer Q containing 100 mM KOAc (Q100). The sample was applied to a UnoQ6 column (Bio-Rad) pre-equilibrated with buffer Q100 at 1.5 ml/min, and the column was washed with 1 column volume of buffer Q100 followed by 5 column volumes of buffer Q400 and eluted with a linear gradient from 400 to 1200 mM KOAc over 10 column volumes. Peak Mediator fractions appeared at 550 mM KOAc.

Mediator fractions were pooled and dialyzed against buffer N (50 mM HEPES-KOH (pH 8.5), 1000 mM KOAc, 0.01% Nonidet P-40, 10% glycerol, 5 mM βME, 0.5× PI mix) containing 10 mM imidazole (N10). Sample was applied to 5 ml of Ni^{2+} resin (HIS-Select, Sigma-Aldrich) pre-equilibrated with buffer N10, and the resin/sample mixture was continuously agitated at 4 °C for 18 h. The resin was allowed to drain and was then washed with 50 ml of buffer N20 over a period of 30 min and eluted with 10 ml of buffer N300 using 1 ml aliquots over a period of 45 min. Sample was dialyzed against buffer S (50 mM HEPES-KOH (pH 7.6), 5% glycerol, 5 mM βME, 0.5× PI mix) and adjusted to the conductivity of buffer S containing 100 mM KOAc (S100). Sample was applied to a 0.16-ml UNO-S column (Bio-Rad) pre-equilibrated with buffer S100 at 0.35 ml/min and eluted using 5 ml of buffer S700.

Production of Antibodies—Polyclonal antibodies against the Med17 subunit were generated (Covance, PA) by inoculating rabbits with the peptide DNDKNLKFLKNKDSLV (Med17 amino acids 72–87) conjugated with KLH. Polyclonal antibodies against the HA epitope were generated (at The Scripps Research Institute) by inoculating rabbits with two copies of the HA peptides, CPDYAGYPYDVPDYAGYPYDV, conjugated with KLH. Antibodies were affinity-purified by glutathione *S*-transferase-HA column as described (39). Production of polyclonal antibody against the Med18 subunit is described elsewhere.[3]

Western Blot Analysis—5-μl samples were subjected to 4–20% gradient SDS-PAGE (Bio-Rad). Western blot analysis was carried out as described (38) and probed with anti-CTD (8WG16 monoclonal, Abcam, Cambridge MA), anti-Med17, anti-Med18, and anti-HA antibodies.

[3] Y. Takagi and R. D. Kornberg, submitted for publication.

Small Scale Immunoaffinity Purification of Mediator and Stability of the RNAPII-Mediator Holoenzyme—A small amount of whole-cell extract was prepared as described above but using the following low-salt extraction method. 50 g of yeast cells (from YT110 strain) were lysed using the blender method and extracted with 50 ml of 2× low-salt lysis buffer (100 mM HEPES-KOH (pH 7.6), 400 mM KOAc, 2 mM EDTA, 20% glycerol, 0.01% Nonidet P-40, 10 mM βME, and 2× PI mix). The salt concentration of the lysis buffer was carefully adjusted so that the final conductivity of the WCE was close to that of buffer A300. Half of the WCE obtained was loaded onto a 0.6 ml anti-HA antibody column (Sigma-Aldrich) pre-equilibrated with buffer A300 and incubated for 4 h at 4 °C. The resin was washed with buffer A300 until no protein could be detected in the washes and then divided into two 0.3-ml columns. One of the 0.3-ml resin fractions was washed again with buffer A300. The other 0.3-ml resin fraction was washed with a total of 5 column volumes of buffer A600, and 5 fractions were collected. The resin was further washed with a total of 5 column volumes of buffer A1000, and 5 more fractions were collected. Finally, the resin fraction subjected to high-salt washes was equilibrated with buffer A300. Elutions from both low- and high-salt washed resin fractions were carried out by incubation with elution buffer containing 0.5 mg/ml 2× HA peptide for 15 min at 30 °C. Fractions from the 600 and 1000 mM washes, as well as peak elution fractions, were analyzed by Western blot using anti-CTD, anti-Med17, and anti-Med18 antibodies. Both sets of elutions were further analyzed by quantitative Western blot to estimate the amount of RNAPII and Mediator, using purified RNAPII and recombinant Med17 and Med18 proteins[3] as standards. From the quantitative Western blot results, the free Mediator to holoenzyme molar ratio was estimated by assuming that the signal from RNAPII represents holoenzyme and that the Mediator signal arises from the combined signal from free Mediator and holoenzyme.

Specific Transcription and CTD Phosphorylation Assays—Reconstituted transcription was performed essentially as described (3) with minor modifications. UnoS fractions containing purified free Mediator were dialyzed against a buffer containing 50 mM HEPES-KOH (pH 7.6), 150 mM KOAc, 20% glycerol, and 5 mM βME for 1 h at 4 °C. All factors (RNAPII, TFIIF, TFIIB, TBP, TFIIE, TFIIH, and for some experiments, Mediator and/or GCN4) and the DNA template were mixed and preincubated for 5 min. Transcription was initiated by the addition of a nucleotide mix containing ATP, CTP, and [γ-^{32}P]UTP. This reaction mixture was incubated for an additional 45 min at 24 °C, and the final concentration of cold UTP was adjusted to 10 μM. Transcripts were resolved by denaturing gel electrophoresis followed by autoradiography. The CTD phosphorylation assay was carried out as described previously (3) except that 4–15% SDS-PAGE was used to resolve the phosphorylated Rpb1 subunit.

