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The *Caenorhabditis elegans* Ortholog of TRAP240, *CeTRAP240/let-19*, Selectively Modulates Gene Expression and Is Essential for Embryogenesis*

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Mediator complexes are large multiprotein assemblies that function in the regulation of eukaryotic gene transcription. In yeast, certain mediator subunits appear to comprise a subcomplex that acts in the regulation of a specific subset of genes. We investigated in a metazoan, *Caenorhabditis elegans*, the roles and interactions of two of those subunits, *CeTRAP240/let-19* and *CeTRAP230/dpy-22*. We found that *CeTRAP240/let-19* contains four domains that are conserved in the human TRAP240 protein and that one of those domains displays intrinsic transcriptional repression activity. Using RNA interference, we found that reduced expression of *CeTRAP240/let-19* displayed a high penetrance of embryonic lethality in F1 progeny; animals that escaped embryonic arrest showed mutant phenotypes such as burst vulva and molting defects. *CeTRAP240/let-19* appeared to affect specific genes, as *CeTRAP240/let-19*(RNAi) led to selectively reduced expression of a subset of reporter genes examined. Genetic experiments supported the view that *CeTRAP240/let-19* and *CeTRAP230/dpy-22*, like their *Drosophila* and yeast counterparts, can operate on common pathways. Thus, a male tail phenotype caused by the *pal-1*(*e2091*) mutation was suppressed not only by *CeTRAP230/dpy-22* mutants, as reported previously, but also by reduced expression of *CeTRAP240/let-19*. Additionally, *CeTRAP240/let-19*(RNAi) in a *CeTRAP230/dpy-22* mutant background produced a strong synthetic lethal phenotype. Overall, our results establish specific roles of *CeTRAP240/let-19* in *C. elegans* embryonic development and a functional interaction between *CeTRAP240/let-19* and *CeTRAP230/dpy-22*. Interestingly, whereas this interaction has been conserved from yeast to mammals, the subcomplex modulates metazoan-specific genetic pathways, likely in addition to those also controlled in yeast.

The Mediator complex is a multiprotein assembly that participates in both positive and negative regulation of transcription by RNA polymerase II in eukaryotes; it was first identified in *Saccharomyces cerevisiae* using biochemical and genetic approaches (1–4). The Mediator complex serves as a vehicle for physical and functional communication between DNA-bound regulatory factors and the transcription initiation apparatus; this communication can include enzymatic modifications as well as simple contacts (5–11). Although still a matter of some debate (12, 13), the Mediator complex can be conceptualized as a component of many functional regulatory complexes that assemble at response elements and modulate initiation by RNA polymerase II.

Biochemical approaches have yielded numerous distinguishable mammalian Mediator-related complexes. For example, CRSP was isolated by conventional chromatography based on its ability to potentiate activation by Sp1 (14). Other complexes were identified by their association with various transcriptional regulatory factors, such as thyroid hormone receptor (TRAP), vitamin D3 receptor (DRIP), SREBP-1a (ARC), and E1A (15–19). Still others, such as SMCC (20) and NAT (21), were isolated by affinity purification using antibodies directed against mammalian orthologs of yeast Mediator components. Many of these complexes activate transcription from naked DNA or chromatin templates *in vitro* (14, 18, 22, 23), and SMCC and NAT have been shown to mediate repression *in vitro* (8, 21); ARC has been fractionated into two complexes, one of which, ARC-L, lacks apparent activation activity *in vitro* (24).

Whether the compositional or functional differences among mammalian Mediator-related complexes observed *in vitro* reflect the existence of multiple complexes *in vivo* remains to be determined. However, in yeast, biochemical evidence for certain subcomplexes of Mediator components is underpinned by genetic data, and it appears that some of those subcomplexes mediate the transcriptional regulation of distinct subsets of genes (8, 9). Interestingly, the twenty identified mammalian Mediator subunits are conserved across the eukaryotes (25), and certain inferred mammalian Mediator subcomplexes parallel those previously validated in yeast. For example, TRAP230 (also called ARC240/DRIP240), TRAP240 (also called ARC250/DRIP250), cyclin-dependent kinase 8, and cyclin C are found in ARC-L, but are absent from the smaller CRSP complex (24), reminiscent of the subcomplex comprised of their respective yeast orthologs, Srb8, Srb9, Srb10, Srb11 (26). Similarly, a Mediator complex purified from murine cells lacking TRAP100 is also devoid of TRAP95 and SUR2 (27), suggesting that these three subunits may form a subcomplex; consistent with this view, the Mediator isolated from murine cells lacking SUR2 correspondingly lacks TRAP95 and TRAP100 (28). However, although TRAP95 and SUR2 are conserved from yeast to mammals, TRAP100 appears to be a metazoan-specific Mediator subunit (25).

It is intriguing to speculate that Mediator complexes of differing composition may have acquired new roles during evolution, acting in concert on subsets of genes that accom-

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modate the specialized needs of metazoans while maintaining many of the functions evolved earlier. To examine this possibility, we have undertaken an analysis of Mediator complexes, subcomplexes, and component subunits in *Caenorhabditis elegans*, with its genetic manipulability, defined cellular lineage, and limited tissue complexity. In this report, we focused on the *C. elegans* ortholog of TRAP240 and Srb9, *CeTRAP240/let-19*.[1] We set out to isolate the full-length *CeTRAP240/let-19* cDNA and to characterize its expression pattern. We also sought to assess the *in vivo* functions and potential genetic interactions of *CeTRAP240* with *C. elegans* TRAP230 and Srb8 ortholog, *CeTRAP230/dpy-22*.

EXPERIMENTAL PROCEDURES

Strains—*C. elegans* (N2 wild type and derived mutant strains) was grown at room temperature. The following integrated GFP[2] reporter strains were used: nhr-2::GFP (29), *pha-4*::GFP (30), *hlh-1*::GFP (31), *pes-10*::GFP (32), *med-1*::GFP (33), *end-1*::GFP (34), *elt-5*::GFP (35), and *sur-5*::GFP (36). Mutant alleles *sop-1*(*bx93*) and *pal-1*(*e2091*) was described previously (37).

