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Novel Mediator Proteins of the Small Mediator Complex in *Drosophila* SL2 Cells

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The Mediator complex is generally required for transcriptional regulation in species ranging from yeast to human. Throughout evolution, the functional diversity of the Mediator complex has been enhanced to meet the increasing requirements for sophisticated gene regulation. It is likely that greater structural complexity is thus required to accomplish these new, complex regulatory functions. In this study, we took systematic steps to examine various types of Mediator complexes in *Drosophila melanogaster*. Such efforts led to the identification of three distinct forms of Mediator complexes. In exploring their compositional and functional heterogeneity, we found that the smallest complex (C1) is highly enriched in a certain type of *Drosophila* cells and possesses novel Mediator proteins. The subunits shared among the three Mediator complexes (C1, C2, and C3) appear to form a stable modular structure that serves as a binding surface for transcriptional activator proteins. However, only C2 and C3 were able to support activated transcription *in vitro*. These findings suggest that different cell types may require distinct Mediator complexes, some of which may participate in nuclear processes other than the previously identified functions.

Eukaryotic gene transcription is driven by RNA polymerase II (pol II)[1] and ancillary basal transcription factors and is controlled at multiple levels by several classes of multisubunit co-regulator complexes (1). The Mediator complex is one such co-regulator complex and in general is required for transcriptional regulation throughout evolution (2). Mediator was originally purified from yeast (3, 4) and subsequently from metazoans (5–12). Intriguingly, metazoan Mediator complexes are more intricate than yeast Mediator and show structural diversity that may reflect the elaborate mechanisms for transcriptional regulation observed in higher eukaryotes (13).

To fully decipher the mechanisms by which Mediator regulates transcription, two aspects of the heterogeneity of Mediator complexes (functional and compositional) must be considered. With respect to functional multiplicity, Mediator complexes behave as both positive and negative transcriptional signal integrators. In yeast, individual activators and repressors interact with distinct subunits of Mediator (14, 15). Thus, both the positive and negative regulatory functions appear to coexist in the same complex. Human Mediator complexes were also shown to carry out diverse transcription-related functions (6, 11), but these functions have not yet been fully elucidated (7, 16). It is also not known whether these individual functions are present in a single metazoan Mediator complex.

Heterogeneity of Mediator complexes is also detectable in their subunit compositions. Mammalian Mediator complexes share some subunits, but also have complex-specific components (6, 8–10, 17) and show variations in their total molecular masses. Recently, a small core form of Mediator was identified in yeast nuclear extracts (18). However, whether the identification of this small Mediator in human and yeast indeed reflects the heterogeneity of Mediator complexes *in vivo* has yet to be determined.

Drosophila embryo nuclear extracts have been shown to contain a 2-MDa complex (dMediator) that is homologous to the larger yeast and human Mediator complexes (12). Whether distinct *Drosophila* Mediator complexes that differ from dMediator in subunit composition and/or function exist in these nuclear extracts is unknown. In this study, we scrutinized nuclear extracts prepared from different types of *Drosophila* cells in an attempt to identify as yet uncharacterized Mediator complexes. Such efforts led to the discovery of three chromatographically distinct Mediator complexes. We present the biochemical properties of these Mediator complexes and the identification of novel Mediator proteins that are conserved in human cells.

EXPERIMENTAL PROCEDURES

Purification of Mediator Protein-containing Complexes from Cultured SL2 Cells—SL2 (Schneider line-2) cells were grown in serum-free medium to a density of 8×10^6 cells. SL2 cell nuclei were isolated as described previously (19). The nuclei were resuspended in 4 packed nuclear volumes of buffer containing 15 mM HEPES-KOH (pH 7.6), 110 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 2 mM dithiothreitol, and 1 mM sodium metabisulfite. An aliquot (0.1 volume) of 4 M ammonium sulfate (pH 7.0) was added to the nuclear suspension, which was then mixed by rotation for 20 min. After centrifugation at 35,000 rpm for 1 h in a Ti-45 rotor (Beckman Instruments), the supernatant was decanted, mixed with 0.3 g of ammonium sulfate/1 ml of supernatant, and stirred. After

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[1] The abbreviations used are: pol II, RNA polymerase II; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; GST, glutathione *S*-transferase; SNF, soluble nuclear fraction; TF, transcription factor.