Electron Microscopy Analysis—Samples were diluted to 35 μg/ml with 50 mM HEPES-KOH (pH 7.6), and 3.2-μl samples were applied to carbon-coated 400-mesh copper and rhodium grids (Ted Pella, Redding, CA) that were glow-discharged in the presence of amyl amine for 1 min. After 1 min of absorption, the samples were blotted dry and washed three times with a 1% solution of uranyl acetate. The sample was then immersed in 1% uranyl acetate, a second layer of carbon was applied to the top (40, 41), and the samples were dried. Samples were imaged using a Phillips CM120 transmission electron microscope outfitted with a LaB_6 filament and operated at 100 kV. Images were recorded on Kodak SO-163 film at ×60,000 magnification and digitized using a 7 μm step size on a Zeiss SCAI scanner.

Mass Spectroscopy Analysis—5 μg of protein in 25 μl of buffer S700 was precipitated by adding an equal volume of 20% trichloroacetic acid and incubated for 30 min on ice. Samples were pelleted by spinning at 13,000 × g for 10 min and washed three times in ice-cold acetone. The washed pellets were resuspended in 100 mM Tris-HCl (pH 8.5), denatured with 8 M urea, and reduced and alkylated by incubation with 5 mM tris(2-carboxyethyl)phosphine for 30 min at room temperature followed by incubation with 10 mM iodoacetamide for 30 min at room temperature. Samples were divided into three aliquots and subjected to three separate digests. For trypsination, the urea concentration was adjusted to 2 M using 100 mM Tris-HCl (pH 8.5), trypsin (10 ng/μl) was added in the presence of 2 mM $CaCl_2$, and the mixture was agitated overnight at 37 °C. For elastase treatment, the urea concentration was reduced to 2 M as above, elastase was added to a final concentration of 5 ng/μl, and the sample was incubated overnight at 37 °C. For subtilysin digests, urea concentration was reduced to 4 M, and 10 ng of subtilysin was added. The reaction was allowed to proceed for 1 h at 37 °C. Samples were loaded on a 4-cm, 250-micron internal diameter column packed with 5 μm reverse-phase beads coupled to a 3-cm column with a strong cation-exchange resin and a final 10-cm section of reverse-phase media.

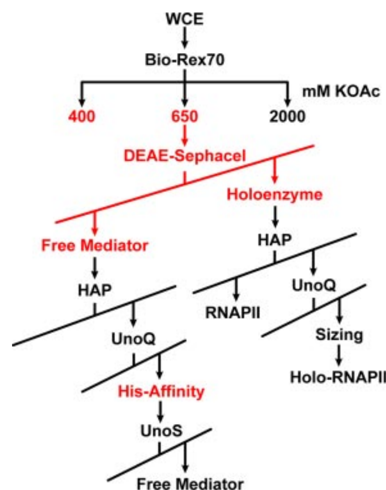

FIG. 1. **Protocol for purification of free Mediator and Mediator-RNAPII holoenzyme by ion-exchange chromatography.** A scheme for purification of RNA polymerase II holoenzyme and free Mediator was derived as part of a general scheme for purification of components of the yeast transcription machinery (45). Changes to the protocol that made possible the reproducible purification of free Mediator are indicated in *red*. *Horizontal* and *diagonal lines* indicate stepwise and gradient elution, respectively.

Peptides were eluted directly to the electrospray ionization tandem mass spectrometer using a gradient of 100 to 0% acetonitrile followed by stepwise elution using 10, 25, 35, 50, 65, 80, and 100% 500 mM ammonium acetate. Electrospray ionization was done at 2.5 kV, and a Thermo Finnigan LTQ was used to acquire data during the entire elution procedure. The nine largest spectral peaks were searched against the *S. cerevisiae* genome data base using the SEQUEST program and were sorted and filtered using the DTASelect program for each protein sequence containing at least three identified peptide fragments (42, 43).

RESULTS AND DISCUSSION

Monitoring Mediator in WCE and Optimization of WCE Preparation—Attempts to implement a Mediator immunoaffinity purification protocol from a crude whole-cell extract through introduction of TAP (44) and/or HA (39) tags in Mediator subunits have been only partially successful.[4] The purity of the resulting Mediator fractions was not sufficient for structural characterization studies, and more importantly, Mediator purified by TAP tag exhibited a significantly lower specific activity in transcription assays.[5] This prompted a reexamination of the original Mediator purification protocol based on conventional chromatography, which was developed in the context of a global effort to fractionate yeast WCE for purification of yeast general transcription factors (45). A protocol for purification of free Mediator was derived from this original scheme (Fig. 1), but optimization of the early chromatographic steps in the procedure had been hampered by the fact that none of the antibodies raised against Mediator subunits for use in Western blot analysis were specific enough to efficiently detect Mediator in WCE.[5] It seemed likely that although an affinity tag was not enough to efficiently separate Mediator from WCE, it could potentially be used to trace Mediator more efficiently during the early steps in the conventional chromatographic purification process, thereby allowing for the optimization of the purification procedures. Whereas a strain engineered with a triple HA epitope tag (3xHA) into the C terminus of the Med8 Mediator subunit and a His_{10} tag engineered on the N terminus of subunit Med17 could not be successfully used for simple affinity purification, the HA tag could be used to track the elution pattern of Mediator early in the purification protocol.

