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Caenorhabditis elegans lin-25: A Study of Its Role in Multiple Cell Fate Specification Events Involving Ras and the Identification and Characterization of Evolutionarily Conserved Domains

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ABSTRACT

Caenorhabditis elegans lin-25 functions downstream of *let-60 ras* in the genetic pathway for the induction of the 1° cell fate during vulval development and encodes a novel 130-kD protein. The biochemical activity of LIN-25 is presently unknown, but the protein appears to function together with SUR-2, whose human homologue binds to Mediator, a protein complex required for transcriptional regulation. We describe here experiments that indicate that, besides its role in vulval development, *lin-25* also participates in the fate specification of a number of other cells in the worm that are known to require Ras-mediated signaling. We also describe the cloning of a *lin-25* orthologue from *C. briggsae*. Sequence comparisons suggest that the gene is evolving relatively rapidly. By characterizing the molecular lesions associated with 10 *lin-25* mutant alleles and by assaying *in vivo* the activity of mutants *lin-25* generated *in vitro*, we have identified three domains within LIN-25 that are required for activity or stability. We have also identified a sequence that is required for efficient nuclear translocation. We discuss how *lin-25* might act in cell fate specification in *C. elegans* within the context of models for *lin-25* function in cell identity and cell signaling.

MANY cell-cell interaction events in a wide variety of organisms are mediated by Ras/MAP kinase signal transduction pathways. Much progress in the understanding of Ras-mediated signaling has come from genetic analyses, in particular, from studies with *Caenorhabditis elegans* and *Drosophila melanogaster* (Sternberg and Han 1998). In *C. elegans*, a signal transduction pathway involving Ras and MAP kinase is required for a number of different signaling events that occur during development. Several of these are required for correct cell fate specification. In the hermaphrodite, these include the induction of the primary fate during vulval development (Beitel *et al.* 1990; Han and Sternberg 1990), the induction of the excretory duct cell fate in the embryo (Yochem *et al.* 1997), the induction of the uv1 cell fate during development of the uterus, and induction of the P12 cell fate in the L1 larva (Jiang and Sternberg 1998; Chang *et al.* 1999). Ras-mediated signaling is also required in the hermaphrodite for the proper migration of the sex myoblasts (Sundaram *et al.* 1996), for proper body morphology, and for germ cells to exit the pachytene stage of meiosis and mature into oocytes (Church *et al.* 1995). In the *C. elegans* male, Ras-mediated signaling is required for inductive signaling both in the preanal ganglion equivalence group and during development of the male tail (Chamberlin and Sternberg 1994; P. Sternberg, personal communication).

The Ras-mediated signaling event that has been most extensively studied in *C. elegans* is that between the anchor cell (AC) and a group of ventral hypodermal (epidermal) cells that leads one of them, P6.p, to adopt the 1° fate (Sundaram and Han 1996; Kornfeld 1997; Sternberg and Han 1998). In the *C. elegans* hermaphrodite, P6.p is one of a group of six cells, P3.p–P8.p, that form the vulval equivalence group (Sulston and Horvitz 1977). In wild-type worms, P6.p adopts the 1° fate; P5.p and P7.p adopt the 2° fate; and P3.p, P4.p, and P8.p adopt the 3° fate. The cells generated by P6.p, P5.p, and P7.p give rise to the vulva whereas those generated by P3.p, P4.p, and P8.p join the hypodermal syncytium, hyp7. Collectively, P3.p–P8.p are referred to as the vulva precursor cells (VPCs). The signal sent by the AC to induce the 1° fate is LIN-3, a protein similar in sequence to mammalian epidermal growth factor (EGF; Hill and Sternberg 1992). LIN-3 functions by activating LET-23, a putative receptor type tyrosine kinase of the EGF receptor subfamily (Aroian *et al.* 1990). In turn LET-23, through its interaction with SEM-5, a protein containing src homology domains (Clark *et al.* 1992), activates a pathway involving a Ras protein, LET-60 (Beitel *et al.* 1990; Han and Sternberg 1990), LIN-45 Raf (Han *et al.* 1993), the MEK-2 mitogen-activating protein (MAP) kinase kinase (Kornfeld *et al.* 1995; Wu *et al.* 1995), and the SUR-1/MPK-1 MAP kinase (Lackner *et al.* 1994; Wu and Han 1994). Dominant hypermorphic mutations in genes in this pathway cause

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a Multivulva phenotype: the cells that normally adopt a nonvulval (3°) fate, P3.p, P4.p, and P8.p, are induced instead to adopt a vulval (1° or 2°) fate. MPK-1 functions in part by regulating the activities of the proteins LIN-1 and LIN-31 within P6.p. *lin-1* and *lin-31* have been shown to function downstream of *mpk-1* in the genetic pathway for induction of the 1° fate (Miller *et al.* 1993; Beitel *et al.* 1995), and LIN-1 and LIN-31 proteins are both biochemical subtrates for MPK-1 MAP kinase *in vitro* (Jacobs *et al.* 1998; Tan *et al.* 1998). *lin-1* encodes a putative transcription factor in the ETS family (Beitel *et al.* 1995), and *lin-31* encodes a putative winged helix transcription factor similar to mammalian HNF3 and *D. melanogaster* Forkhead (Miller *et al.* 1993). LIN-1 and LIN-31 have been shown to form a heterodimer that may act as an inhibitor of the 1° fate in the absence of signaling by the AC (Tan *et al.* 1998). It is thought that in response to LIN-3, MPK-1 MAP kinase phosphorylates LIN-31 and thereby disrupts the complex; phosphorylated LIN-31 appears to function as an activator that promotes the 1° cell fate.

