Requirement of TRAP/Mediator for Both Activator-Independent and Activator-Dependent Transcription in Conjunction with TFIID-Associated TAF$_{II}$S

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The multiprotein human TRAP/Mediator complex, which is phylogenetically related to the yeast SRB/Mediator coactivator, facilitates activation through a wide variety of transcriptional activators. However, it remains unclear how TRAP/Mediator functions in the context of other coactivators. Here we have identified a previously uncharacterized integral subunit (TRAP25) of the complex that is apparently metazoan specific. An antibody that is specific for TRAP25 allowed quantitative immunodepletion of essentially all TRAP/Mediator components from HeLa nuclear extract, without detectably affecting levels of RNA polymerase II and corresponding general transcription factors. Surprisingly, the TRAP/Mediator-depleted nuclear extract displayed severely reduced levels of both basal and activator-dependent transcription from DNA templates. Both activities were efficiently restored upon readdition of purified TRAP/Mediator. Moreover, restoration of basal and activator-dependent transcription to extracts that were simultaneously depleted of TRAP/Mediator and TFIID (TBP plus the major TAF$_{II}$s) required addition of both TBP and associated TAF$_{II}$s, as well as TRAP/Mediator. These observations indicate that TAF$_{II}$s and Mediator are jointly required for both basal and activated transcription in the context of a more physiological complement of nuclear proteins. We propose a close mechanistic linkage between these components that most likely operates at the level of combined effects on the general transcription machinery and, in addition, a direct role for Mediator in relaying activation signals to this machinery.
TRAP/Mediator purification. Affinity purification of TRAP/Mediator from nuclear extract was performed essentially as previously described (11). Eluted 5-ml aliquots of nuclear extracts prepared from cell lines expressing FLAG-tagged CDK8 (13), FLAG-tagged NUT2 (27), or FLAG-tagged TR (9) were adjusted to 300 mM KCl and 0.5% NP-40 and incubated with 100 μl of M2-agarose beads (IBI/Kodak) at 4°C for 6 h with rotation. After four 5-ml washes with BC300-0.1% NP-40, proteins were eluted from beads by incubation at 4°C for 30 min with 100 μl of BC100-0.05% NP-40 plus 0.2 mg of FLAG peptide/ml. Highly purified TRAP/Mediator was obtained from nuclear extract prepared from cell lines expressing FLAG-tagged NUT2 (27). Briefly, after precipitation with 33% ammonium sulfate, the suspended protein was loaded on a phos- phocellulose P11 column at BC40. Protein fractions eluting between 0.5 and 0.85 M KCl were collected and dialyzed to BC300. Finally, affinity purification of TRAP/Mediator on M2 agarose was performed as described above.

In vitro transcription. Transcription reactions were carried out in a final volume of 25 μl and contained 50 ng of each supercoiled plasmid DNA template, 2 or 5 μl of HeLa nuclear extract (at ~10 mg/ml), 20 mM HEPEs (pH 8.2), 11 to 16% glycerol, 4 mM MgCl2, 60 mM KCl, 8 mM dithiothreitol, 0.5 mM ATP and CTP, 5 μM UTP, 0.1 mM 3′-O-methyl-GTP, 16 μCi (0.6 MBq) of [α-32P]UTP, 0.4 mg of bovine serum albumin per ml, and 20 μl of RNAse (Promega). Reaction mixtures were incubated at 30°C for 1 h, at which time the UTP concentration was increased to 25 μM and 15 μl of RNase T1 was added. After 30 min of incubation at 30°C, the reactions were extracted with phenol/ chloroform in the presence of 150 μl of stop solution (0.4 mM sodium acetate [pH 5.2], 13 mM EDTA, 0.33% SDS, and 0.67 μg of yeast tRNA per ml). The aqueous layer was precipitated by ethanol and analyzed by gel electrophoresis followed by autoradiography. Correctly initiated transcripts were quantitated by densitometry. To perform immunodepletion from nuclear extract, since recombinant His-tagged TRAP25 was totally insoluble in E. coli, the His-tagged TRAP25 was purified with a nickel-nitritotriacetic acid-agarose column under denaturing conditions (6 M guanidine HCl, 0.05 M NaCl, 20 mM HEPEs [pH 7.6], 100 mM EDTA). The purified His-tagged TRAP25 was cross-linked under the above conditions with CNBr-activated Sepharose 4B (Pharmacia) that had been pretreated with 1 mM HCl. After mixing for 2 h at room temperature, the cross-linking reaction was blocked by adding Tris buffer (pH 8.0) to a final concentration of 0.1 M. The resin was washed three times with 0.1 M sodium acetate (pH 4.0)–0.5 M NaCl and three times with equilibration buffer (0.1 M Tris [pH 8.0], 0.5 M NaCl) before use.