[4] J. A. Davis, Y. Takagi, and F. J. Asturias, unpublished observation.
[5] Y. Takagi, unpublished observation.

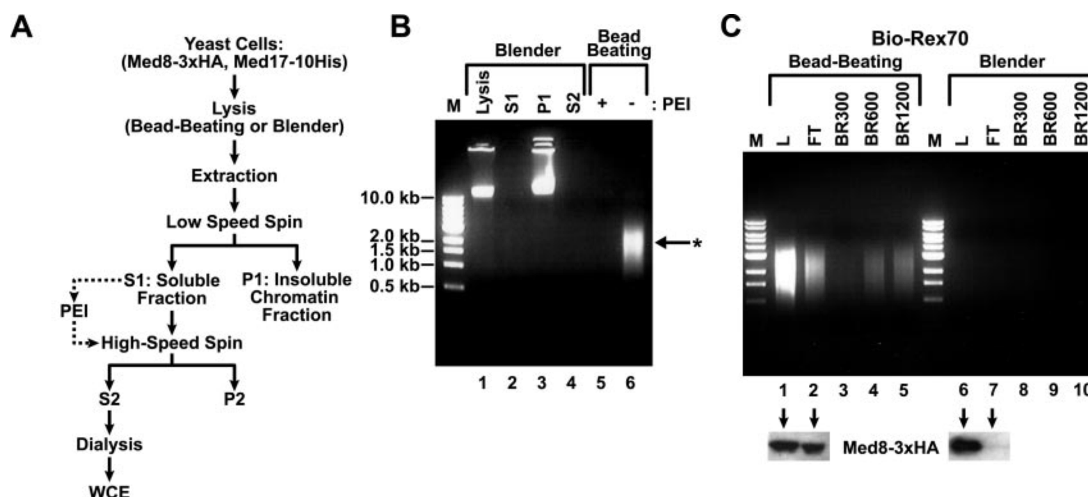

FIG. 2. **Preparation of yeast whole-cell extract and separation of genomic DNA.** *A*, a scheme for preparation of yeast WCE using the blender method or the more traditional bead-beating method (described in detail under "Materials and Methods"). *B*, analysis of genomic DNA fragments generated by the blender (*lanes 1–4*) and bead-beating methods (*lanes 5* and *6*). Genomic DNA present in the WCEs generated by either the blender method or the conventional bead-beating method was extracted from ~50 ml of each WCE and analyzed by 1% agarose gel electrophoresis followed by staining with ethidium bromide. *Lanes*: *M*, 1.0-kb ladder DNA marker; *Lysis*, crude lysate prior to low speed centrifugation; *S1*, supernatant after low speed centrifugation; *P1*, pellet after low speed centrifugation; *S2*, supernatant after ultracentrifugation of S1. Genomic DNA from S1 fraction generated by bead-beating method with (*lane 5*) or without (*lane 6*) PEI treatment. *C*, analysis of fractions from Bio-Rex70 column chromatography of WCEs generated by the blender and bead-beating methods. Genomic DNA from Bio-Rex70 fractions originated from WCE generated by the bead-beating (*lanes 1–5*) or the blender method (*lanes 6–10*) were extracted and analyzed by 1% agarose gel electrophoresis followed by staining with ethidium bromide. Load (*lanes 1* and *6*) and flow-through (*lanes 2* and *7*) fractions were subjected to Western blot analysis probed by anti-HA antibody to track the Med8–3xHA subunit of Mediator as indicated below. *Lanes: M*, 1.0-kb ladder DNA marker; *L*, load; *FT*, flow-through; *BR300*, Bio-Rex70 300 mM KOAc eluate; *BR600*, 600 mM KOAc eluate; *BR1200*, 1200 mM KOAc eluate. (Note that the salt concentrations listed correspond to those used in the original Mediator purification protocol.)

Monitoring Mediator elution from the Bio-Rex70 resin, the first step of purification (Fig. 1), indicated that ~50% of cellular Mediator did not bind to the Bio-Rex70 resin, despite the larger ratio of resin to WCE (data not shown). As the Bio-Rex70 resin provided the largest share of the WCE fractionation, it became imperative to address this issue. The original protocol for preparation of WCE called for lysis of yeast cells by extended beating with glass beads at low (~4 °C) temperature (Fig. 2*A*). Because of the tendency of this method to shear genomic DNA, it was suspected that this sheared DNA might interfere with binding of Mediator to the Bio-Rex70 resin. In fact, a considerable amount of sheared DNA fragments (1.0–2.0 kb) was generated during bead-beating cell lysis (Fig. 2*B*, *lane 6*). Although the cationic polymer polyethylenimine (PEI) could be used to remove the sheared DNA fragments (Fig. 2*B*, *lane 5*), the amount of PEI required to remove the sheared DNA resulted in the loss of a significant portion of the soluble Mediator (~50% under the extraction and PEI treatment condition used; data not shown). These problems were avoided by using an alternative lysis method that did not generate sheared genomic DNA fragments. Yeast cells were lysed in a stainless steel blender in a mixture of liquid nitrogen and dry ice crystals (46). This procedure resulted in high lysis levels (90~95%) and had several advantages. Because lysis was carried out at liquid nitrogen temperature, the problem of proteolysis was minimized. In addition, unlike the bead-beating method, genomic DNA was left mostly intact (Fig. 2*B*, compare *lanes 1* and *6*) and could be effectively removed by centrifugation without the use of PEI (Fig. 2*B*, *lanes 1–4*). Perhaps more importantly, WCE prepared by the blender method had a better binding affinity to Bio-Rex resin. When the bead-beating cell lysis method was used to prepare WCE and the PEI precipitation step was eliminated (to avoid loosing Mediator), sheared genomic DNA interfered with the Bio-Rex chromatography step and was carried through the Bio-Rex washes and elution (Fig. 2*C*, *lanes 1–5*). Early attempts at removing this DNA by the standard method of high ionic strength DEAE resin elution (47) were unsuccessful.[5] However, when lysis was carried out by the blender method and the genomic DNA was simply removed by centrifugation, little or no DNA was applied to the Bio-Rex column (Fig. 2*C*, *lanes 6–10*). Once the interference from DNA was eliminated, the binding capacity of the Bio-Rex resin and the washing and elution buffer concentrations could be optimized by using the anti-HA antibodies to monitor the HA-tagged Mediator (see "Materials and Methods").