Isolation of CeTRAP240/let-19 cDNA—The full-length *CeTRAP240/let-19* cDNA was isolated by reverse transcription (RT)-PCR using mix-staged N2 total RNA. RT-PCR was performed according to the manual of the SuperScript one-step RT-PCR for long template kit (Invitrogen). Total RNA was isolated using Tri-reagent (Molecular Research Inc.) according to manufacturer's protocol. The *CeTRAP240/let-19* cDNA was isolated as three fragments. (i) The 5′ end was isolated by RT-PCR using an SL1 primer (GGTTTAATTACCCAAGTTTGAG) and a primer that encompasses a KpnI site in *CeTRAP240* (AAGGTACCAAATGTTCGATGC); this RT-PCR product was amplified again by primers GGGGTACCATGTCCTCCGCCAAGGATAG and AAGGTACCAAATGTTCGATGC. (ii) The middle region was amplified by RT-PCR using primers TTGGTACCTTGGCTCAGAAAG and TTCTGCAGCAAATGTTCGATGCGGCGCC. (iii) The 3′ end was amplified by RT-PCR using primers ACCTGCAGAAACTCCATTTGA and AACTGCAGCTAGGCAAGTAGACGAGCC. The three fragments were digested by KpnI, KpnI/PstI, and PstI, respectively, and then sub-cloned into pBluescript KS (+) plasmid (Stratagene) for sequence analysis.

Identification of CeTRAP240/let-19 Expression Pattern—The *CeTRAP240/let-19* promoter to first exon was amplified by PCR of cosmid K08F8 (provided by the Sanger Center) using primers AATCTAGAATTACGTTGGTTAAAAG and CGCGGATCCGCCAAAATGTATTTGGAGAATTATATT. This genomic fragment was digested by XbaI and BamHI and subcloned in-frame into the pPD95.75 plasmid. This construct (50 ng/μl) was then injected into N2 hermaphrodites along with pRF4 (*rol-6*, 50 ng/μl) as a marker. Multiple extrachromosomal transgenic arrays were generated; multiple animals were examined in each transgenic strain, and all displayed similar GFP expression patterns.

RNA Interference—For *CeTRAP240/let-19*(RNAi), sequences corresponding to nucleotides 4105–5386 of *CeTRAP240/let-19* cDNA were amplified by PCR and subcloned into pBluescript KS (+). This plasmid was digested by PvuII, and single strand RNAs were synthesized using the Ambion MEGA-script T3 and T7 *in vitro* transcription kit. To make dsRNA, equal amounts of sense and antisense strand RNA were mixed well and incubated at 95 °C for 5 min followed by 65 °C for 5 min, 50 °C for 5 min, 37 °C for 5 min, and 25 °C for 5 min. The dsRNA was then injected into N2 hermaphrodites. The injected worms were placed on plates to recover for 18–24 h. After the recovery period the worms were moved to fresh plates for embryo collection. Worms were allowed to lay eggs for 8–12-h intervals and then transferred to a new plate. Embryonic arrest was measured 18–24 h after the end of the egg-laying interval. Larval growth was monitored daily, and larvae that did not progress to the adult stage by day 4 post-hatch were scored as larval arrest. To confirm our *CeTRAP240/let-19*(RNAi) results, an additional dsRNA that corresponds to the C terminus of *CeTRAP240/let-19* cDNA (yk398g7) was also used for RNA interference (RNAi) experiments. Although of lower potency, the latter dsRNA produced results consistent with those reported here.

For *CeTRAP230/dpy-22*(RNAi), the EST clone yk222f3 was digested with either PvuI or PvuII, and single-stranded RNAs were synthesized using the Ambion MEGA-script T3 and T7 *in vitro* transcription kit. Production of dsRNA and RNAi experiments were performed as described above. To confirm the results from *CeTRAP230/dpy-22*(RNAi), dsRNA synthesized from the PvuII fragment of another EST clone, yk294f12, was used and yielded results similar to those using yk222f3 dsRNA.

For bacterial feeding RNAi experiments, cDNA corresponding to the nucleotide sequence 3541–5386 of *CeTRAP240* cDNA was amplified by PCR and subcloned into the KpnI and PstI sites of plasmid pAD12 (provided by Andy Dillon, University of California, San Francisco), which contains two T7 promoters in inverted orientation. This construct was then transformed into an IPTG-inducible *Escherichia coli* strain, HT115, which lacks double-strand-specific RNase III. Single colonies of HT115 containing pAD12 were picked and grown in culture for 18–24 h with 120 μg/ml carbenicillin and tetracycline and seeded on NGM-lite plates containing 1 mM IPTG and 50 μg/ml ampicillin. L1 hermaphrodites were placed on these plates for 2 days; L4 or young adults were then transferred to new plates seeded with the same bacteria. The worms were allowed to lay eggs for 24 h. Embryonic arrest was assessed 24 h later, and larval growth was monitored daily.

Immunostaining—Immunostaining of *C. elegans* embryos using antibody against Ser(P)-2 (H5) of polymerase II C-terminal domain (CTD) was as previously described (38).

Transient Transfection, Luciferase Assay, and Cell Culture—To construct an expression plasmid that encodes the C terminus of CeTRAP240/LET-19, the nucleotide sequences of CeTRAP240/LET-19 corresponding to amino acids 2229–2863 were amplified by PCR using the primer pairs CGGGATCCGTTGGAAACAGCGCGACACTC and ACTCTAGACTAGGCAAGTAGACGAGCC. This PCR fragment was then digested by BamHI and XbaI and subcloned into pSG424 plasmid (39). Construction of GAL4-TLE1 and (GAL4)5E1BLuc reporters (40, 41) and assays for β-galactosidase and luciferase activity (42) have been described previously. A549 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Transfection of A549 lung adenocarcinoma cells employed LipofectAMINE 2000 (Invitrogen) according to the technical manual; cells were harvested 24 h post-transfection.

RESULTS

CeTRAP240 cDNA and Expression Pattern—A Blast search identified a single *C. elegans* ortholog of hTRAP240 protein (*C. elegans* clone K08F8.6). We amplified the full-length *CeTRAP240* cDNA by RT-PCR using total RNA prepared from mixed-stage animals. To confirm our results, we sequenced an EST clone (yk288e10) encoding the 3′ end of *CeTRAP240/let-19*. Amino acid sequence alignment of CeTRAP240 and hTRAP240 revealed four homologous domains, as shown in Fig. 1, *A–E*. Interestingly, our RT-PCR also identified a shorter *CeTRAP240/let-19* cDNA that lacks the fourth and most C-terminal homologous domain. These results suggest that the CeTRAP240 transcription unit may be subject to alternative splicing in a manner that yields two protein isoforms.

To visualize the expression pattern of *CeTRAP240/let-19*, a DNA fragment extending 2.0 kilobases upstream of the translation start site was cloned in-frame into a GFP-encoding plasmid, pPD95.75. This construct was microinjected into N2 worms along with the pRF4 (*rol-6*) plasmid as a transformation marker. Ubiquitous GFP expression was observed in the embryo (Fig. 2*A*, *panel a*). In larvae and adults, GFP expression was observed in most tissues, including vulva (Fig. 2*A*, *panel b*), tail neurons (Fig. 2*A*, *panel c*), skeletal muscle (Fig. 2*A*, *panel c*, arrow), hypodermal cells (Fig. 2*A*, *panel c*, *yellow arrow*), the H-shaped excretory canal (Fig. 2*A*, *panel d*) and pharynx (data not shown).