For immunodepletion of TRAP/Mediator, anti-TRAP25 antisera were purified by passage through a column containing His-tagged TRAP25 protein, which was covalently cross-linked to CNBr-activated Sepharose 4B (Pharmacia). Bound anti-TRAP25 antibodies were eluted with 200 mM glycine (pH 2.5). The antibodies were further purified and concentrated by binding to protein A-Sepharose (Pharmacia) and then cross-linked to protein A with dimethylmethyl- date (Sigma). One milliliter of HeLa nuclear extract in BC100 was passed four times over a 1-ml cross-linked anti-TRAP25–protein A-Sepharose column. The flow-through fractions were collected and used in vitro transcription. TBP and TAFs were depleted in BC500 and depleted extracts were dialyzed in BC100 essentially as described (34).

MS. Protein sequencing using mass spectrometry (MS) and tandem MS (MS/ MS) was carried out as described previously (35), with the exception that 180-labeled H2O was omitted. Briefly, the Coomassie blue-stained protein band was in-gel digested with trypsin and the recovered peptides were analyzed using an electrospray ion trap mass spectrometer (LCQ, Finnm modular MAT, San Jose, Calif.) coupled on-line with a capillary high-pressure liquid chromatograph (LC) (C18 Magic MS column, Magic 2002; Michrom BioResources, Auburn, Calif.). The flow was split with a Magic precolumn capillary splitter assembly (Michrom BioResources). The LC/MS was programmed to run in a data-dependent fash- ion, in which the mass spectrometer switched to the MS/MS mode to acquire collision-induced dissociation spectra, once an ion signal was detected to exceed a preset value in the MS mode during the entire LC run. Data derived from the collision-induced dissociation spectrum were used to search a compiled protein database that was composed of the nonredundant protein database and a six-reading-frame translated EST database to identify the protein.

RESULTS

Molecular cloning of a full-length cDNA encoding TRAP25. Our earlier analysis (27) of the polypeptide composition of the TRAP/Mediator complex revealed, in addition to the subunits whose sequences were previously identified (13, 17), two or
three polypeptides in the 20- to 25-kDa range that coeluted with the complex regardless of whether it was purified from a FLAG-NUT2- or a FLAG-SRB10/CDK8-expressing cell line (13, 27). These bands were also consistently observed in PC2 preparations that had been chromatographed over several columns (27), strongly suggesting that they represent authentic TRAP/Mediator subunits. To determine the identities of these bands, we electrophoretically resolved a TRAP/Mediator preparation that was affinity purified from the FLAG-SRB10/CDK8-expressing cell line (13) (Fig. 1A). The bands (a to f) corresponding to these polypeptides were excised and subjected to mass-spectrometric sequence analysis.