Separation of Free Mediator from Mediator-RNAPII Complexes—The second chromatographic step in the general scheme for purification of components of the transcription apparatus is fractionation over DEAE-Sephacel (Fig. 1), previously carried out by washing the column with a buffer containing 200 mM KOAc (DE200) and performing a step elution at 550 mM KOAc (DE550) thought to elute all of the general transcription factors. This step was also reevaluated and optimized. The column was washed with a buffer containing 100 mM KOAc, and elution was carried out using a linear gradient from 100 to 550 mM KOAc. Western blot analysis of the fractions from this elution gradient was carried out using anti-Med17 (Srb4) and anti-HA antibodies to track Mediator and an anti-CTD (against the C-terminal domain of Rpb1, the largest RNAPII subunit) antibody to track RNAPII. A Mediator peak eluting around 410 mM KOAc partially overlaps with an RNAPII peak eluting around 500 mM KOAc (Fig. 3*A*) indicating independent elution profiles.

The RNAPII-free portion of the Mediator peak identified by Western blot analysis of DEAE-Sephacel fractions constituted the first reproducible experimental indication of an RNAPII-free Mediator fraction. Previous experience indicated that Mediator and RNAPII could not be separated by subsequent chromatographic steps (hydroxyapatite (HAP), UnoQ, UnoS, or heparin; data not shown) in the purification scheme. Therefore, it seemed appropriate to pool elution fractions from the DEAE-Sephacel column into two sets. Early (low ionic strength) Mediator fractions were pooled, with care taken not to include any of the RNAPII-containing fractions. Late (high ionic strength)