CeTRAP240/let-19 RNAi Phenotypes—We used RNAi to compare the developmental consequences of down-regulating *CeTRAP240/let-19*; phenotypes were scored in F1 animals. Injection of a *CeTRAP240* double-stranded RNA (dsRNA, 2 μg/μl) that corresponds to the middle region of the *CeTRAP240/*

[1] During the course of this work H. Sawa (RIKEN, Center for Developmental Biology, Kobe, Japan) showed that *CeTRAP240* is identical to the *let-19* gene and *CeTRAP230* and *sop-1* are identical to the previously identified *dpy-22* gene.

[2] The abbreviations used are: GFP, green fluorescence protein; RT, reverse transcription; dsRNA, double-stranded RNA; IPTG, isopropyl-1-thio-β-D-galactopyranoside; CTD, RNA interference; RNAi, RNA interference; GR, glucocorticoid receptor; pol, polymerase.

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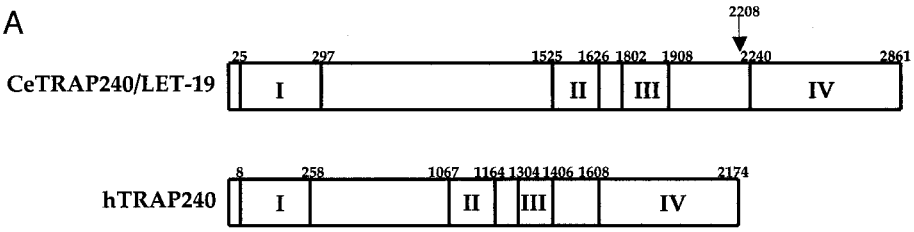

B

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CeTRAP240: 25   NGGSLEDCISNVYALLELPGIKWKCY--RTKPNAPRGVALT-ADFVLKAYSKCLLDGILC 81
               NG SLEDC  N++ L +L GIKWK Y  +   +AP    +T  D +L ++S+CL   +L
hTRAP240:  8    NGASLEDCHCNLFCLADLTGIKWKKYVWQGPTSAPILFPVTEEDPILSSFSRCLKADVLG 67

CeTRAP240: 82   TWRRKPLPPTDNKETPKLTNFSNDAPKELWVFWYDDEPALLQKNCEGLDNDDELSSANQM 141
                WRR   P                  +ELW+FW+ ++P LL      +    +     +M
hTRAP240:  68   VWRRDQRP----------------GRRELWIFWWGEDPVLL--TLFTMTYQKKKMECGRM 109

CeTRAP240: 142  NIVSYEVRTIIFKALHVVLERDLTKDGFVRFGRWFTVPYDARENYLHFTYPSHSPAIRFN 201
                +     V     KA+H +LER L    FVR G+WF  PY+  E  ++    S   +  F
hTRAP240 : 110  DFPMNAV-LCFSKAVHNLLERCLMNRNFVRIGKWFVKPYEKDEKPIN---KSEHLSCSFT 165

CeTRAP240: 202  FFVHGSSTICASVQAQRQPTLITLARRHLECKTKR---VVVVVGPWSMRGQLVQDQIALL 258
                FF+HG S +C SV+  +   +  L+  H+    +      V++ P+ + G L
hTRAP240 : 166  FFLHGDSNVCTSVEINQHQPVYLLSEEHITLAQQSNSPFQVILCPFGLNGTLTGQAF--- 222

CeTRAP240: 259  ADPKIQESAEKEW-NQWKDYLQMEEKEPENVSEERQKSPD 297
                   K+ +SA K+   +WK +  +     E +SEE+Q+  D
hTRAP240 : 223  ---KMSDSATKKLIGEWKQFYPISCCLKE-MSEEKQEDMD 258
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C

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CeTRAP240: 1525  QGESLTIAIVLSDTLLDLHFDSVFDACPICSCSVSIRSRDIGMYITPHTVLYSTERGHTN 1584
                 +  SL + ++LS+++++L  D   D+C IC C+++I+  D+G+YI   T
hTRAP240 : 1067  EAHSLYVNLILSESVMNLFKDCNSDSCCICVCNMNIKGADVGVYIPDPT----------- 1115

CeTRAP240: 1585  MREHTTGPWSGFNVNSATNCTCGFSAIRHRYLSCCSGLFEED 1626
                               +   CTCGFSA+ +R     SGLF ED
hTRAP240 : 1116  -------------QEAQYRCTCGFSAVMNRKFGNNSGLFLED 1144
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D

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CeTRAP240: 1802  LDGPMTWKQIVTKSNRGKPPADDEEDFSLPEPVPAIMRALTKEAIRAAPNIEQYYEQAGL 1861
                 + GP+TW+Q    + RG    D+  +   P P+P  +     + +  +P    Y+E+  L
hTRAP240 : 1304  VQGPLTWQQFHKMAGRGSYGTDESPE---PLPIPTFLLGYDYDYLVLSPFALPYWERLML 1360

CeTRAP240: 1862  GPVDQPKDVMYITVIPDDDDIYSRTVEFMDTLTRTYEKMRLGRHIP 1907
                  P    +D+ Y+ + P+++ + +    F   LT  YE  RLG+H P
hTRAP240 : 1361  EPYGSQRDIAYVVLCPENEALLNGAKSFFRDLTAIYESCRLGQHRP 1406
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FIG. 1. *A*, comparison of CeTRAP240/LET-19 and hTRAP240 proteins. Four regions (I, II, III, and IV) of strong similarity are indicated together with the primary amino acid sequences. The identity/similarity/gap between these two proteins are: region I, 29/46/12; region II, 35/54/23; region III, 27/48/2; region IV, 29/45/20. The short form of CeTRAP240/LET-19 contains 2208 amino acids, indicated by an *arrow*. *B*, the sequence comparison between CeTRAP240/LET-19 and hTRAP240 region I. *C*, the sequence comparison between CeTRAP240/LET-19 and hTRAP240 region II. *D*, the sequence comparison between CeTRAP240/LET-19 and hTRAP240 region III. *E*, the sequence comparison between CeTRAP240/LET-19 and hTRAP240 region IV.

let-19-coding region resulted in a >90% embryonic lethality. Using RT-PCR, we confirmed that accumulation of *CeTRAP240/let-19* transcripts was selectively affected, because levels of *CeRgr1* mRNA, for example, were unchanged (data not shown). Most embryos were arrested at the 200–300 cell stage, and thus, lacked differentiated cells (Fig. 2*B*, *panel a*); a small proportion of the embryos were arrested at later stages, such as the comma, 2- or 3-fold stages. As a control, injection of full-length rat glucocorticoid receptor (GR) dsRNA did not produce significant mutant phenotypes.