Bands a and f appear, on the basis of derived sequences, to be proteolytic degradation products of the previously reported TRAP150. Band d contained sequences from a polypeptide that was reported both in PC2 (referred to as p37 in reference 27) and in CRSP (referred to as CRSP34 in reference 41). Band b corresponded to immunoglobulin light chain derived from the affinity resin. Additional sequences not previously found in Mediator preparations were obtained from bands c, e, and f. The sequence YEYHWADGTNIK in band e identified ESTs (AI393039 and AI557507) that encode the human ortholog of yeast Mob1, which has been linked to the CCR4-NOT complex (21). However, several lines of evidence (data not shown) suggest that Mob1 is not an authentic, stoichiometric component of TRAP/Mediator. The sequence YLRPPNTSLFVR in band e identified ESTs (AF047448 and AF067730) that encode TASSR-2 (translocated in liposarcoma-
associated serine-arginine protein 2). TASR-2 may be involved in RNA splicing via the Pol II C-terminal domain (15, 47). TASR-2 was not further characterized but, like Mob1, is unlikely to be an authentic TRAP/Mediator subunit. However, it remains possible that these proteins are representative of a variety of cellular components (other than the basic transcription apparatus) with which TRAP/Mediator normally interacts.

Band f yielded the additional sequence IGQETVQDIVYR, which identified an EST (AI806623) that encodes a hitherto-uncharacterized protein (termed TRAP25 based on evidence presented below) with a predicted molecular weight of 20 kDa (Fig. 1B). A Drosophila TRAP25 ortholog (CG17183 gene product), which exhibited localized identity of 35% and similarity of 53% with human TRAP25 (Fig. 1B), was found in the database. S. cerevisiae and Caenorhabditis elegans do not appear to possess an equivalent protein, potentially implicating this subunit in signaling processes restricted to higher metazoans. A Northern blot analysis (Fig. 1C) indicated ubiquitous expression of a 1-kb TRAP25 mRNA, although elevated levels were seen in heart, placenta, and skeletal muscle. This pattern is qualitatively similar to that previously reported for other TRAP/Mediator subunits (17).

TRAP25 is a bona fide component of TRAP/Mediator. Toward establishing that TRAP25 is a bona fide subunit of TRAP/Mediator, we first expressed this protein in bacteria and raised antisera against the purified recombinant protein. Immunoblot analysis (Fig. 2A) with the TRAP25-specific antiserum revealed the presence of TRAP25 in all preparations of TRAP/Mediator regardless of how they were obtained, either through association with FLAG-TR or through intrinsic FLAG-tagged subunits (NUT2 or SRB10/CDK8). Furthermore, the amount of TRAP25 relative to other authentic subunits (TRAP80, MED6, and SRB7) was the same for the purified complexes and for unfractionated nuclear extract, strongly suggesting that TRAP25 is a stoichiometric component of TRAP/Mediator. To further demonstrate that TRAP25 is stably associated with TRAP/Mediator complex, TRAP/Mediator purified from epitope-tagged SRB10/CDK8 cells was subjected to gel filtration chromatography on Superose 6 (Fig. 2B). Consistent with previous reports (13), immunoblot analysis of the resulting fractions with antibodies against representative subunits revealed that the majority of the TRAP/Mediator eluted as a 1.8-MDa complex. Importantly, the bulk of the complex coeluted with TRAP25 (Fig. 2B, lanes 5 and 6). This result provides additional strong evidence for the presence of TRAP25 in the TRAP/Mediator complex.

In order to prove definitively that TRAP25 is an integral component of TRAP/Mediator, we used our TRAP25 antiserum to show that depletion of TRAP25 from nuclear extract simultaneously resulted in depletion of other TRAP/Mediator subunits (Fig. 2C). As expected, immunoblot analysis of extract...
that had been passed through an anti-TRAP25 antibody column revealed that TRAP25 was quantitatively removed by this treatment (compare lanes 1 and 2 with lane 3). Furthermore, as predicted, the majority of TRAP/Mediator subunits were also efficiently depleted. Exceptions included SRB11/cyclin C, which was only partially depleted; this likely reflects its presence not only in TRAP/Mediator but also in a kinase-cyclin pair with SRB10/CDK8 that can exist in an unassociated form (13, 27). We therefore conclude that TRAP25 is a bona fide TRAP/Mediator subunit.