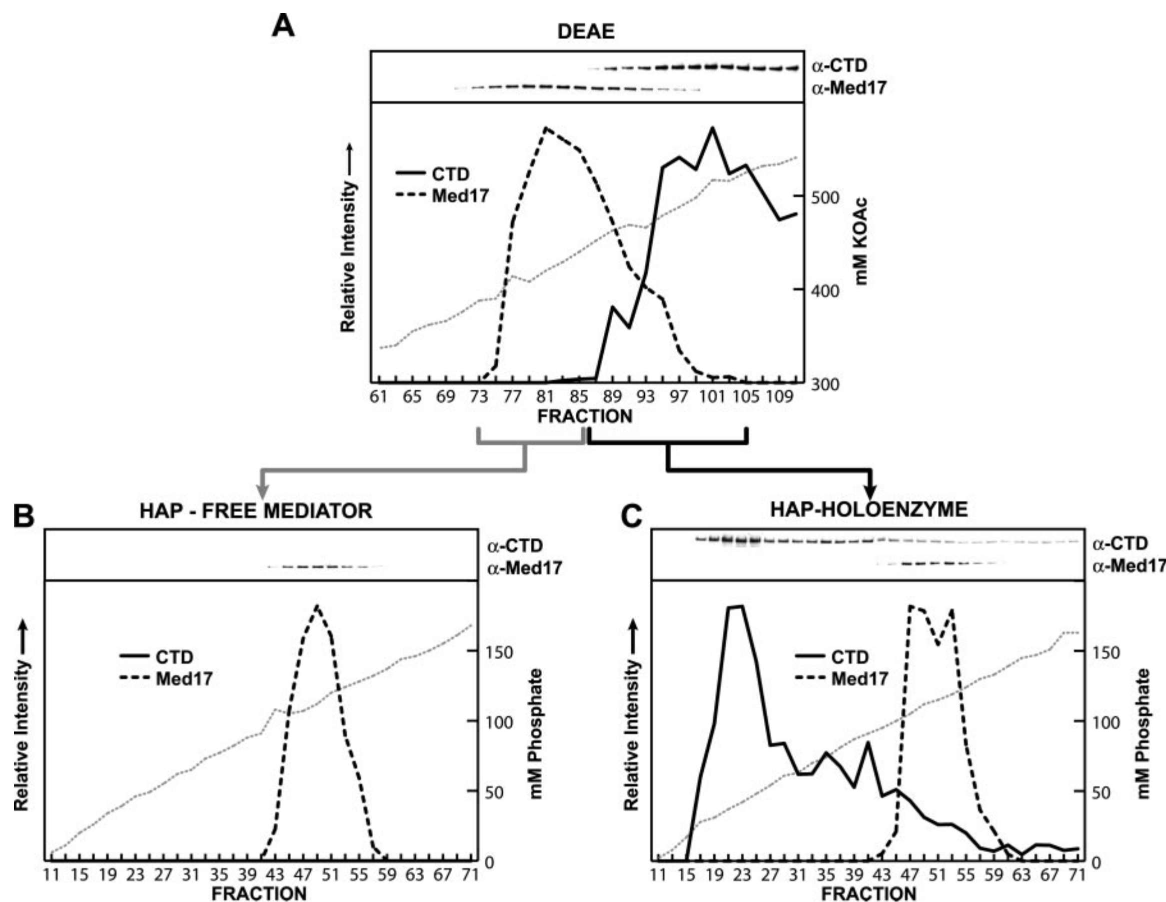

FIG. 3. **Resolution of free Mediator from holoenzyme on DEAE-Sephacel.** *A*, free Mediator was separated from the holoenzyme fraction by the DEAE column chromatography. The 650 mM KOAc eluate from the Bio-Rex70 column was further fractionated on a DEAE-Sephacel column. Proteins were eluted using a linear gradient from 100 to 550 mM KOAc. Each fraction (10 μl) was analyzed by 4–20% SDS-PAGE, transferred onto nitrocellulose, and probed with anti-CTD and anti-Med17 antibodies. Western blot profiles for Mediator (indicated by anti-Med17 antibody signal) and RNAPII (indicated by anti-CTD signal) are shown at the *top*. Signals from Western blot were quantified and plotted in the column profile: *broken line*, Med17; *solid line*, CTD. Free Mediator peaked at ~410 mM KOAc and the holoenzyme at ~460 mM KOAc. *B* and *C*, profiles of free Mediator (*B*) and the holoenzyme (*C*) on a HAP column. Free Mediator and the holoenzyme fractions from the DEAE column were pooled and fractionated (as indicated by the *brackets*), respectively, by the HAP column (fractions analyzed as in *A*). The free Mediator fraction peaked around 110 mM phosphate. The holoenzyme fraction yielded a core RNAPII peak (~50 mM phosphate) followed by a holoenzyme peak (~110 mM phosphate). This profile (*panel C*) represents the "classic" profile for core RNAPII and the holoenzyme (3).

fractions were also pooled. Both sets of fractions were then separately fractionated on the HAP resin. Western blot analysis of HAP fractions originating from the early (RNAPII-free) DEAE-Sephacel fractions rendered, as expected, a single, RNAPII-free Mediator peak (Fig. 3*B*). Fractions originating from the late DEAE-Sephacel fractions resolved into a broad RNAPII peak, with the trailing end overlapped with a Mediator peak that presumably indicated the presence of a Mediator-RNAPII holoenzyme (Fig. 3*C*). The results from Western blot analysis of HAP fractions originating from late (high ionic strength) DEAE-Sephacel fractions are essentially identical to those obtained when all DEAE-Sephacel fractions are pooled together (550 mM KOAc step elution used in the previously published (3) purification protocol illustrated in Fig. 1), emphasizing the point that Mediator and RNAPII cannot be resolved on the HAP resin. Moreover, contrary to what was reported previously (4), Mediator and RNAPII cannot be separated by any of the subsequent ion-exchange chromatography steps in the published Mediator purification protocol. Separation of free Mediator from RNAPII-containing fractions at the DEAE step is the crucial step for purification of RNAPII-free Mediator. Integration of the Western blot signals from the DEAE column indicates that ~70% of the total Mediator signal (anti-Med17) comes from fractions in which Mediator is not associated with RNAPII. Approximately half of the total Mediator in the crude WCE (pre-centrifugation) is tightly associated with DNA (presumably representing the portion of Mediator actively involved in transcription) and is lost when genomic DNA is separated by centrifugation (data not shown). However, despite a 5-fold molar excess of RNAPII to Mediator in a yeast cell (48), in the fraction of Mediator that is soluble, free Mediator appears to outnumber by more than 2:1 Mediator complexed as the holoenzyme.

Stability of the RNAPII-Mediator Holoenzyme—A number of Mediator-containing complexes of varying composition have been reported in the literature, and it has been argued that this variability might be related to conditions during purification (49). Therefore, the stability of the RNAPII-Mediator holoenzyme and the molar ratio of free Mediator to holoenzyme were tested under the mildest conditions possible. WCE was prepared and assayed, using a gentle extraction protocol developed for characterization of the relatively unstable Mediator isolated from a Med17 temperature-sensitive mutant strain[3] in which the final WCE lysate salt concentration corresponds to only 300 mM KOAc (instead of the customary 600 mM). This gentle extraction was immediately followed by a single affinity purification step on an anti-HA resin (Sigma-Aldrich) to which Mediator could bind through the 3xHA tag engineered into Med8. The free Mediator/holoenzyme molar ratio was tested under low-salt conditions (300 mM KOAc), and after extensive