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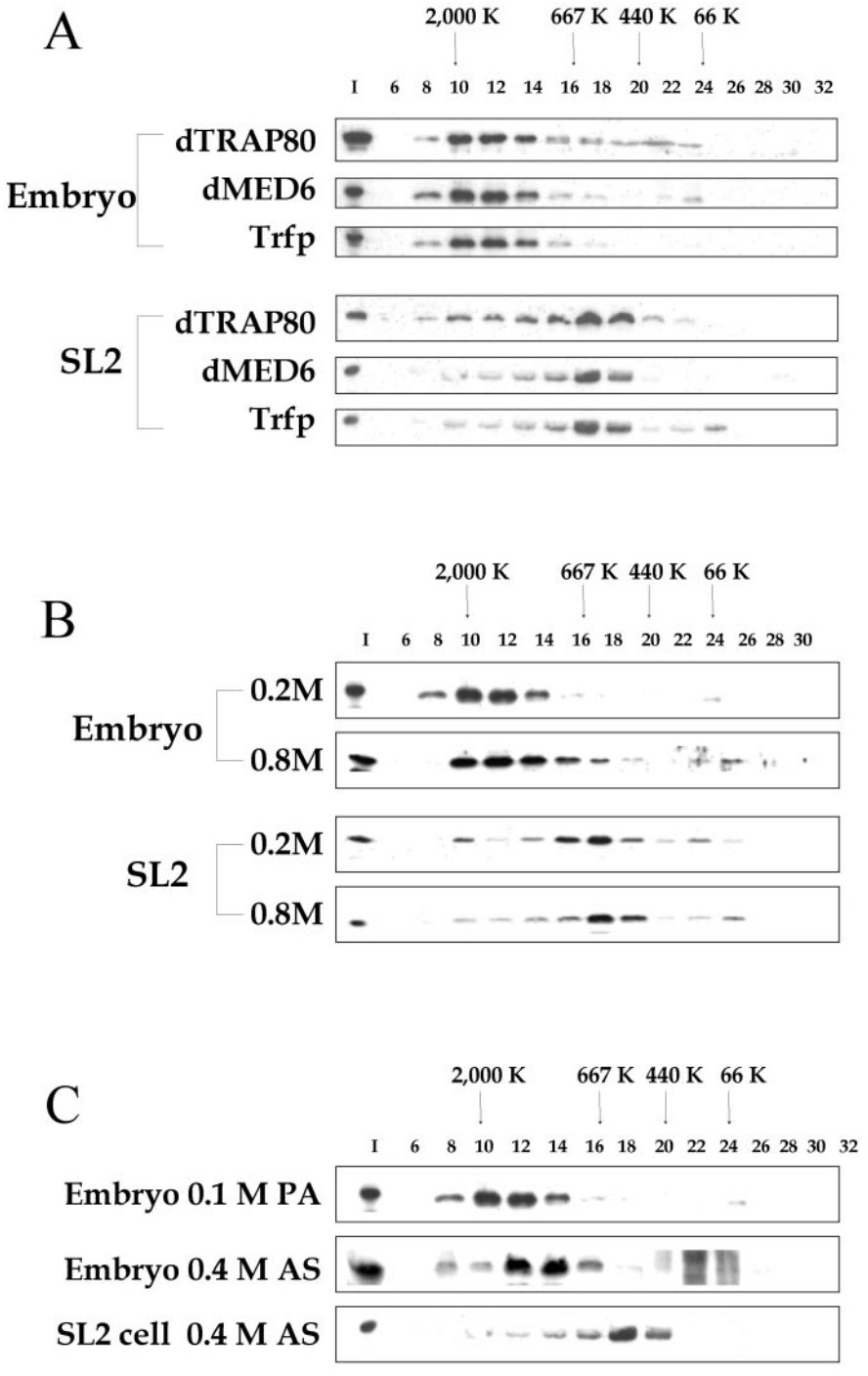

FIG. 1. **Multiplicity of Mediator complexes in *Drosophila*.** Nuclear fractions prepared from *Drosophila* embryos or SL2 cells were subjected to gel filtration chromatography on a Superose 6 column. Fractions were resolved on SDS-polyacrylamide gels and analyzed by immunoblotting. The elution positions of standard markers (in kilodaltons) are indicated by *arrows*. Mediator complexes were visualized by Western analysis with the antibodies indicated on the left (*A*) or with anti-dMED6 antibody (*B* and *C*). *A*, shown are the results from gel filtration analysis of SNFs of embryos and SL2 cells. *B*, SNFs of embryos and SL2 cells were chromatographed on a Superose 6 column in 200 mM (*0.2M*) or 800 mM (*0.8M*) potassium acetate-containing buffer. *C*, shown are the results from Superose 6 analysis of nuclear extracts prepared from embryos or SL2 cells with extraction buffer containing 100 mM potassium acetate (*PA*) or 0.4 M ammonium sulfate (*AS*). The gel filtration result for embryonic SNFs is shown for comparison.

centrifugation at 15,000 rpm for 30 min in a JA-25.50 rotor (Beckman Instruments), the pellet was resuspended in buffer containing 50 mM HEPES-KOH (pH 7.6), 10% glycerol, 1 mM EDTA, 100 mM potassium acetate, 1 mM dithiothreitol, and protease inhibitors. Mediator complexes enriched in the SL2 cell extracts were purified by a series of column chromatography steps as described (12). The peak fractions of Mediator proteins were pooled and incubated with anti-dSOH1 (where "d" is *Drosophila*), anti-dMED6, or anti-Trfp antibody beads. The beads were washed with buffer containing 20 mM HEPES-KOH (pH 7.6), 10% glycerol, 0.1 mM EDTA, and 300 mM potassium acetate containing 0.2% Nonidet P-40, and the bound proteins were eluted twice with 0.1 M glycine (pH 2.5). The eluted fractions were neutralized with 0.1 volume 1.0 M Tris-HCl (pH 8.8).

The gel slices of Coomassie Blue-stained protein bands were excised and subjected to in-gel tryptic digestion. Peptide mixtures were analyzed by MALDI-TOF mass spectrometry using a delayed ion extraction and ion mirror reflector mass spectrometer (Voyager-DE STR, Applied Biosystems, Inc.). For interpretation of the mass spectra, we used the Ms-Fit program.[2]

In Vitro Protein-Protein Binding Assay—pol II complexes were purified from SL2 cells expressing FLAG-tagged dRPB9 by conventional and affinity chromatography. Mediator protein-containing complexes in the Mono S fractions were incubated with purified core pol II in buffer A (25 mM HEPES-KOH (pH 7.6), 20% glycerol, 0.1 mM EDTA, 12.5 mM magnesium acetate, 100 mM potassium acetate, 1.5 mM dithiothreitol, 0.2% Nonidet P-40, and protease inhibitors) for 5 h. Then, 10 μl of anti-FLAG antibody M2-agarose pre-equilibrated with buffer A was added and allowed to interact with FLAG-tagged protein complexes for 12 h at 4 °C with constant inversion. The resin was washed with buffer A, and bound proteins were eluted with SDS gel sample buffer.

GST fusion proteins used in GST pull-down assays were expressed in

[2] Available at www.prospector.ucsf.edu/.