When we reduced the concentration of injected *CeTRAP240/let-19* dsRNA 10-fold (0.2 μg/μl), a greater proportion of animals escaped arrest and displayed a range of mutant adult phenotypes, including abnormal body morphology (Fig. 2*B*, *panel b* and *c*), withered tail (Fig. 1*C*, *panel d*), defective cuticle shedding (Fig. 2*B*, *panel d*), multivulva (Fig. 2*B*, *panel e*), and dumpy and burst vulva (data not shown). All of these mutant phenotypes are characteristic of defects in hypodermal development.

Importantly, *CeTRAP240/let-19*(RNAi) caused a more severe phenotype (embryonic *versus* larval lethality) than the *let-19* homozygous allele, which also showed molting defects and multivulva phenotypes (data not shown).[3] Thus, these results suggest that the *CeTRAP240/let-19* homozygous allele is not a null mutant. The *CeTRAP240/let-19* mutant lacks the conserved hTRAP240 C-terminal domain (Fig. 1, *A* and *E*). We fused the C-terminal domain to the GAL4 DNA binding domain to test whether this domain contains intrinsic transcriptional regulatory activity. Interestingly, this GAL4 DNA binding domain fusion protein (GAL4-LET-19/C) inhibited transcription from a

[3] H. Sawa, personal communication.

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E

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CeTRAP240: 2240 PFPSSNQPPVAFEAAGSP-DTDAYS-TLPHVIVVYVVNPFSYGPEGHSALHMRIAILAFI 2297
                P P  ++  +  +  G P D D+++ T P  IVVY+++PF+Y     S     +  L  +
hTRAP240 : 1608 PHPDVSESTMDRDKVGIPTDGDSHAVTYPPAIVVYIIDPFTYENTDESTNSSSVWTLGLL 1667

CeTRAP240: 2298 RAFNSIMCKIPYEKRPQLQLEIVGMEGMDNVAKPIPDYFNDAKIPFDLLNDRPIRVERPG 2357
                R F  ++  +P   +  + ++I                      IP   L  +P++ E
hTRAP240 : 1668 RCFLEMVQTLPPHIKSTVSVQI----------------------IPCQYLL-QPVKHE--D 1703

CeTRAP240: 2358 ESVQGELARSLSIAVYTHPRVFFPDVYKSASARCMTAFGPGSQLMNTINKIEALNKDSFA 2417
                  +  +  +SL+ + +T  R   P    S + + +T FGPG  +
hTRAP240 : 1704 REIYPQHLKSLAFSAFTQCRRPLP---TSTNVKTLTGFGPGLAM---------------- 1744

CeTRAP240: 2418 RMAKRSKTTLDTMDMYRHPGMIQAQQSTEKKNYIAYRVPSNIAVLAPPPMVYQMDEKGKA 2477
                      +T L + D                          P  I + APP ++  + +K
hTRAP240 : 1745 ------ETALRSPDR----------------------PECIRLYAPPFILAPVKDKQTE 1775

CeTRAP240: 2478 IMNQLDE-----QTLFISYCLVGTD-FLVATATDAQGKLIDNCISNI----KPRRQSNQV 2527
                +     E       LF+ YCL     +++A+ TD  G+L++ CI NI    + RR+ +
hTRAP240 : 1776 LGETFGEAGQKYNVLFVGYCLSHDQRWILASCTDLYGELLETCIINIDVPNRARRKKSSA 1835

CeTRAP240: 2528 YRYRNKTQILDGMGKLWSFILGIMASETKNWRLVVGRLGRIGHGEFRAWTHLLNKTSLLR 2587
                 ++        G+ KLW + LG++   +  WR+V+GRLGRIGHGE + W+ LL++ +L
hTRAP240 : 1836 RKF--------GLQKLWEWCLGLVQMSSLPWRVVIGRLGRIGHGELKDWSCLLSRRNLQS 1887

CeTRAP240: 2588 YSGSLKDICGACRSMPSVIGTPAILSACLITLEPEPSIRIMPE--------FHDQELSTKK 2640
                 S  LKD+C  C    S   +P+ILSACL+ +EP+ S  IMP+       F        +
hTRAP240 : 1888 LSKRLKDMCRMCGI--SAADSPSILSACLVAMEPQGSFVIMPDSVSTGSVFGRSTTLNMQ 1945

CeTRAP240: 2641 SFLFQTPGDLSCTHILTFPVGTEI----------NLEVQDQTADTKADENWEFGDLDIME 2690
                +    TP D SCTHIL FP    +          NL++     +  AD    F  LD  +
hTRAP240 : 1946 TSQLNTPQDTSCTHILVFPTSASVQVASATYTTENLDLAFNPNNDGADGMGIFDLLDTGD 2005

CeTRAP240: 2691 GLDDGDTEIMKDLGLETP--SSAAIRQTGGPS--------MFFSEDSSSI-EIQNQPLAS 2739
                 LD     I+      +P  S  +    GG +        +  +E    +  I  QPLA
hTRAP240 : 2006 DLDPDIINILPASPTGSPVHSPGSHYPHGGDAGKGQSTDRLLSTEPHEEVPNILQQPLAL 2065

CeTRAP240 : 2740 GYYISTAPAPELPAWFWATCPSAKRHSPVHLKSSLHIHISEVKNDDIAMESTXXXXXXX 2799
                 GY++STA A  LP WFW+ CP A+   P+ LK+SLH+H+  V++D++
hTRAP240 : 2066 GYFVSTAKAGPLPDWFWSACPQAQYQCPLFLKASLHLHVPSVQSDEL-------------  2112

CeTRAP240: 2800 XXXXXAHPLESRQTEEVLRHVLESYNALSWLNLNRQTGDRYSCLPIHIQHLLRLYHSVARLL 2861
                      +HPL+S QT +VLR VLE YNALSWL  +  T DR SCLPIH   L +LY+ +  +L
hTRAP240 : 2113 LHSKHSHPLDSNQTSDVLRFVLEQYNALSWLTCDPATQDRRSCLPIHFVVLNQLYNFIMNML 2174
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FIG. 1—*continued*

(GAL4)5E1BLuc reporter in A549 human lung adenocarcinoma cells (Fig. 2*C*); the repression activity of GAL4-LET-19/C was as potent as a fusion of GAL4 to TLE1, a human ortholog of the *Drosophila* Groucho corepressor (Fig. 2*C*). How the repression activity of the CeTRAP240/LET-19 C-terminal domain relates to the phenotypes of *CeTRAP240/let-19* has not yet been explored.