Two other genes that lie downstream of *let-60 ras* and *mpk-1* MAP kinase in the genetic pathway for induction of the primary fate are *lin-25* and *sur-2* (Singh and Han 1995; Tuck and Greenwald 1995; Lackner and Kim 1998). Null mutations in either of these genes reduce the efficiency with which induction occurs and suppress the Multivulva phenotype caused by mutations that constitutively activate Ras or MPK-1 (Singh and Han 1995; Tuck and Greenwald 1995). *lin-25* encodes a 130-kD protein whose predicted sequence is not significantly similar to others in the public databases. *sur-2* is predicted to encode a protein of 180 kD of unknown biochemical activity. During vulval development, LIN-25 is expressed first in all six cells in the vulval equivalence group, P3.p–P8.p (Nilsson *et al.* 1998). The pattern of expression, therefore, is consistent with a role for LIN-25 in allowing cells to respond to LIN-3. The expression of LIN-25 is not affected by mutations in the *let-60 ras* or *mpk-1* MAP kinase but is dependent upon *sur-2* activity. Hermaphrodites homozygous for *sur-2(ku9)*, a mutation that is thought to reduce or eliminate *sur-2* activity (Singh and Han 1995), contain approximately 10-fold lower levels of LIN-25 than wild-type hermaphrodites (Nilsson *et al.* 1998). Genetic and molecular studies strongly suggest that LIN-25 and SUR-2 are mutually dependent upon one another for their respective activities and that they function in the same cellular process (Nilsson *et al.* 1998). A protein that is significantly similar in sequence to the predicted *C. elegans* SUR-2 protein has recently been identified in human cells by virtue of its ability to bind to the transcriptional activator E1A (Boyer *et al.* 1999). Human SUR-2 was also shown to associate with a large multiprotein complex termed Mediator, which associates with the C-terminal domain (CTD) of RNA polymerase II (Boyer *et al.* 1999). Mediator is thought to mediate signaling between proteins that regulate transcription and the general transcription machinery (Bjorklund *et al.* 1999). The biochemical activity of LIN-25, however, is as yet unknown: prior to this study no *lin-25* homologues had been identified in other species.

From previous genetic experiments, two different models have been proposed for how *lin-25* might function during VPC fate specification. First, *lin-25* might be involved in specifying the proper identity of the VPCs such that they are competent to respond to extracellular signals (Sternberg 1993; Tuck and Greenwald 1995; Greenwald 1997); second, LIN-25 might be part of the machinery that allows cells to respond to signals generated by Ras and MAP kinase (Tuck and Greenwald 1995; Nilsson *et al.* 1998). The Multivulva phenotype caused by dominant mutations in *let-60 ras* is suppressed not only by mutations in genes encoding the kinases acting downstream of Ras (and by mutations in *lin-25)* but also by reduction-of-function mutations in *lin-39*, which encodes a homeobox protein (Clandinin *et al.* 1997; Maloof and Kenyon 1998). *lin-39* is needed at several steps during development of the vulva. One function is to allow the VPCs to be generated (Clark *et al.* 1993; Wang *et al.* 1993). A second is to allow the 1° and 2° fates to be executed (Clark *et al.* 1993; Maloof and Kenyon 1998). LIN-39 protein is also expressed in the VPCs themselves, and, by analogy with the activity of other homeotic proteins, LIN-39 might help to specify the identities of the VPCs (Clandinin *et al.* 1997). *lin-39* might, for example, be required for the correct expression of components of the signaling pathway in the VPCs such as LET-60 itself. Based on the results of previous studies, therefore, one plausible explanation for the *lin-25* mutant phenotype might be that LIN-25 acts as a cofactor for LIN-39 in the VPCs to allow the expression of proteins activated by the pathway. Alternatively, LIN-25 might play a more intimate role in the signaling process, functioning either as a target of the pathway itself or as part of a complex of proteins regulated by MPK-1.

We show here that, besides its role in vulval development, *lin-25* is also involved in the specification of the fates of other cells known to require Ras-mediated signaling. In particular we show that *lin-25* is required for the efficient induction of the excretory duct and P12 cell fates. In the male, *lin-25* also functions in the specification of induced fates in the preanal ganglion equivalence group and for the specification of anterior fates in the tail region. Our results suggest that *lin-25* participates in a number of Ras-mediated signaling events in *C. elegans*. To help elucidate the biochemical activity of LIN-25 we have sought to identify regions within the protein that are important for activity. We have identified four evolutionarily conserved domains by cloning *lin-25* from a related nematode, *C. briggsae*, and shown that these are required *in vivo*. We have also identified

a sequence that is required for efficient translocation of the protein into the nucleus.