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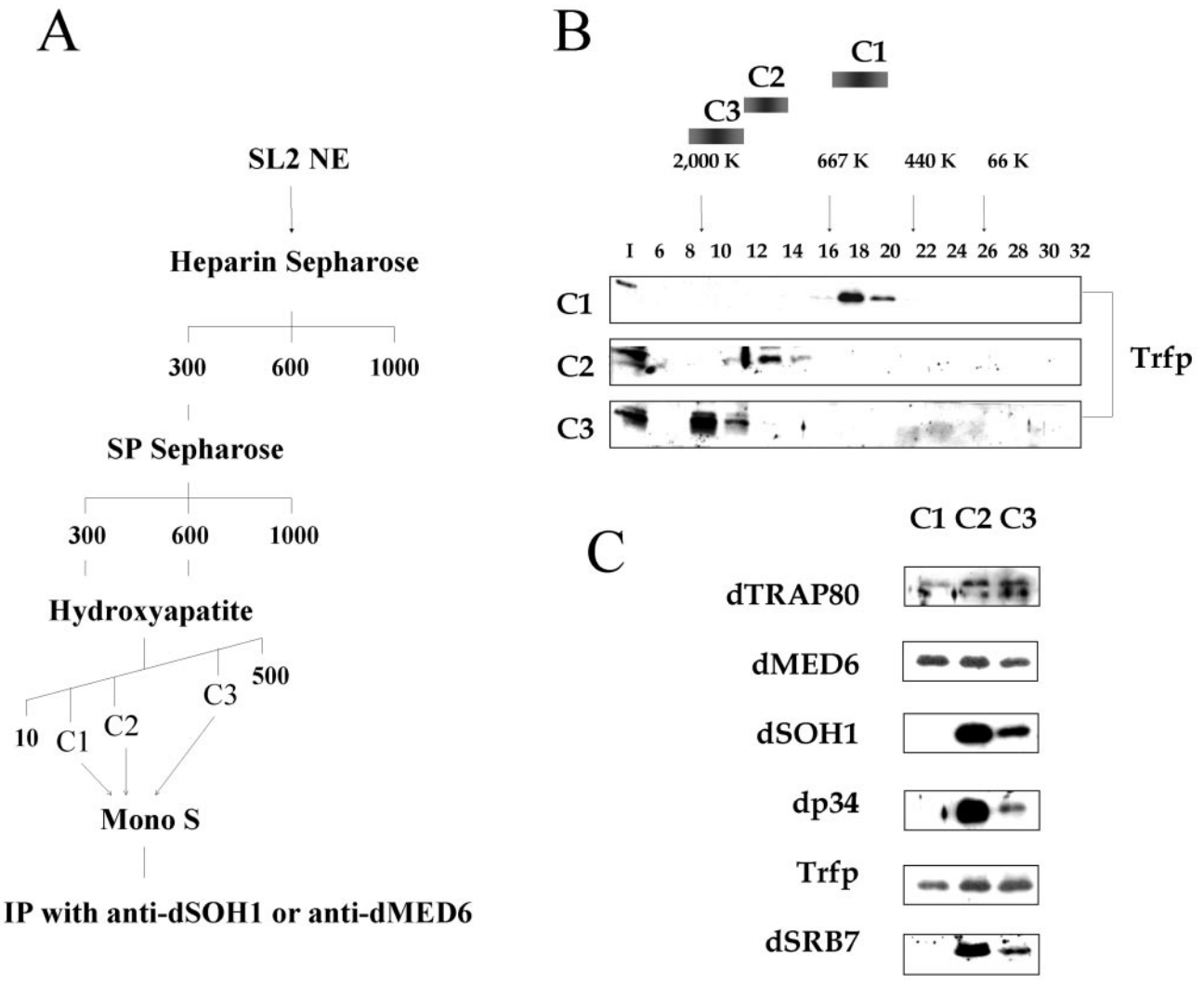

FIG. 2. **Purification of distinct Mediator complexes from SL2 cells.** *A*, purification scheme for Mediator complexes. *B*, Superose 6 chromatography of purified individual Mediator complexes. Results from Western analysis of Mediator proteins with anti-Trfp antibody are shown. *C*, immunoblot analysis of purified Mediator complexes with antibodies against the proteins indicated. *NE*, nuclear extract; *Ip*, immunoprecipitation.

Escherichia coli and purified on glutathione-Sepharose resin (Amersham Biosciences) as described (20). Ten microliters of the beads, which retained 1 μg of each GST fusion protein, was incubated with ^{35}S-labeled proteins synthesized in TNT reticulocyte lysates (Promega). The beads were washed, and bound proteins were analyzed by autoradiography.

In Vitro Transcription Assay—*In vitro* transcription using *Drosophila* embryo soluble nuclear fractions (SNFs) (21) and immunodepletion experiments were carried out as previously described (12). For reconstituted transcription assays, *Drosophila* TFIIA, TFIIB, TFIID, TFIIE, TFIIF, TFIIH, and pol II were included in the reactions. TFIIA, TFIIB, and TFIIE were expressed in and purified from *E. coli* BL21(DE3). TFIIF was expressed in Sf9 cells infected with baculovirus expression constructs. TFIID, TFIIH, and pol II were purified from *Drosophila* embryo nuclear extracts as described (22).

Generation and Treatment of Double-stranded RNA for RNA Interference—Individual DNA fragments that were ~0.5–1.0 kb in length were inserted into pBluescript KSa(+) (Stratagene) and then digested with *Bss*HII to generate 5′-overhangs so that we could obtain runoff transcripts. After purification of the DNA fragments, *in vitro* transcription reactions were performed with the RiboMAX RNA production kit (Promega), and the reaction mixtures were incubated at 37 °C for 4 h. RNA products were purified by ethanol precipitation and annealed by incubating at 65 °C for 30 min, followed by slow cooling to room temperature. Cellfectin (Invitrogen) was used to transfect double-stranded RNA (2.5 μg) into SL2 cells, which were plated at a density of 1×10^6 cells/well of a six-well dish. The cells were incubated for 5 h at 25 °C, and then the medium was replaced with 2 ml of fresh medium. After an additional 5 days of incubation, the protein composition of the cell lysate was analyzed by immunoblotting.