We also examined if the TRAP25 antibody depleted Pol II and its associated GTFs. Immunoblotting analysis showed negligible reductions in the amounts of Pol II, TFIIA, TFIB, TFIIID, TFIIH, TFIIIE, and TFIIIF in the supernatant (Fig. 2C), despite the fact that this depletion experiment was performed under mild salt conditions (125 mM KCl). Therefore, a majority of TRAP/Mediator appears not to be tightly associated with Pol II and other GTFs, consistent with previous results (13, 43). However, as minor differences would not be evident from the present analysis, it remains possible that a subpopulation of TRAP/Mediator complexes is associated with Pol II (S. Malik and R. G. Roeder, unpublished results).

**Function of TRAP/Mediator in nuclear extract.** Our previous functional analyses of TRAP/Mediator (13, 17, 27) employed an in vitro assay system that was reconstituted from highly purified Pol II, GTFs, and the USA-derived positive cofactor, PC4. The Mediator-depleted extract described above now provided an opportunity to assess the function of TRAP/Mediator in a less purified system that presumably contains a more normal complement of both positively and negatively acting nuclear factors.

We thus tested the Mediator-depleted extract for its ability to support activation by GAL4-p53 (Fig. 3A) and GAL4-VP16 (Fig. 3B), which consist of the DNA binding domain of the yeast activator GAL4 fused, respectively, to the activation domains of the tumor suppressor p53 and the viral activator VP16. Standard transcription assays contained two templates. The G5HML plasmid has five copies of a GAL4 binding site upstream of a hybrid core promoter and permits us to assay GAL4-fused activation domains. The MLΔ53 plasmid contains only the core promoter sequences (−53 to +10) from the adenovirus major late promoter and allows us to monitor basal (activator-independent) transcription. The G5HML is thus a weaker core promoter and yields lower levels (circa fourfold) of basal activity relative to the MLΔ53 promoter (Fig. 3, lanes 1 and 2) (46). Under our conditions, GAL4-p53 (Fig. 3A, lanes 1 and 2) activated transcription 72-fold and GAL-VP16 (Fig. 3B, lanes 1 and 2) activated transcription 13-fold in untreated extracts. By contrast, in the Mediator-depleted extract (ΔMED) (Fig. 3) the absolute levels of activated transcription were dramatically reduced for both GAL4-p53 (ca. 7.5-fold) (Fig. 3A, lane 2 versus lane 4) and GAL4-VP16 (ca. 15-fold) (Fig. 3B, lane 2 versus lane 4). In a control extract, which had been passed over a column containing preimmune antiserum, the levels of activated transcription were essentially unaltered (Fig. 3, lanes 2 versus lanes 6). We conclude that depletion of Mediator from nuclear extract severely compromises its ability to support high levels of activated transcription.

We also noted that the level of basal transcription, as monitored from both the MLΔ53 template and the G5HML template in the absence of added activators, was reduced 6- to 18-fold following depletion of Mediator (compare lanes 1, 3, and 5 in Fig. 3A and, especially, 3B). Thus, despite the low absolute levels, the level of activation appears to remain unchanged in the presence and absence of Mediator. While this residual activation might possibly be attributed to an alternative mechanism (involving a distinct coactivator activity), the underlying reason is currently unclear. It may arise as well from trace amounts of TRAP/Mediator that are not detected by immunoblot analysis but whose effects would be evident in the more sensitive transcription assay. The slight reduction in transcription from the MLΔ53 template upon activator addition (e.g., lane 1 versus lane 2) is normal and has previously been
attributed to competition between the two templates for limiting factors.

We next asked if purified TRAP/Mediator could restore the ability of Mediator-depleted extract to support activation by GAL4-p53 and GAL4-VP16. For this purpose, TRAP/Mediator that had been affinity-purified from nuclear extract from our epitope-tagged NUT2 cell-line, and which additionally had been fractionated over phosphocellulose P11 (see Materials and Methods), was added back to transcription reactions performed with the depleted extract (Fig. 4A and B). In this experiment, TRAP/Mediator was added over a concentration range that, as determined by immunoblot analysis (data not shown), either was just under (Fig. 4A and B, lanes 3 and 4) or actually approximated (lane 5) the concentration of Mediator in standard nuclear extracts.