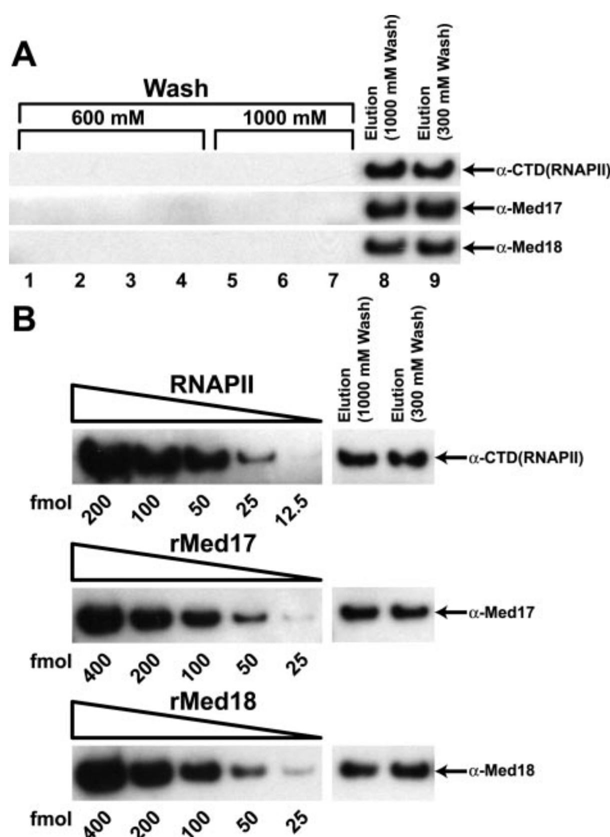

FIG. 4. **Stability of the RNAPII-Mediator holoenzyme complex.** *A*, effect of high-salt washes on integrity of the holoenzyme complex. Whole-cell lysate extracted with a low-salt buffer (300 mM KOAc) was applied to an anti-HA affinity column to which Mediator could bind through a 3xHA tag on the Med8 subunit. The column was washed with buffers containing 600 and 1000 mM KOAc (*lanes 1–4* and *lanes 5–7*, respectively), eluted with 2xHA peptide (*lane 8*), and probed for RNAPII (anti-CTD) and Mediator (anti-Med17 and anti-Med18). These results were compared with those from a similarly prepared column that was not subjected to high-salt washes (*lane 9*). *B*, quantitative comparison of the effect of high- and low-salt washes on stability of the RNAPII-Mediator holoenzyme. Composition of immunoaffinity-purified free Meditator and the holoenzyme were estimated by quantitative Western blot analysis. Purified RNAPII (12.5, 25, 50, 100, 200 fmol) and recombinant Med17 (25. 50, 100, 200, 400 fmol) and Med18 (25, 50, 100, 200, 400 fmol) were used as standards. The amount of RNAPII and Mediator remained constant (at ~30 and 100 fmol, respectively) regardless of washing conditions.

washing of the immobilized Mediator with 300, 600, and 1000 mM KOAc. Exposure to high-salt conditions did not disrupt the holoenzyme complex (Fig. 4*A*, compare *lanes 8* and *9*), and in all cases the free Mediator/holoenzyme ratio (assayed by quantitative Western blot analysis) remained constant (free Mediator/holoenzyme = 2.3) regardless of whether the complex was exposed to only 300 mM KOAc or extensively washed with up to 1000 mM KOAc (Fig. 4*B*). The ratio of Mediator/holoenzyme (~2.3) detected from this mild purification was essentially the same as that detected from the analysis of the DEAE-Sephacel fractions (~70% free Mediator, ~30% holoenzyme, or ~2.3), indicating that the free Mediator to holoenzyme ratio in the DEAE fractions does not result from the higher salt extraction during WCE preparation. This is in agreement with a previous report indicating that both free Mediator and a Mediator-RNAPII complex can be isolated from yeast nuclear extract (49). However, although the conditions for Mediator isolation from nuclear extract were characterized as "gentle," they included extended exposure to Zymolase 100T (an enzymatic preparation that contains proteases and is used to disrupt the cell wall) followed by a long incubation in 500 mM ammonium sulfate and

TABLE I
Purification of free Mediator from 2.0 kg of yeast

Fraction[a]	Total protein	Total Mediator amount	Total Mediator to total protein ratio[b]	Yield
	mg	*mg*	$\times 10^3$	*%*
WCE	13,387	NA[c]		
Bio-Rex 70	1,802	NA		
DEAE-Sephacel	137	~0.8	5.83[d]	100
HAP	8.11	NA	NA	NA
UnoQ6	3.03	~0.36	118	45
Ni^{2+}-NTA	0.242	0.242	1000	30
UnoS	~0.242			30

[a] Bio-Rex70 (650 mM KOAc eluate), DEAE-Sephacel (410 mM KOAc eluate), HAP (~110 mM potassium phosphate eluate), UnoQ6 (~550 mM KOAc eluate), Ni^{2+}-NTA (300 mM imidazole eluate), and UnoS (700 mM KOAc eluate).

[b] The transcriptional activity of Mediator cannot be assayed without several additional components of the transcription machinery. Therefore, the "Total Mediator to total protein ratio" is presented as an indirect measurement of purification efficiency. Mediator amounts in the DEAE-Sephacel and UnoQ fractions were estimated based on quantitative Western blot analysis using an anti-Med17 antibody as described under "Materials and Methods."

[c] NA, not applicable.

[d] The values presented are based on the amount of protein and Mediator present in the DEAE-Sephacel fraction, because Western blot signals from the WCE and Bio-Rex70 could not be determined reliably.

ammonium sulfate precipitation (~3 M ammonium sulfate) (50). Perhaps as a result of this treatment, the free Mediator identified in that previous study contained only a limited number of the subunits now known to be present in all Mediator complexes (5, 12). It seems likely that these harsh isolation conditions resulted in degradation of the Mediator, which in turn would explain why the same study reported that exposure of the holoenzyme isolated from nuclear extract to 600 mM KOAc leads to the loss of RNAPII (49). The results presented here strongly suggest that free Mediator predominates in yeast and demonstrate conclusively that, with a full complement of Mediator subunits present, the Mediator-RNAPII holoenzyme is very stable; exposure of the Mediator-RNAPII holoenzyme to as much as 1000 mM KOAc (arguably a much higher ionic strength than that to which the holoenzyme would be exposed in the nucleus) does not result in disruption of the holoenzyme complex.