RESULTS

Identification of Multiple Mediator Complexes—Mammalian Mediator complexes isolated at different laboratories have shown similar but distinct subunit compositions. Some of these discrepancies may arise from differences in the purification procedures used in each laboratory, but some may result from the isolation of distinct Mediator complexes with divergent functions in transcriptional regulation. As our source of Mediator, we used transcriptionally active *Drosophila* embryo SNFs (21), which we subjected to gel filtration on a Superose 6 column. Immunoblot analysis of known Mediator proteins in the fractions from the column showed that the SNFs were highly enriched in a 2-MDa Mediator complex, but had only small amounts of Mediator proteins in fractions that corresponded to lower molecular masses (Fig. 1*A*). This result suggests that, even if there are multiple Mediator complexes in *Drosophila* embryos, nearly all are in the 2-MDa size range. However, *Drosophila* embryos consist of diverse types of differentiated embryonic cells; thus, the possibility that smaller Mediator complexes exist in a tiny fraction of the cells could not be ruled out.

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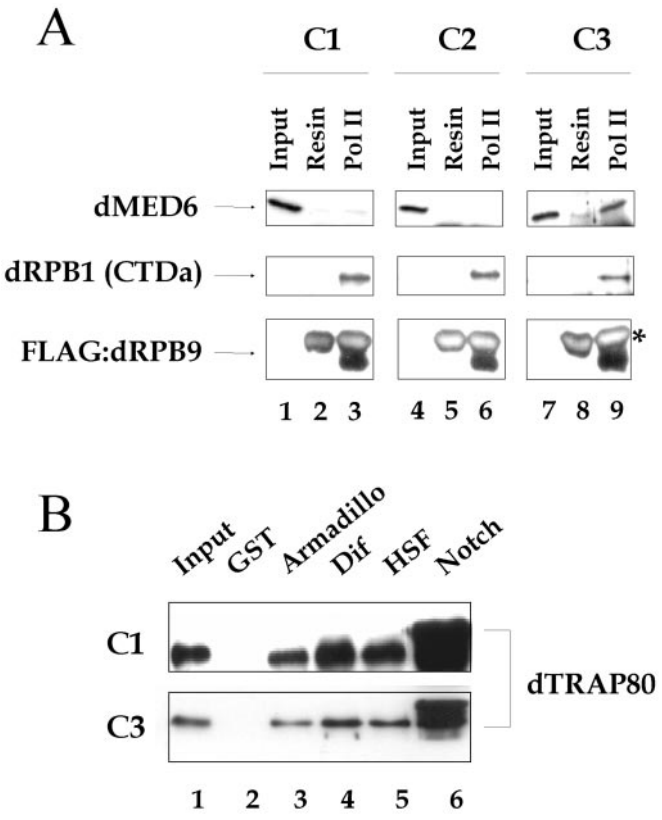

FIG. 3. **Comparison of the properties of the Mediator complexes.** *A*, physical interaction of purified Mediator complexes with immobilized FLAG epitope-tagged pol II. After immunoprecipitation with anti-FLAG antibody beads, proteins bound to the beads were analyzed by immunoblotting with the antibodies indicated on the left. The input sample contained 20% of the amount of the proteins used for the binding assay. The *asterisk* indicates a nonspecific band detected by anti-FLAG antibody. *B*, physical interaction of purified Mediator complexes with *Drosophila* transcriptional activators. GST-fused transcriptional activation domains of the *Drosophila* activators indicated at the top were mixed with the purified Mediator complexes, and the interactions were analyzed by GST pull-down assay, followed by immunoblot analysis with anti-dTRAP80 antibody. The input sample contained one-sixth of the amount of the proteins used for the binding assay. *FLAG:dRPB9*, FLAG-tagged dRPB9. *CTDa*, carboxy-terminal domain a.

To overcome this problem, we purified Mediator complexes from SL2 cells and compared their Superose 6 profile with that of the embryos. Immunoblot analysis of the Superose 6 fractions from the SL2 SNF revealed a pattern of Mediator components that was quite different from that of the embryonic SNF. Mediator proteins in the SL2 SNF eluted broadly in fractions that ranged in size from 2 to 0.5 MDa, with the majority of the Mediator proteins eluting in the 0.5-MDa range (Fig. 1*A*). It was possible that this difference in the Mediator protein profiles resulted from a purification artifact due to unstable structural integrity of the Mediator complexes. Therefore, we monitored the relative abundances of the various Mediator complexes in embryonic and SL2 SNFs under two different salt concentrations (0.2 and 0.8 M potassium acetate) using Superose 6 column chromatography. As shown in Fig. 1*B*, high salt conditions had no major effect on the overall size profiles of the Mediator complexes in either type of SNF; the majority of embryonic Mediator complexes were >1.5 MDa in size, and the broad migration profile of the SL2 Mediator complexes was unchanged. When we prepared nuclear extracts from embryos and SL2 cell nuclei under more stringent extraction conditions (the extraction buffer contained 0.4 M ammonium sulfate), the embryo- and SL2-specific patterns of Mediator complex sizes were unchanged (Fig. 1*C*). These results indicate that the 0.5-MDa Mediator complex is not a breakdown product of a larger Mediator complex and that it exists in higher concentrations in SL2 cells than in the embryonic cell population.

Although the high salt extraction had no major effect on the Mediator size profiles of the two cell populations, we did observe a small shift in the Superose 6 peak fraction of embryonic Mediator proteins toward lower molecular mass positions (Fig. 1*C*). The shift of the Superose 6 peak fraction also occurred as a result of high salt concentrations used in the gel filtration column (Fig. 1*B*). Taken together, these results show that it is highly likely that the 1.5-MDa complex is a subcomplex generated by a partial loss of some unstable components from the intact 2-MDa dMediator.

Purification and Properties of Distinct Mediator Complexes—To purify the differently sized Mediator complexes, we fractionated the SL2 SNF as shown Fig. 2*A*. Column chromatography fractions were examined for the presence of Mediator complexes by immunoblotting with antibodies specific to six known components of dMediator (dTRAP80, dMED6, dSOH1, dp34, Trfp, and dSRB7). During the multiple chromatography steps, the Mediator proteins were resolved into three distinct co-fractionated complexes, tentatively termed C1, C2, and C3, respectively, according to the order of elution from the hydroxylapatite column. On the basis of the elution profile of the hydroxylapatite column, C3 likely corresponds to dMediator purified from embryonic extracts (12). The molecular mass of purified C3 was comparable to that of dMediator, as determined by Superose 6 chromatography (Fig. 2*B*). The Superose 6 analyses of purified C1 and C2 showed that their molecular masses are 0.5 and 1.5 MDa, respectively, indicating that C1 may correspond to the 0.5-MDa complex that is specifically enriched in SL2 cells, whereas C2 may represent the 1.5-MDa subcomplex, which is probably generated from dMediator by a partial disruption of some components during the purification procedure (Figs. 1*B* and 2*B*).