Upon addition of increasing amounts of TRAP/Mediator to Mediator-depleted extract, in which activation by GAL-p53 (Fig. 4A, lane 2) and GAL-VP16 (Fig. 4B, lane 2) was virtually undetectable, activated transcription was enhanced in an essentially linear dose-dependent fashion (lanes 3 to 5). Indeed, for both GAL4-p53 and GAL4-VP16, full recovery of activity was observed when the normal concentration of Mediator in nuclear extracts was reached (compare lanes 5 and 1 in Fig. 4A and B). These results confirm that diminution of coactivator activity following depletion of Mediator is due solely to its absence and not to depletion of associated factors.

We also observed that basal transcription from the MLΔ53 template was fully restored upon complementation of the depleted extract with TRAP/Mediator. This is especially evident in Fig. 4B (compare lane 5 and lane 1). Nonetheless, to ensure that these Mediator effects on basal transcription are entirely independent of activators, we repeated the depletion-and-complementation experiment in the absence of any exogenous activators (Fig. 4C). Once again, basal transcription activity from both GSHML and MLΔ53 templates was both severely reduced in Mediator-depleted extract (Fig. 4C, lane 2) and fully restored by complementation with purified TRAP/Mediator (lane 5 versus lane 1). Together, these results demonstrate that, in nuclear extracts, Mediator plays a dominant role in regulating both activator-dependent and activator-independent transcription.

**Joint requirement of TRAP/Mediator and TAF II5s in TFIID for activated transcription.** We also made use of our ability to deplete Mediator from nuclear extracts to address the outstanding question of whether the coactivator activities of Mediator and TAF II5s within TFIID are functionally redundant or synergistic. We therefore prepared HeLa nuclear extract lacking both TFIID and Mediator by sequential passages over columns containing immobilized antibodies against TBP and TAFII100 (to deplete TFIID) (34) and against TRAP25 (to deplete Mediator). As judged by immunoblot analysis of representative TFIID subunits (TBP, TAFII135, and TAFII100), and consistent with previous results (34), the extract (ΔMED/ΔHID) was efficiently depleted of TFIID (Fig. 5A, lane 2 versus lane 1). Similarly, the extract was simultaneously depleted of Mediator. Importantly, none of the other GTFs tested showed any significant decrease. Our strategy was to supplement these extracts with Mediator and either TBP or intact TFIID, either singly or in combination. Thus, together with an extract (ΔMED) that was selectively depleted of only Mediator

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**FIG. 4. Restoration of activated and basal transcription by exogenously added purified TRAP/Mediator.** (A) Increasing amounts of purified TRAP/Mediator (lanes 3 to 5) were added to anti-TRAP25 antibody-depleted nuclear extract (ΔMED) in vitro transcription reactions. Reaction mixtures were as for Fig. 3 except that they contained 20 μg of each nuclear extract. As determined by immunoblotting, the amount of TRAP/Mediator added to the reactions corresponded to approximately 20% (lane 3); 70% (lane 4), or 100% of the TRAP/Mediator concentration in untreated nuclear extract. GAL4-p53 was used as the activator in all reactions. Lane 1 shows the product of a control reaction with untreated extract. (B and C) In vitro transcription reactions were performed as for panel A except that GAL4-VP16 was used as the activator (B) or no activator was added (C).
above) (Fig. 5A, lane 3), this provided a system for assessing the relative contribution of TFIID-associated TAF\textsubscript{II}s and TRAP/Mediator in transcription. These assays were performed both with the above-described G5HML template (Fig. 5B and D) and with a template (G5E1b) in which the GAL4 sites are located upstream of the adenovirus E1b core promoter, which contains a relatively weak TATA box (Fig. 5C and E).

As expected, ΔMED/ΔIID extract failed to support both basal and activated transcription by GAL-p53 and GAL-VP16 (Fig. 5B to E, lanes 7 and 8 versus lanes 1 and 2). Furthermore, whereas the activated transcription in ΔMED extracts could be readily recovered upon supplementation with purified TRAP/Mediator in this experiment (Fig. 5B to E, lanes 6 versus lanes 2 and 4), consistent with earlier data (Fig. 4A and B), this was not sufficient to restore activity to the ΔMED/ΔIID extract.