Effects of Mediator Components on Expression from Reporter Gene Promoters—To investigate whether the requirement of *CeTRAP240/let-19* in embryogenesis reflects a role in general embryonic gene transcription, we first analyzed phosphorylation of the RNA polymerase II (pol II) large subunit CTD, which consists of heptapeptide repeats, YSPTSPS; full polymerase activity requires phosphorylation of Ser-2 and Ser-5 within these repeats. As shown in Fig. 2*A*, depletion of the pol II large subunit (AMA-1) by RNAi leads to a dramatic reduction in detection of transcriptionally active pol II by an antibody specific for phosphorylated Ser-2 (38). In contrast, although *CeTRAP240/let-19*(RNAi) caused an embryonic lethal phenotype, like *ama-1*(RNAi), reduced expression of *CeTRAP240/let-19* did not influence the phosphorylation status of CTD Ser-2 in embryos (Fig. 3*A*); similarly, *CeTRAP230/dpy-22*(RNAi), which resulted in a 20–30% of embryonic lethal phenotype in F1 progeny, did not influence CTD Ser-2 phosphorylation (Fig. 3*A*). These results suggest that *CeTRAP240/let-19* and *CeTRAP230/dpy-22* act selectively in gene expression rather than being required for general transcription.

To examine whether *CeTRAP240/let-19* is only required for the expression of specific genes, we injected *CeTRAP240/let-19* dsRNA into *C. elegans* strains bearing a series of integrated reporter::GFP fusion genes. We first tested the requirement of *CeTRAP240/let-19* on *pes-10*::GFP, which is expressed at the onset of embryonic transcription (Fig. 3*B*, *panel 1*) and ubiquitously expressed *sur-5*::GFP (Fig. 3*B*, *panel 2*). Interestingly, reduction of *CeTRAP240/let-19* activity by RNAi did not influence the expression of these two reporters. We next tested the effect of *CeTRAP240/let-19*(RNAi) on reporter constructs containing the promoters from genes that are expressed in certain cell types or developmental stages. Reduction of *CeTRAP240/let-19* diminished *hlh-1*::GFP (a muscle cell marker, Fig. 3*B*, *panel 3*), *end-1*::GFP (an endodermal marker, Fig. 3*B*, *panel 4*), and *elt-5*::GFP expression (an epidermis/seam cell marker, Fig. 3*B*, *panel 5*) but not *nhr-2*::GFP (an early developmental maker that begins to express at 16-cell stage but disappears during the initial stages of morphogenesis; Fig. 3*B*, *panel 6*), *pha-4*::GFP (a digestive tract maker, Fig. 3*B*, *panel 7*), and *med-1*::GFP (a mesendodermal marker; Fig. 3*B*, *panel 8*). Although we have not determined if these genes are direct targets of *CeTRAP240/let-19* activity, our results suggest that *CeTRAP240/let-19* is responsible for the expression of a specific subset of genes rather than for general embryonic gene expression. Together, our results suggest that *CeTRAP240/let-19* is selectively involved in multiple developmental pathways.

Suppression of the pal-1(2091) Phenotype by Inhibition of Mediator Expression—*CeTRAP240/let-19* is the *C. elegans* ortholog of Srb9, which in yeast forms a subcomplex with

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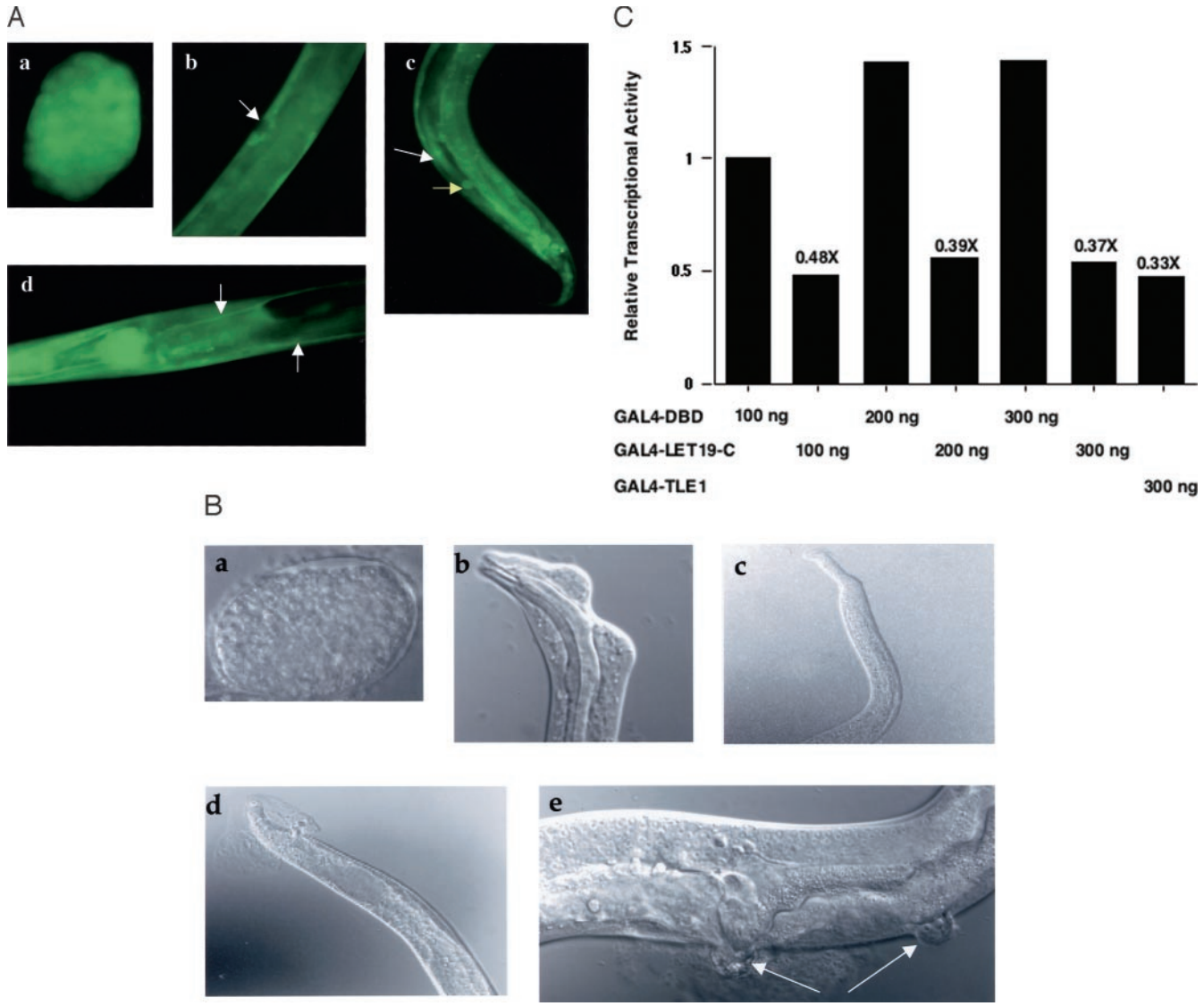