All three Mediator complexes were then subjected to ion exchange chromatography on a Mono S column, and the column fractions were subjected individually to immunoprecipitation with anti-dMED6, anti-Trfp, and anti-dSOH1 antibodies to purify each complex to homogeneity. C1 was efficiently immunoprecipitated by anti-dMED6 antibody and, to a lesser extent, by anti-Trfp antibody, but not by anti-dSOH1 antibody. In contrast, C2 and C3 were efficiently immunoprecipitated by all three antibodies (data not shown). Immunoblot analysis of the immunoprecipitated complexes showed that all six Mediator-specific antibodies were able to detect their cognate Mediator components in C2 and C3, whereas only dTRAP80, dMED6, and Trfp were detected in C1 (Fig. 2*C*).

Functions of the Mediator Complexes in Transcriptional Regulation—Next, we explored the functional properties of the three Mediator complexes. In our previous study, dMediator was shown to play a critical role in transcriptional activation *in vitro* and to interact with pol II (12). Here, we first checked the abilities of C1, C2, and C3 to associate with pol II. Purified core pol II containing a FLAG-tagged dRPB9 subunit was mixed with each of the Mediator complexes. The mixture was then incubated with anti-FLAG antibody M2-agarose beads, and the bead-bound proteins were analyzed by immunoblotting. C3 was able to form a complex with core pol II, whereas C1 and C2 were not (Fig. 3*A*). Therefore, it is likely that the Mediator proteins present in C3, but not in C1 and C2, are required to form an interface for stable binding to pol II.

We then examined whether C1 and C3 are able to interact with various transcriptional activator proteins. Each of the purified Mediator complexes was mixed individually with four

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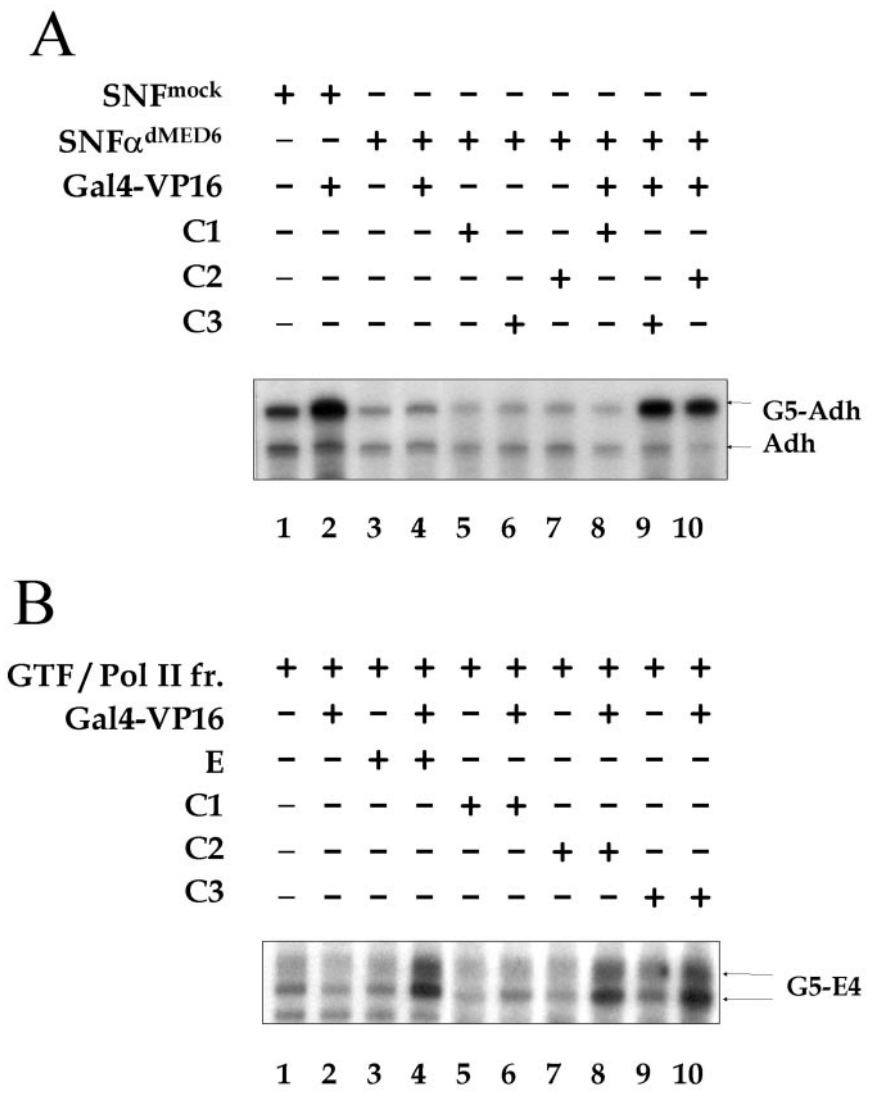

FIG. 4. **Transcriptional functions of the Mediator complexes.** Shown are the results from the *in vitro* transcription assay of the Mediator complexes (C1, C2, and C3). *A*, the Gal4-VP16 protein and SNF immunodepleted with anti-β-galactosidase (SNF^{mock}) or anti-dMED6 ($SNF\alpha^{dMED6}$) antibody were used in transcription reactions using the G-less template-containing *Adh* promoter with or without five copies of the Gal4-binding sites (*G5-Adh* and *Adh*, respectively). Each immunopurified Mediator complex was added to the reaction. *B*, shown are the results from reconstituted transcription reactions with each purified Mediator complex. Transcription reactions contained all basal factors and the E4 promoter constructs with five copies of the Gal4-binding sites (*G5-E4*). The amount of the transcribed RNA was measured using primer extension assay, followed by autoradiography. *GTF*, general transcription factor; *fr.*, fraction; *E*, dMediator complex purified from embryos.

recombinant GST-fused activator proteins (Armadillo (23), Dx (24), heat shock transcription factor HSF (25), and Notch (26)), and their associations were examined using a GST pull-down assay, followed by immunoblot analysis with anti-dTRAP80 antibody. Although only C3 interacted with pol II, both C1 and C3 interacted strongly with all of the activator proteins tested (Fig. 3*B*). Therefore, the Mediator proteins shared by C1 and C3 can function as transcriptional activator-binding subunits.