**FIG. 5.** Synergistic effect of TRAP/Mediator and TFIID-associated TAF\textsubscript{II}s on transcription activation in vitro. (A) Immunoblot analysis of TFIID- and TRAP/Mediator-depleted HeLa nuclear extract (Material and Methods) with the indicated antibodies. Lane 1, mock-depleted extract; lane 2, nuclear extract depleted with anti-TBP, anti-TAF\textsubscript{II}100, and anti-TRAP25 antibodies (ΔMED); lane 3, anti-TRAP25-depleted nuclear extract (ΔMED). Each lane was loaded with 20 μg of each nuclear extract and analyzed on a 4-to-20% gradient SDS-PAGE gel, followed by immunoblotting. (B and C) In vitro transcription reaction mixtures contained the indicated nuclear extract plus 25 ng each of pML\textsubscript{G5} and either pG5HML (B) or pG5E1b (C) in a 12.5-μl reaction volume. GAL4-p53 (50 ng), recombinant TBP (rTBP, 3 ng), affinity-purified TFIID containing an equivalent molar amount of TBP (as determined by immunoblotting), and an amount of TRAP/Mediator corresponding to that in standard nuclear extract were added where indicated. (D and E) In vitro transcription reactions were performed as for panels B and C, respectively, except that Gal4-VP16 (50 ng) was the activator.
We also added affinity-purified TFIID to the ΔMED/ΔIID extract in amounts that were empirically determined to support baseline levels (in the absence of exogenous Mediator) of transcription equivalent to those seen with the ΔMED extract (Fig. 5B to E, compare lanes 12 with lanes 4). This resulted in the addition of an amount of TFIID whose TBP content was only about a fifth of that in standard extract, which is to be expected in view of the fact that TBP is shared among several nuclear multiprotein complexes. Under these conditions, addition of an equivalent molar amount of TBP had negligible effects (Fig. 5B to E, compare lanes 9 and 10 with lanes 7 and 8). When the ΔMED/ΔIID extract was supplemented with both TFIID and TRAP/Mediator, the resulting transcription levels for both GAL-p53 (Fig. 5B and C) and GAL-VP16 (Fig. 5D and E) were virtually indistinguishable from those observed with control extract (lanes 18 versus lanes 2).

In sharp contrast, when the ΔMED/ΔIID extract was supplemented with TBP and TRAP/Mediator, the absolute transcription levels remained low (less than 5% of that with intact TFIID) (Fig. 5B to E, lanes 16 versus lanes 18). Similarly, although TBP could support low-level (circa three- to sixfold activation) activated transcription (Fig. 5B to E, lanes 15 and 16 versus lanes 17 and 18) in the presence of TRAP/Mediator, the results clearly show that TAF16 contribute an additional potential that allows a further 5-fold enhancement (8- to 28-fold activation in the presence of TFIID and TRAP/Mediator).
significantly as a result of the combined effects of TFIID and TRAP/Mediator (Fig. 5B to E, lanes 17 versus lanes 7) but much less so with TBP and TRAP/Mediator (lanes 15 versus lanes 7), the final extent of transcription is significantly higher in the former situation. We note, however, that the TBP- and TFIID-nucleated basal transcription in this system is critically dependent on the amount of exogenously added factor. Thus, the levels of basal transcription reconstituted with f:TFIID and TRAP/Mediator were similar to those with rTBP and TRAP/Mediator when a fivefold excess of exogenous f:TFIID and rTBP was added to the reaction mixtures (data not shown).

As indicated above, these assays were performed with templates containing either combined TATA and initiator elements (G5HML) or a relatively weak TATA (G5E1b) element within the core promoter. The fact that the two templates showed qualitatively similar results indicates that, at least with respect to the limited number of core promoters tested, the observed synergy between TFIID-specific TAFIIs and Mediator is not a function of core promoter sequences.