FIG. 2. *A*, expression patterns of *CeTRAP240/let-19*. A reporter construct containing a 2.0-kilobase fragment encompassing the *CeTRAP240/let-19* promoter and regulatory region-driving expression of GFP was injected into N2 hermaphrodites. Expression was monitored in different stages, tissues and cells: *a*, 200-cell stage embryo; *b*, vulva (*arrow*); *c*, neurons in tail, hypodermal cell (*arrow*), and skeletal muscle (*yellow arrow*); *d*, H-shaped excretory canal (*arrow*). *B*, *CeTRAP240/let-19*(RNAi) phenotypes. Depending on dosage of dsRNA, disruption of *CeTRAP240/let-19* function by RNAi resulted in substantial embryonic lethality; survivors developed several phenotypes: *a*, embryonic arrest at 200–300 cell stage; *b*, abnormal head morphology; *c*, abnormal tail morphology; *d*, withered tail and cuticle shedding defect; *e*, multivulva. *C*, the C terminus of CeTRAP240/LET-19 is an intrinsic transcriptional repressor. A549 cells were transfected with various amounts of expression plasmids, as indicated, that encode either GAL4 DNA binding domain, GAL4-LET-19/C, or GAL4-TLE1 together with a (GAL4)5E1BLuc reporter (150 ng). The RSV-LacZ plasmid was also transfected to normalize transfection efficiency. Results shown here are representative of three independent experiments.

Srb8, -10, and -11 (26). Thus, we sought to determine whether *CeTRAP240/let-19* interacts during *C. elegans* development with other components of its orthologous subcomplex. Previous work had been done on the *C. elegans* ortholog of Srb8, *CeTRAP230/dpy-22*; several mutant alleles of that gene, such as *sop-1*, suppress a mutant male tail phenotype of *pal-1*(*e2091*) (37). Animals carrying the *pal-1*(*e2091*) mutation fail to develop V6 rays in the adult male tail (Ref. 37, Fig. 4*A*). We first confirmed that *CeTRAP230/dpy-22*(RNAi) in the *pal-1*(*e2091*) strain results in 80–90% rescue of the male tail phenotype (Ref. 37, Fig. 4*D*). We then tested the effect of *CeTRAP240/let-19*(RNAi) on the mutant male tail phenotype of *pal-1*(*e2091*). Indeed, *CeTRAP240/let-19*(RNAi) yielded 57% of F1 male *pal-1*(*e2091*) worms with fully (Fig. 4*B*) or partially (Fig. 4*C*) recovered tails (Fig. 4*D*). As a control, GR dsRNA injected into *pal-1*(*e2091*) produced little or no suppression of the male tail phenotype, similar to that seen in uninjected *pal-1*(*e2091*) mutants (Fig. 4*D*). Thus, both *CeTRAP240/let-19* and *CeTRAP230/dpy-22* are likely involved in *pal-1* gene expression.

Notably, *sop-1* mutants alone do not exhibit significant phenotypes (37), whereas the *dpy-22* mutant is sick and displays some larval lethality (43). Injection of a low concentration (0.2 μg/μl) of *CeTRAP230/dpy-22* dsRNA into N2 worms did not result in any mutant phenotype, whereas a higher concentration (2 μg/μl) produced ~15% embryonic and larval arrest and mutant adult phenotypes in about half of the remainder. The predominant mutant phenotype from *CeTRAP230/dpy-22*(RNAi) was protruding/burst vulva; some animals also displayed dumpy, abnormal body morphology and molting defects (data not shown). All of these mutant phenotypes are characteristic of defects in hypodermal development and

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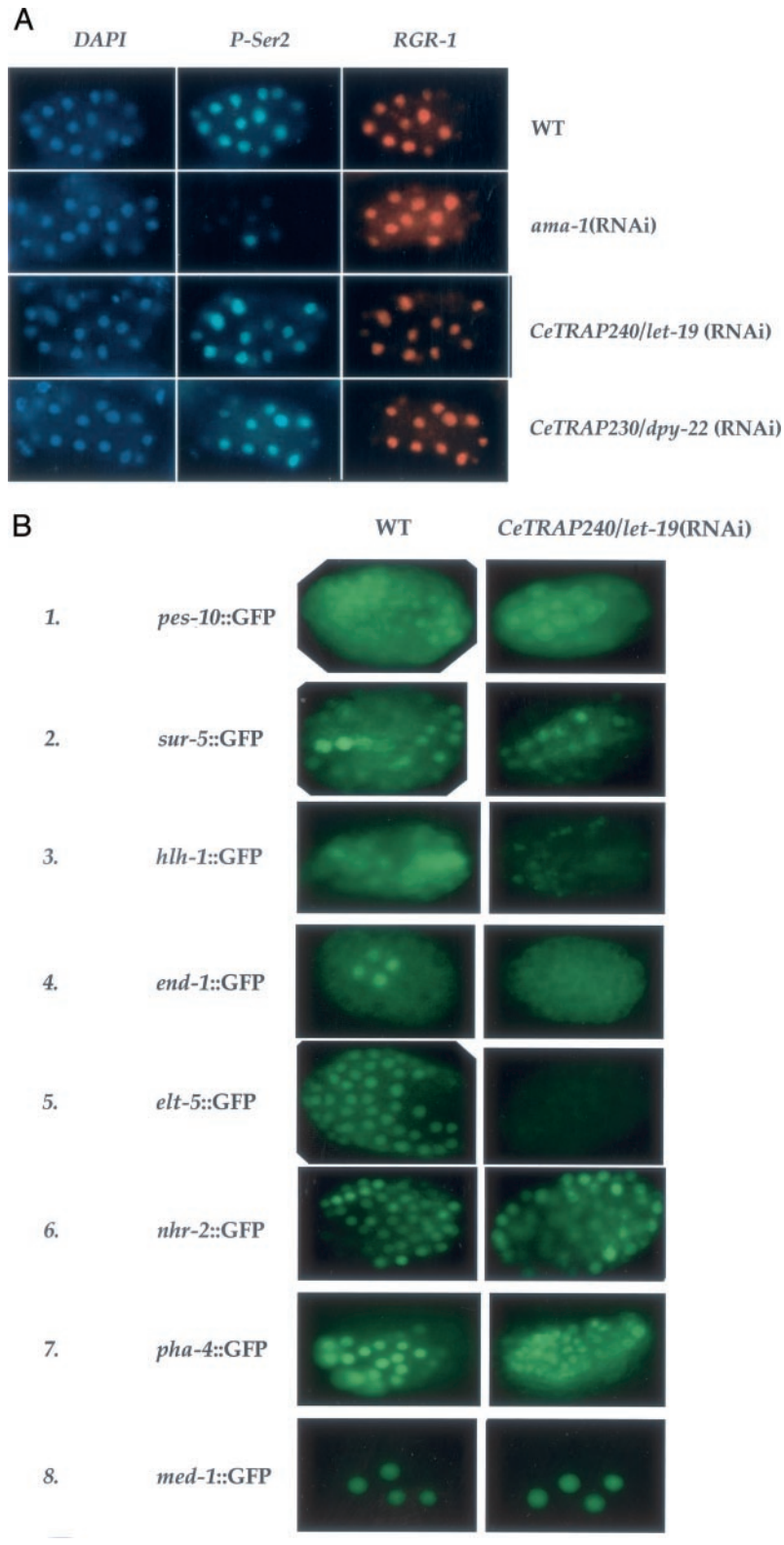