Further Purification and Functional Characterization of Free Mediator—In an attempt to purify free Mediator to near homogeneity as required for biochemical and high-resolution electron microscopy studies, the HAP fractions containing free Mediator were subjected to UnoQ6 column chromatography, with the Mediator peak eluting around 550 mM KOAc. UnoQ6 Mediator fractions were then bound to a Ni^{2+}-NTA resin through the His_{10} tag on the Med17 subunit. Multiple, slow wash steps using a high-salt buffer were used to remove stubborn residual contaminants (data not shown), and a lengthy equilibration time during elution finally resulted in purification of Mediator to near homogeneity as assayed by silver-stained SDS-PAGE and electron microscopy analysis. As a final concentration step, the pooled eluates from the Ni^{2+}-NTA column were applied to a small (0.16 ml) UnoS polishing column and eluted using a step gradient (700 mM KOAc). The relative efficiency and yield of the different chromatographic purification steps are summarized in Table I.

As evidenced by silver-stained SDS-PAGE analysis (Fig. 5*A*) and electron microscope imaging of purified free Mediator particles preserved in stain (uranyl acetate) (Fig. 5*B*), the purification protocol described here results in isolation of homogenous free Mediator. We found that it was critical to confirm the purity and homogeneity of Mediator preparations by electron microscope imaging, because quite often Mediator preparations

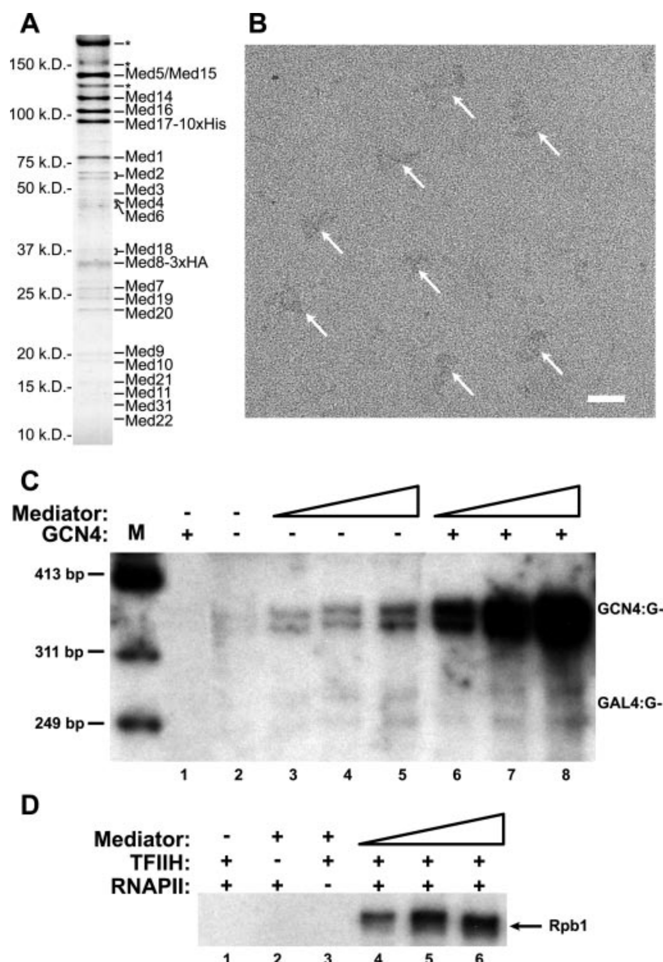

FIG. 5. **Physical and functional characterization of free Mediator.** *A*, SDS-PAGE analysis of purified Mediator by silver staining, 10 μl of UnoS fraction was subjected to 4–20% gradient SDS-PAGE analysis and visualized by silver staining (trace contaminant bands are marked by *asterisks*). *B*, electron microscope images of Mediator. Individual Mediator particles are indicated by *arrows*. *C*, effect of purified free Mediator on basal and activated transcription. Reconstituted transcription assays were carried out as described (see "Materials and Methods") from templates containing GCN4-binding sites (*GCN4:G-*) and the GAL4-binding site (*GAL4:G-*), in the presence or absence of recombinant GCN4 and with increasing amounts of pure free Mediator (from dialyzed UnoS fractions) (0 ng in *lanes 1* and *2*; 70, 140 and 280 ng in *lanes 3–5* and *6–8*). Basal transcription was stimulated by purified Mediator (compare *lane 2* with *lanes 3–5*), and Mediator also enabled response to an activator (compare *lane 1* with *lanes 6–8*). ^{32}P-Labeled PhiX174/Hinf I DNA markers are shown in the *leftmost lane* (*M*). GCN4 alone had no effect on transcription (*lane 1*). *D*, stimulation of the CTD kinase activity of TFIIH by purified free Mediator. Approximately 100 ng of RNAPII was incubated with TFIIH in the presence of [γ-^{32}P]ATP with increasing amount of purified free Mediator (0, 70, 140, and 280 ng) to monitor phosphorylation of CTD as described (see "Materials and Methods"). Phosphorylated Rpb1 subunit was resolved by 4–15% SDS-PAGE followed by autoradiography. Control experiments established that phosphorylation is TFIIH-dependent (*lane 2*) and that the target of phosphorylation is the Rpb1 CTD (*lane 3*).