DISCUSSION

The main conclusions of this work are (i) that TRAP25 is a novel, integral subunit of human Mediator, (ii) that Mediator is required for both basal (activator-independent) and activated transcription in the context of nuclear extract, and (iii) that Mediator and TAFIIs within TFIID function synergistically in both basal and activated transcription in this system.

Role of metazoan Mediator in basal transcription. Initial characterization of the yeast Mediator complex had pointed to three distinct associated biochemical activities. It was found to play a critical role as a coactivator (the function most closely identified with the Mediator) and to stimulate basal transcription activity in reaction mixtures reconstituted with Pol II and GTFs, leading to the hypothesis that the two activities may be interdependent (19). Functions in both basal and activated transcription were also evident from analyses of extracts from yeast with a deletion mutation in the SRB5 subunit of Mediator (44). In vitro studies also showed that Mediator can stimulate the Pol II carboxy-terminal repeat domain kinase activity of TFIIH (19). Although our earlier functional analysis of the various human Mediator complexes (13, 17) clearly revealed their coactivator potential for a wide range of activators (including the thyroid hormone receptor in conjunction with the retinoid X receptor, GAL4-p53, and GAL4-VP16), the effects on activator-independent transcription were not as marked. Our previous reconstituted transcription system relied on an additional coactivator, PC4, that, in addition to promoting activated transcription, strongly suppresses basal transcription (11, 26). Similarly, in experiments in which ARC (33) and DRIP (38) coactivator effects were seen only in the context of chromatin templates, basal transcription may have been suppressed (either by histones or by negative cofactors in the crude chromatin assembly extracts) to such an extent that it was refractory to stimulation by Mediator alone. By contrast, the unfractonated nuclear extract-based assays employed here and by Mittler et al. (31) have helped uncover a prominent basal function of human Mediator. By thus extending our initial characterization, these findings now suggest that, consistent with the phylogenetic conservation of some subunits, the fundamental mechanisms by which the Mediator functions may also be conserved from yeast to humans.

It is likely that basal effects of Mediator are readily observed in an unfractonated nuclear extract because transcription in such a system is subject to a much broader range of both positively and negatively acting cofactors than exist in the purified reconstituted systems. Thus, it may be that net transcription, both basal and activated, in less purified systems entails a major antirepression component and that one function of Mediator is to help overcome restrictions imposed by negatively acting cofactors. These cofactors are presumably distinct from histones and PC4 but could include cofactors such as NC2 (reviewed in reference 25). It perhaps is relevant that in pure systems, basal transcription (in the absence of PC4) is relatively high, providing another possible reason why basal effects of Mediator have been undetected (26).

Alternative, but not mutually exclusive, mechanisms by which Mediator could promote basal transcription involve direct interactions with the basal transcription machinery. Indeed, in yeast, genetic interactions of SOH1 with Pol II and TFIIH have been described (reviewed in reference 5). However, it should be noted that while SOH1 is an integral subunit of the human Mediator (13; Malik and Roeder, unpublished), it has not been documented as a yeast Mediator subunit. Furthermore, in vitro recruitment experiments have revealed that the yeast holoenzyme (consisting minimally of Pol II and Mediator) is dependent on both TBP and TFIIIB for incorporation into the preinitiation complex formation (39). It is conceivable, therefore, that the Mediator could directly stabilize the preinitiation complex. A more dynamic effect of the Mediator on basal transcription also might be exerted via its ability to modulate TFIIH activities (19). Although it has been proposed that the SRB10/CDK8 kinase subunit of the human Mediator down-regulates TFIIH activity by phosphorylating its cyclin H subunit (1), the yeast Mediator preparation that was shown to stimulate TFIIH carboxy-terminal repeat domain kinase activity lacked the SRB10 kinase (19).