FIG. 3. *A*, effects of *CeTRAP240/let-19*(RNAi) on phosphorylation state of Ser-2 of pol II CTD. Representative wild type (*WT*) or RNAi embryos that have not reached terminal developmental arrest are shown. These embryos were stained with H5 antibody, which specifically recognizes Ser(P)-2 (*P-Ser-2*) of the pol II CTD, 4′,6′-diamidino-2-phenylindole hydrochloride (*DAPI*) to visualize DNA and with RGR-1 antibody as a control. The *yellow arrow* indicates a mitotic cell, which is known to cross-react with the H5 antibody in a manner not associated with transcription. *B*, effects of *CeTRAP240/let-19*(RNAi) on reporter::GFP constructs. Wild type and *CeTRAP240/let-19*(RNAi) embryos were visualized by differential interference microscopy or fluorescence microscopy. For *pha-4*::GFP, *hlh-1*::GFP, and *sur-5*::GFP strains, the expression of GFP was verified at 200–300-cell stage embryos. For *nhr-2*::GFP, *med-1*::GFP, *end-1*::GFP, and *pes-10*::GFP, the expression of GFP was verified at 100-cell stage embryos.

overlap with those observed in *CeTRAP240*(RNAi) animals, suggesting that both of these Mediator subunits function in that pathway.

Genetic Interaction between CeTRAP230/dpy-22 and CeTRAP240/let-19—To further investigate the potential relationship between *CeTRAP230/dpy-22* and *CeTRAP240/let-19* functions, we tested for a synthetic lethal relationship between these two genes. We used the bacterial feeding RNAi method for these experiments, in which *CeTRAP240/let-19* dsRNA expression was dependent upon IPTG (44, 45). Approximately 20% of wild type N2 worms grown in bacteria producing *CeTRAP240/let-19* dsRNA displayed embryonic and larval arrest (Table I). In the absence of *CeTRAP240/let-19* dsRNA (no IPTG control), the rate of embryonic or larval arrest was 4% (Table I). Thus, the effect of bacterial feeding RNAi is significant, but it provides a weaker effect than the direct injection of *CeTRAP240/let-19* dsRNA into worms. We then tested the effect of *CeTRAP240/let-19*(RNAi) on *sop*-1(*bx*93) mutants. When

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sop-1(*bx*93) was fed with uninduced *CeTRAP240/let-19* dsRNA bacteria, ~32% embryonic and larval arrests were observed (Table I). Upon IPTG induction, more than 78% of F1 *sop*-1(*bx*93) progeny were arrested at the embryonic or larval stages or developed mutant phenotypes (Table I). Thus, *CeTRAP240/let-19*(RNAi) and a *CeTRAP230/dpy-22* mutant display substantial synthetic lethality.

DISCUSSION

In this study, we demonstrated important roles for *CeTRAP240/let-19* in *C. elegans* embryonic development and showed that it is likely involved in regulating the expression of a specific subset of genes. The reduced expression of *CeTRAP240/let-19* by RNAi is unlikely to disrupt the integrity of the Mediator complex because *in vitro* studies of mammalian and yeast mediator complexes have demonstrated the presence of complexes lacking cyclin-dependent kinase 8-cyline c-TRAP230-TRAP240. However, we have not excluded the possibility that decreased expression of *CeTRAP240/let-19* may influence the association of cyclin-dependent kinase 8, cyclin C, and CeTRAP230/DPY-22 within the Mediator complex. Two other *C. elegans* Mediator components, *CeMed6* and *CeMed7*, are similarly required for the expression of certain developmental but not ubiquitously expressed genes (46), whereas *CeRgr1* functions more globally in gene transcription (38). Thus, it appears that two broad classes of components may reside in Mediator complexes, one represented by *CeRgr1*, which affects the expression or regulation of all or most RNA polymerase II genes, and a second, represented by *CeTRAP240/let-19*, *CeTRAP230/dpy-22*, *CeMed6*, *CeMed7*, and *sur-2*, which acts more selectively in gene regulation.

This concept is consistent with genetic data from yeast and mammalian Mediator complexes. In yeast, various Mediator subunits seem to function selectively on distinct subsets of genes, whereas Srb4 and Srb6 appear to participate in the expression of almost all genes (9). In mammalian cells TRAP100 is broadly required (27), whereas TRAP220 and SUR-2 play specific roles in transcriptional regulation. Primary fibroblast cells cultures derived from TRAP220 knock-out mouse embryos show an impaired thyroid hormone response and are defective in peroxisome proliferator-activated receptor γ2-induced adipogenesis but undergo MyoD-stimulated myogenesis normally, and GAL4-RAR/RXR, p53, and VP16 regulation are unaffected (22, 47). Similarly, murine SUR-2 is selectively involved in mediating activation by Elk1 but does not affect many other regulatory proteins such as VP16, ATF-2, the ligand binding domain of GR, p53, or nuclear factor B (p65) (28).

How can we rationalize and begin to understand this apparent complexity of Mediator composition and function? What is apparent is that the Mediator evolved as a device of eukaryotes to accommodate a substantially increased demand for complex networks of transcriptional regulation. This requirement can be seen even in simple eukaryotes, such as yeast, but is most stringent in metazoans, which produce terminally differentiated cells with clearly distinct expression patterns, and must integrate myriad cell signals to produce physiologically rational responses at the level of the whole organism. Strong evolutionary conservation of Mediator components suggest that *C. elegans*, with its powerful genetics, fully sequenced genome, simple anatomical system, and known cell lineage offers multiple approaches to analyzing mechanisms of differential transcriptional regulation that will likely be relevant to all metazoans.