that appeared very clean by SDS-PAGE analysis contained a mixture of intact, partial, and aggregated Mediator complexes.[6] The free Mediator was also subjected to biochemical testing in a reconstituted yeast transcription system to assess its functionality in three critical assays: (i) stimulation of basal transcription, (ii) response to activator-induced transcription, and (iii) stimulation of CTD phosphorylation by TFIIH (3). The transcription machinery was reconstituted with highly purified basal factors and two DNA templates (pJJ470 and pGCN4; see "Materials and Methods") in the presence or absence of Mediator and/or the activator Gcn4. The purified Mediator effectively stimulated basal transcription (Fig. 5*C*, *lanes 1–4*), enabled activated transcription (Fig. 5*C*, *lanes 5–7*), and stimulated CTD phosphorylation by TFIIH (Fig. 5*D*). To assess the composition of the purified Mediator, the sample was subjected to matrix-assisted laser desorption/ionization time-of-flight mass spectrometry analysis. The purified Mediator was found to contain all 21 of the consensus Mediator subunits, including Med31 (Soh1) (6, 51). In agreement with previous reports (4), the four components of the Cdk8/cyclin C module were not associated with the purified free Mediator.

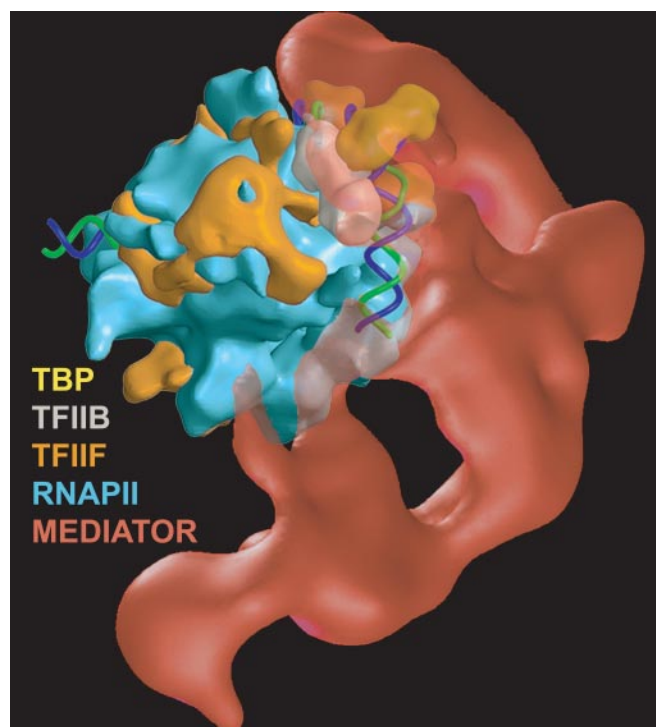

FIG. 6. **Model for a minimal preinitiation complex showing the relative positions of Mediator, RNA polymerase II, and promoter DNA.** Results from chemical cross-linking and cleavage studies (55), macromolecular electron microscopy (52), and x-ray crystallography (56) led to a model of a minimal preinitiation complex including RNAPII, TFIIF, TFIIB, TBP, and promoter DNA. The interaction of this minimal preinitiation complex with Mediator can be deduced from the relative orientation of Mediator and RNAPII in the yeast Mediator-RNAPII holoenzyme complex as revealed by two-dimensional cross-correlation analysis of the holoenzyme structure (18). The resulting model predicts that promoter DNA upstream of the TATA box will run down the back face of RNAPII, between Mediator and polymerase. This would imply that free Mediator and RNAPII must be independently recruited to the promoter. This figure was prepared using the Raster 3D program (54).

Free Mediator and the Mechanism of Regulation—The results described here establish a reproducible protocol for purification of free Mediator using a combination of ion-exchange and affinity chromatography. They also reveal that free Mediator, not the RNAPII holoenzyme, is the predominant form of the complex in yeast. This observation argues against the idea that a preassembled, large, multicomponent holoenzyme complex including Mediator and RNAPII is involved in transcription initiation. Rather, in agreement with results from *in vivo* experiments that have examined the temporal and spatial recruitment of Mediator and RNAPII to a promoter, it appears that Mediator may be recruited independently of RNAPII. The existence and independent recruitment of free Mediator are in fact necessary according to our current understanding of the organization of a minimal preinitiation complex (Fig. 6). It is

[6] F. J. Asturias, unpublished observation.

expected that DNA upstream of the transcription start site would be positioned between Mediator and the back face of the polymerase (19, 52). Integrating the promoter DNA into a preassembled holoenzyme complex would therefore represent a significant structural challenge.

It is interesting to consider the possible implications of the existence of free Mediator for the mechanism of regulation by the complex. Incubation of purified Mediator with purified RNAPII leads to the formation of holoenzyme complexes by the majority of Mediator (7). However, despite the significant stability of the Mediator-RNAPII holoenzyme and a large (~5:1) molar excess of RNAPII with respect to Mediator, in WCE (and arguably also *in vivo*) most of the Mediator (~70%) remains unassociated with RNAPII. It is tempting to speculate that the presence of the majority of Mediator as an RNAPII-free complex could be related to tight regulation of the large conformational transition required for interaction of Mediator with RNAPII (7, 18, 53). An unidentified mechanism for control of Mediator conformation that would prevent Mediator from adopting a conformation conducive to formation of the holoenzyme and preinitiation complex assembly could play a critical role in the regulation of transcription at initiation.

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