To determine whether the affinities of the Mediator complexes for pol II and/or the transcriptional activator proteins correlate with the transcriptional coactivator activity of the complexes, we tested the ability of C1, C2, and C3 to support activated transcription *in vitro*. To this end, SNFs were immunodepleted of endogenous Mediator proteins by incubation with anti-dMED6 antibodies and used for *in vitro* transcription assays (Fig. 4*A*). When the transcriptional activator protein Gal4-VP16 was added to the transcription reaction mixture, it selectively activated, in a Mediator-dependent manner, transcription of the template that contained the Gal4 activator-binding sites (*G5-Adh*), but did not activate the templates that contained only the core promoter (*Adh*) (Fig. 4*A*, *lanes 1–4*). When the Mediator-depleted SNFs were supplemented with a comparable amount of each of the three purified Mediator complexes, C2 and C3, but not C1, restored transcriptional activation by Gal4-VP16 (*lanes 8–10*). In contrast, none of the complexes affected the level of basal transcription (*lanes 5–7*).

The coactivator activities were further confirmed using a highly purified transcription system consisting of recombinant or purified general transcription factors (TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH) and pol II. This reconstituted system supported Gal4-VP16-driven transcriptional activation when embryonic dMediator was added to the mixture (Fig. 4*B*, *lane 4*). Again, in this system, C2 and C3 supported a moderate level of transcriptional activation by Gal4-VP16 (*lanes 8* and *10*), whereas C1 was unable to promote activated transcription (*lane 6*). As was seen with the SNF system, none of the complexes affected the level of reconstituted basal transcription (*lanes 5*, *7*, and *9*). Therefore, our results show that C2 and C3 are able to mediate transcriptional activation *in vitro*, but strong association of the complexes with core pol II sufficient to be detectable in our *in vitro* assay is not necessary for this activation function. In addition, if C2 is indeed a subcomplex of C3 and does not have any unique components, the transcriptional coactivator function of C3 must be dependent on the subset of the subunits that the two complexes have in common. On the other hand, C1 did not show any pol II transcription-related activity (including transcriptional repression) in our *in vitro* assays and may have functions distinct from those of dMediator and other Mediator complexes identified in yeast and human.

Compositional Analysis of the Small Mediator Complex, C1—Although we did not observe transcriptional activation

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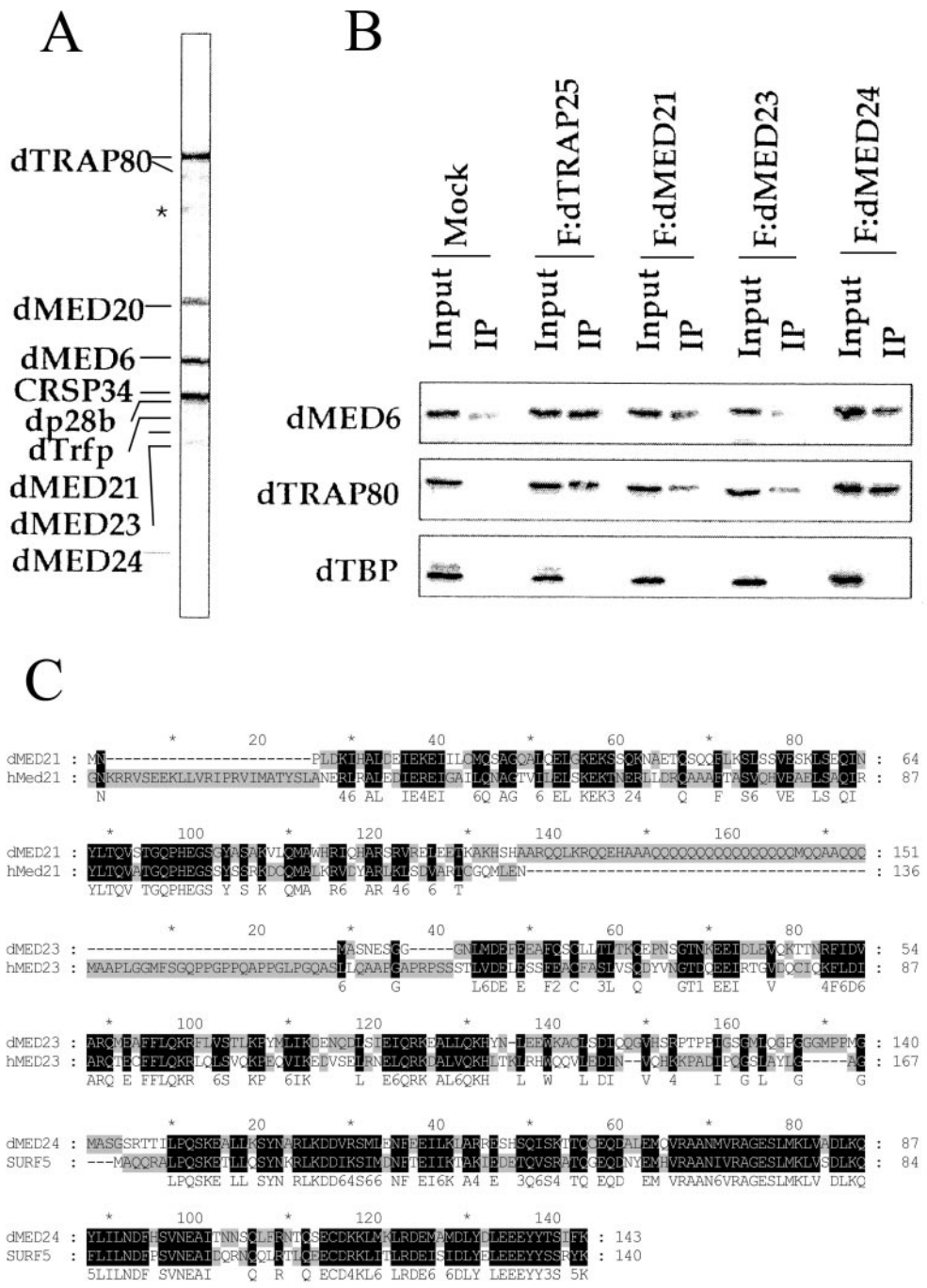