It is well established that the yeast Mediator complex is composed of several subcomplexes. Genetic disruption of the components in distinct subcomplexes leads to common or overlapping phenotypes (5, 9). In metazoans, subcomplexes are implied but not proven. The yeast ortholog of *CeTRAP240/let-19*, Srb9, is part of a module that also contains Srb8, Srb10, and Srb11 (26). Interestingly, recent genetic studies in *Drosophila* reveal that dTRAP240 and dTRAP230, orthologs of yeast Srb9 and Srb8 respectively, act in concert in eye-antennal disc development (48). Our results here suggest that *CeTRAP240/let-19* and *CeTRAP230/dpy-22* likely act together in some biological pathways. Thus, both *CeTRAP240/let-19*(RNAi) and *CeTRAP230/dpy-22*(RNAi) suppressed the *pal-1*(*e2091*) male tail phenotype (Fig. 3). Notably, these results also suggest the functions of *CeTRAP240/let-19* and *CeTRAP230/dpy-22* are not redundant because we can reduce the expression of either one to suppress the *pal-1*(*e2091*) phenotype. Furthermore, we demonstrated a genetic interaction between *CeTRAP240/let-19* and *CeTRAP230/dpy-22* (Table I). Finally, low levels of *CeTRAP240/let-19*(RNAi) yielded defects similar to those seen with *CeTRAP230/dpy-22*(RNAi) (Fig. 1*C* and data not shown). Although we cannot discern whether the effects of *CeTRAP240/let-19*(RNAi) and *CeTRAP230/dpy-22*(RNAi) reflect qualitative or only quantitative differences, the simplest interpretation of current results is that these two Mediator components can act together on subsets of genes. Thus, the functional interaction between these two subunits in the Mediator complex appears to be conserved during evolution. In contrast, a mutation in *sur-2*, an ortholog of yeast Mediator subunit Gal11, failed to suppress

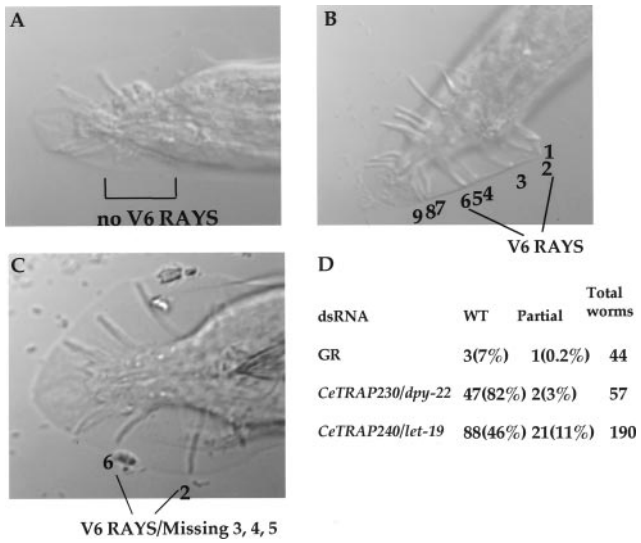

FIG. 4. **Effects of *CeTRAP240/let-19*(RNAi) on suppression of mutant male tail phenotype of *pal-1*(*e2091*).** *A*, the *pal-1*(*e2091*) male tail lacks V6 rays. *B*, the wild type (*WT*) male tail phenotype containing all 5 rays (2–6). *C*, the partial male tail phenotype showing 2 or 3 V6 rays. *D*, ratios of suppression of *pal-1*(*e2091*) by *CeTRAP230/dpy-22*(RNAi), and *CeTRAP240/let-19*(RNAi).

TABLE I
Synthetic lethality between CeTRAP230/dpy-22 and CeTRAP240

		Normal progeny	Dead eggs	Larval arrest	Mutants
N2	No IPTG	27 (96%)	12 (4%)	3	
	Add IPTG	557 (77%)	110 (15%)	33 (5%)	26 (3%)
sop-1 (*bx*93)	No IPTG	182 (67%)	80 (30%)	5 (2%)	4 (1%)
	Add IPTG	164 (22%)	450 (60%)	79 (11%)	55 (7%)

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the *pal-1*(*e2091*) male tail phenotype (49), consistent with the finding that Gal11 interacts neither genetically nor biochemically with Srb8 and Srb9 (5, 9).

It is intriguing that mutations of the yeast Srb8-Srb9-Srb 10-Srb11 subcomplex lead to increased transcription, implying that this subcomplex is inhibitory (5, 50). Furthermore, some human mediator complexes that include cyclin-dependent kinase 8-cyclin C·hTRAP230·hTRAP240 fail to activate transcription *in vitro* and inhibit an activation-competent mediator complex (24). Consistent with this view, we found that the C terminus of CeTRAP240/LET-19 protein confers transcriptional repression. Notably, this domain is conserved between CeTRAP240/LET-19 and hTRAP240, implying that the C-terminal domain of hTRAP240 may also repress transcription; we are currently testing this possibility. Interestingly, human cyclin-dependent kinase 8 can phosphorylate general transcription factor TFIIH and inhibit the transcriptional initiation (51). Thus, if the function of CeTRAP240/LET-19 is conserved during evolution, the metazoan cyclin-dependent kinase 8 subcomplex may inhibit transcription by two independent mechanisms.

In principle, discrete Mediator subcomplexes could explain the differential effects of specific subunits on the regulation of particular genes despite the function of the Mediator as a unit in *in vitro* assays. One model is that different combinations of subcomplexes are recruited to different response elements through protein-protein interactions with regulatory factors. This could account for the multiple forms of Mediator complex that have been described *in vitro* and is supported by findings that regulatory factors interact selectively with specific Mediator components (16). However, there is no direct support for the existence of multiple Mediator complexes *in vivo*, *i.e.* the multiple forms observed *in vitro* might be artifacts of biochemical purification. It should be possible in future studies to determine by chromatin immunoprecipitation the presence or absence of Mediator subunits *in vivo* at specific genes at which they are either required or functionally dispensable.

Subcomplexes appear to be neither functionally dedicated to general expression or specialized genes nor evolutionarily "sequestered" with respect to their subunit composition. Thus, TRAP100 plays a general role in gene regulation and is metazoan-specific, whereas SUR2, which resides in the same subcomplex, acts on a specific subset of genes and is conserved from yeast to mammals (27). This suggests that functions of the Mediator complex cannot be attributed entirely to the differential recruitment of different subcomplexes. Instead, different regulators may confer distinct conformations to one or more Mediator subunits (24), perhaps affecting their functions within the complex or creating novel functional surfaces. According to this model, a given subcomplex might reside in two different regulatory complexes and participate in different regulatory mechanisms. This would be analogous to the behavior of GR and GRIP1, two regulatory factors that can reside at different response elements and use different surfaces to either activate or repress transcription (52).

In this study we have identified several apparent target genes and tissues for *CeTRAP240/let-19* action. It should now be possible to determine whether *CeTRAP230/dpy-22* acts in these same settings and whether the functional surfaces are similar or distinct in the different contexts. By carrying out this investigation in *C. elegans*, we can examine these alternatives throughout the course of development and in all tissues of this metazoan organism.

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The *Caenorhabditis elegans* Ortholog of TRAP240, *CeTRAP240/let-19*, Selectively Modulates Gene Expression and Is Essential for Embryogenesis

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