Synergistic transcriptional activation by Mediator and TAFIIs. Our results also have revealed that, in unfractonated systems, Mediator and TAFIIs (in TFIID) functionally synergize to give high levels of activated transcription that result, albeit only in part, from favorable TAFI- and Mediator-dependent basal effects discussed above. Although at apparent variance (but see below) with earlier results (34) indicating that the TBP subunit of TFIIID could support activated transcription in a similar unfractonated system in the absence of associated TAFIIs, the present experiments have clearly served to define experimental conditions in which functional synergy between Mediator and TFIID-specific TAFIIs can be readily observed. Our use of near-physiological concentrations of exogenously added factors in depleted extracts may account in part for the present results. However, it also remains possible that our particular protocol (depletion of both TFIID and Mediator and subsequent complementation with highly purified preparations of Mediator and TFIID) results in conditions that favor multiple rounds of transcription and thus render the system TAFI-dependent (34). The combined depletion of TFIID and Mediator may also result in depletion of components that facilitate TAFI-independent transcription and that are not present in the highly purified TFIID and Mediator.
preparations. Nonetheless, the synergistic function of TFIID and Mediator is in accord with the coactivator properties ascribed to each (9, 13, 45) and with recently published data showing synergistic effects of TFIID and Mediator in highly purified reconstituted systems (14, 27, 41).

While the role of TFIID-specific TAF_{18} as conventional coactivators remains somewhat controversial, a consensus is emerging that they may play major roles in core promoter selectivity both in yeast and in metazoans (reviewed in reference 12). This is especially evident from studies showing an absolute requirement for metazoan TAF_{18} in basal transcription from promoters that contain divergent core promoter elements, including initiators and downstream promoter elements (DPEs), but often no TATA element (3, 22, 29). TAF_{18} are also required for functional synergy between TATA and initiators or DPEs, indicating promoter-selective functions on composite core promoters (29). Preliminary indications are that, mechanistically, this may involve direct interactions with core promoter sequences, especially in the case of promoters made up of divergent (non-TATA) promoter elements (3).

The recently published results of Park et al. (36), who examined the relative contribution of TAF_{18} and Mediator in activation of several Drosophila promoters, are also in agreement with the hypothesis that TFIID-associated TAF_{18} are primarily involved in core promoter effects. These authors observed that a TATA-box-containing promoter, Adh, could be activated by GAL4-VP16 (through upstream GAL4 sites) with only TBP (in the absence of TAF_{18}) and Mediator. However, activation through the engrailed promoter, which lacks a TATA-box, required a DPE, a signal required intact TFIID and Mediator.

In view of the above results and given that this study, in contrast to an earlier report (34), utilized relatively weak core promoters (the adenovirus E1b promoter and the synthetic HML promoter that contains the TATA box from the human immunodeficiency virus promoter and the initiator element from adenovirus ML promoter), it is likely that the TAF_{18} dependence observed here has its basis in how the transcription apparatus senses the promoter sequences. Together with the newly uncovered basal transcription effect of the Mediator reported here and by Mittler et al. (31), it appears that the abilities of human TFIID and Mediator complexes, originally thought only to mediate activator signals to the basal apparatus, to directly modulate the basal machinery and to process activation signals are inextricably conjoined. This implies that one mechanism by which these coactivators function is to “present” the basal machinery in a form that is now responsive to activators. For TFIID (including TBP and TAF_{18}) this may take the form of appropriately positioning Pol II and GTFs (i.e., in promoter recognition); for Mediator, this could entail any of the mechanisms discussed above. Interestingly, by this view, and on the basis of the results presented here, Mediator would appear to be functionally indistinguishable from any of the GTFs. However, because it is likely the key component that contacts activators (2, 9, 17, 33, 38) and because the level of activation in the presence of Mediator is significantly higher than what would be expected if it were simply contributing as a basal factor, Mediator is also clearly functioning as a coactivator in the conventional sense. Therefore, the activation process must additionally involve a Mediator-dependent signal transduction event. This notion is also supported by studies indicating that mutations in yeast Mediator subunits may alter function without noticeably affecting interactions of Mediator with activator and Pol II (24). Clearly, future studies on a wide variety of naturally occurring combinations of core promoters and upstream activating sequences, together with detailed mutagenesis of the Mediator components, will be required to further substantiate and elaborate these concepts.

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