FIG. 5. **Compositional analysis of the C1 complex.** *A*, shown are the results from SDS-12% PAGE and silver staining analysis of the purified C1 complex. The positions of specific subunits are indicated on the left. The *asterisk* indicates the IgG heavy chain used for immunopurification. *B*, extracts (*Input*) from SL2 cells expressing FLAG (*F*)-tagged components were incubated with anti-FLAG antibody M2-agarose, and then resin-bound proteins were analyzed by immunoblotting with specific antibodies to Mediator and *Drosophila* TATA-binding proteins, as indicated. The input sample contained 10% of the amount of the proteins used for the binding assay. *C*, amino acid sequence comparisons of *Drosophila* (*d*) and human (*h*) homologs are shown. The conserved residues are indicated with *black boxes*. The alignment was performed with GeneDoc software. *IP*, immunoprecipitation.

activity for C1 in an *in vitro* assay, its activator-binding activity and abundance in a specific cell type suggest that C1 may participate in an as yet undefined reaction in SL2 cells that involves interaction with transcriptional regulatory proteins. Therefore, we purified C1 to homogeneity and used MALDI-TOF analysis to determine its entire polypeptide composition. We then confirmed the association of the various C1 subunits in a co-immunoprecipitation assay. Multiple peptide sequences derived from individual C1 subunits revealed the presence of the following proteins: dTRAP80, dMED6, CRSP34, dTrfp, and dp28b. In addition to these previously identified subunits of the dMediator complex, several of the C1 subunits were encoded by novel genes (Fig. 5*A* and Table I). One of these novel genes encodes a 35-kDa polypeptide that was identified as CG17183, which is the *Drosophila* homolog of the recently identified TRAP25 (27). The 20-, 19-, and 15-kDa polypeptides were found to correspond to the CG6884, CG5121, and BACR7A4.6 gene products, respectively; and we named these genes *dMED21*, *dMED23*, and *dMED24*, respectively. Each of these novel Mediator subunits has a highly conserved human homolog (Fig. 5*C*).

To verify that these novel subunits are indeed components of

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TABLE I
Results of MALDI-TOF analysis for C1 complex composition

Protein	MOWSE score	No. of peaks identified	Masses matched	Protein mass	Accession No.
			%	*kDa*	
dTRAP80	2.39e + 009	15	37	71.6	AE003721
dMED20	4.8e + 003	9	30	35.3	AE003469
dMED6	17.3	8	28	28.2	AE003684
CRSP34	6.47e + 004	11	32	33.9	AE003602
dp28b	1.94e + 003	7	29	24.7	AE003422
Trfp	527e + 004	13	46	27.6	AE003619
dMED21	575	4	7	19.6	AE003519
dMED23	521	12	42	21.1	AE003752
dMED24	1.32e + 003	9	34	16.6	AE003419

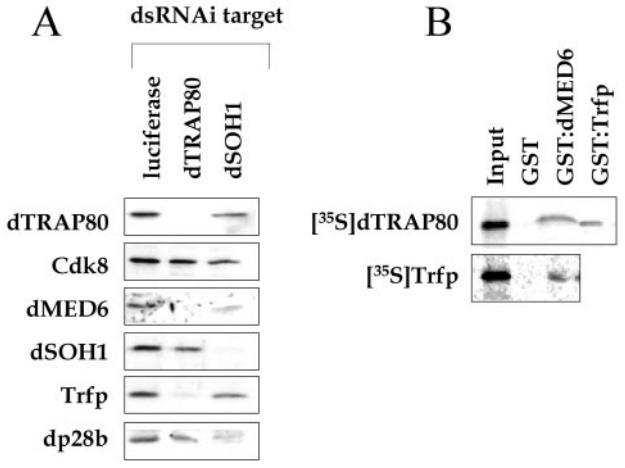

FIG. 6. **Physical interaction of C1 Mediator complex proteins.** *A*, cell lysates prepared from SL2 cells treated with luciferase, dTRAP80, or dSOH1 double-stranded RNA were analyzed by immunoblotting with the antibodies indicated on the left. *B*, shown is the direct interaction between Mediator components. ^{35}S-Labeled dTRAP80 or Trfp was incubated with the bead-bound GST-Mediator fusion proteins indicated at the top, and Mediator proteins in the lysate (*Input*), GST-bound fraction, and GST-Mediator-bound fraction were analyzed by autoradiography. The input sample contained one-twenty-fifth of the amount of the proteins used for the binding assay. *dsRNAi*, double-stranded RNA interference.

the Mediator complex, a FLAG-tagged version of the putative Mediator genes was transfected into SL2 cells, and its association with other Mediator proteins was examined by co-immunoprecipitation. When the transfected cell extracts were immunoprecipitated with anti-FLAG antibody M2-agarose, FLAG-tagged versions of the dTRAP25, dMED21, dMED23, and dMED24 proteins were co-immunoprecipitated with the dTRAP80, dMED6, and Trfp Mediator proteins, but not with the TATA-binding protein (Fig. 5*B*; Trfp data not shown). These results confirmed that the newly identified proteins (dTRAP25, dMED21, dMED23, and dMED24) are genuine components of the *Drosophila* Mediator complex.

To further demonstrate that these subunits are *bona fide* components of C1, we established a stable SL2 cell line expressing FLAG-tagged dTRAP25 and purified a dTRAP25-containing complex from C1-enriched hydroxylapatite fractions using two successive immunoprecipitations with anti-FLAG and anti-dMED6 antibodies. Silver staining analysis revealed that the composition of the purified complex was identical to that of C1 (data not shown). These results confirmed that the C1 Mediator complex is composed of both newly identified Mediator subunits (dTRAP25, dMED21, dMED23, and dMED24) and previously identified dMediator proteins (dTRAP80, dMED6, CRSP34, Trfp, and dp28b).

Physical Interaction of C1 Mediator Complex Proteins—Among the subunits of the C1 Mediator complex, dTRAP80, dMED6, CRSP34, Trfp, and dp28b are shared with dMediator. dMED21, one of the newly identified Mediator subunits, was also found in dMediator by immunoblot analysis performed with anti-dMED21 antibody (data not shown). The presence of the other novel C1 Mediator subunits (dTRAP25, dMED23, and dMED24) in dMediator was not confirmed due to the difficulty in raising functional antibodies to these subunits. However, the identification of a dTRAP25 homolog in the human Mediator complex that corresponds to dMediator suggests that dTRAP25 may also be shared by the C1 and dMediator complexes.

To examine whether these shared subunits are physically associated in cells, we generated dTRAP80 mutant SL2 cells using double-stranded RNA interference (28). Transfection of dTRAP80 double-stranded RNA into SL2 cells inhibited dTRAP80 protein synthesis by causing the specific degradation of *dTRAP80* transcripts. Five days after the double-stranded RNA transfection, the level of dTRAP80 protein dropped significantly, so only a trace amount of dTRAP80 protein was detected by immunoblot analysis (Fig. 6*A*). In the dTRAP80-deficient SL2 cell extracts, we examined the concentrations of the shared Mediator proteins as well as those of the dMediator-specific subunits (Cdk8 and dSOH1) using specific antibodies. Interestingly, the levels of dMED6 and Trfp were reduced to barely detectable levels, and the amount of dp28b protein was reduced slightly. However, the amounts of the dMediator-specific proteins were not changed by the loss of dTRAP80 (Fig. 6*A*). When dSOH1, a dMediator-specific subunit, was removed from cells using a similar approach, none of the shared Mediator proteins were lost from the extracts. These results suggest that when dTRAP80 was not present in the cell extracts, the subunits shared between C1 and dMediator, including dMED6 and Trfp, were lost from both types of Mediator complexes, implying that these proteins interact physically. GST pull-down analysis confirmed the direct interaction between dMED6, Trfp, and dTRAP80 (Fig. 6*B*). Therefore, these subunits shared by the two Mediator complexes may form a distinct modular structure, which could be C1 itself, to mediate interaction with transcriptional regulatory proteins.

DISCUSSION

In this study, we examined *Drosophila* nuclear extracts for the presence of alternative Mediator complexes that are distinct from the previously identified dMediator (12). Even though several Mediator complexes have been identified in human, it is unclear whether these complexes represent the actual repertoire of Mediator complexes *in vivo*. This is because the subunit composition of the assorted human Mediator complexes varies according to the purification and assay methods used (13). Instead of pursuing specific functional activities, we analyzed *Drosophila* embryonic and SL2 nuclear extracts by column chromatography and immunoblot analysis to trace the

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entire spectrum of Mediator proteins. Such an approach permitted us to recognize the existence of smaller complexes containing dMediator components and ultimately to isolate the novel complex whose existence cannot be detected by conventional approaches.

Our analysis provides strong circumstantial evidence that C2 is a subcomplex that separated from dMediator. This mode of Mediator diversification is not unprecedented and actually is reminiscent of the human PC2 complex, which is regarded as a submodule of human Mediator TRAP/SMCC (17). Although it is still unclear whether C2 is the *Drosophila* counterpart of human PC2, both C2 and PC2 can act as transcriptional coactivators *in vitro*. Although we could not detect an interaction between pol II and C2, addition of C2 was sufficient to support transcriptional activation in Mediator-deficient SNFs and in a defined transcription system reconstituted with general transcription factors and pol II (Fig. 4, *A* and *B*). This implies that, at least for transcriptional activation *in vitro*, a tight association between pol II and Mediator proteins is dispensable (or that the conditions used in the *in vitro* binding assay were not ideal for detection of weak *in vitro* binding of C2 and pol II). These experiments also show that the dMediator components that are absent in C2 are not essential for the mediation of transcriptional activation by Gal4-VP16. Hence, C2 may function as a core coactivator, whereas other Mediator proteins may serve as auxiliary molecules that are critical in the transcriptional regulation of specific subsets of genes.

Our observations that C1 exists separate from dMediator, does not support transcriptional activation by Gal4-VP16 *in vitro*, and does not associate with core pol II raise several possibilities regarding the significance of the existence and function of C1. First, novel subunits may endow C1 with a distinctive character different from that of dMediator. The putative process that requires the function of C1 is not necessarily confined to the transcriptional activation arena. C1 may be involved in gene transcription, but may be required by only specific types of transcriptional regulators. Another possibility is that C1 is a subcomplex of dMediator, but can exist on its own or in a complex with other proteins depending on the developmental process or physiological conditions. The fact that dMED6 and Trfp interact with dTRAP80 and disappear along with dTRAP80 in RNA interference experiments is reminiscent of the modular structure of the yeast Gal11 module proteins (Gal11, Med2, and Hrs1). It is intriguing that both of these modular domains in yeast and metazoans are involved in activator binding. Thus, C1 may be an alternative form of dMediator that is regulated by developmental signals, and the shared subunits may interact with regulatory proteins to function (mediate or squelch regulatory processes) in response to specific signals. Further studies of the functions of C1 are needed to extend our understanding of the physiological roles of metazoan Mediator complexes.

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Novel Mediator Proteins of the Small Mediator Complex in*Drosophila* SL2 Cells

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