MATERIALS AND METHODS

Nematode strains: *C. elegans* and *C. briggsae* nematode strains were cultivated using standard methods developed initially for *C. elegans* (Brenner 1974). Experiments were carried out at 20° unless otherwise noted. *C. elegans* var Bristol N2 is the wild-type parent for all *C. elegans* strains used in this study. The *C. briggsae* strain used (AF16) was kindly provided by the Caenorhabditis Genetics Center and is derived from an isolate from Ahmedabad, India (Fodor *et al.* 1983). The *lin-25* mutations used in this study were as follows: *e1446*, *n545*ts (Ferguson and Horvitz 1985), *ar90*, *ga67*, *ku70*, *ku77*, *ku78*, *n1063*, *n1722*ts (Tuck and Greenwald 1995), and *ga67sv17*.

Other strains used were *LG I*, *let-23(n1045)* (Ferguson and Horvitz 1985); *clr-1(e1745)* (Clark and Baillie 1992); *sur-2(ku9)* (Singh and Han 1995); *LG III*, *lin-39(n1760)* (Clark *et al.* 1993); *LG IV*, *dpy-20(e1362)* (Clark *et al.* 1995); *him-8(e1489)* (Hodgkin *et al.* 1979); *let-60(s1124)* (Clark *et al.* 1988); *let-60(s1155)* (Clark *et al.* 1988); *let-60(n1046)* (Ferguson and Horvitz 1985); *let-60(n2021)* (Beitel *et al.* 1990); *unc-24(e138)* (Riddle and Brenner 1978); *LG V*, *lon-3(e2175)* (Brenner 1974); *sma-1(e30)* (Brenner 1974); *vab-8(e1017)* (Hedgecock *et al.* 1987); *LG X*, *egl-15(n484)* (Stern and Horvitz 1991); *lin-15(n309)* (Ferguson and Horvitz 1985).

Phenotypic analysis of *lin-25* double and single mutants: *Growth arrest phenotype:* To examine the effect of *lin-25* mutations on the growth arrest phenotype caused by *let-60*, we constructed hermaphrodites of the genotype *unc-24(e138) let-60(n2021)/dpy-20(e1362); lon-3(e2175) lin-25(ar90)/sma-1(e30) vab-8(e1017)*. Maternally rescued Lon-3 Unc-24 individuals segregating from this strain gave rise to rod-shaped dead larvae that had a fluid-filled morphology. To examine the mutant larvae more closely, gravid Lon-3 Unc-24 hermaphrodites segregating from the parental strain were cut open, and the eggs released were allowed to develop. Individual larvae were mounted onto agarose pads within an hour after hatching and examined by Nomarski differential interference contrast microscopy. In all larvae examined (n = 47), fluid quickly began to accumulate in the region immediately anterior to the excretory cell nucleus (where the excretory duct cell is normally positioned) and the larvae gradually filled with liquid. Since the excretory duct cell itself is not always easy to score in newly hatched wild-type larvae, it was difficult to score the absence of the duct cell in *let-60; lin-25* double-mutant worms with certainty. Nevertheless, besides accumulating liquid, 79% (37/47) of the animals examined lacked a duct. Since 48% of *let-60(n2021)* single mutants survive to become adults (that presumably contain a duct cell) we believe that *lin-25* mutations enhance the penetrance of the growth arrest phenotype caused by *let-60(n2021)* by affecting the specification of the duct cell fate. In 21% (10/47) of *let-60(n2021); lin-25(ar90)* double-mutant larvae a duct of sorts was visible. One possibility is that in these larvae the duct cell fate was partially specified but the cell failed to differentiate properly and the duct formed was not fully functional. Such a possibility might also account for the low penetrance growth defect caused by *lin-25* single mutants. Six percent of hermaphrodites homozygous for *lin-25(ar90)* arrest in the L1 or L2 stage with a rod-like morphology (Tuck and Greenwald 1995), and these larvae appear to contain a duct. This early growth arrest phenotype caused by *lin-25* null mutations is suppressed by *let-60(gf)*: <1% (2/235) of *let-60(n1046); lin-25(ar90)* double mutant hermaphrodites die as L1 or L2 larvae. The effect of *lin-25* mutations on the growth arrest phenotype caused by *let-23(n1045)* (Ferguson and Horvitz 1985) was examined by picking non-Unc, Egl individuals [of the genotype *let-23(n1045); lin-25(ar90)*] segregating from a strain of the genotype *let-23(n1045); +/nT1(IV); lin-25(ar90)/nTI[unc(754) let](V)*.

Exit of germ cells from pachytene: Hermaphrodites homozygous for *let-60(s1124)* or *let-60(s1155)* derived from a hermaphrodite parent of the genotype *let-60/+* invariably die as rod-shaped L1 larvae (Clark *et al.* 1988; Beitel *et al.* 1990; Han *et al.* 1990). Both *let-60(s1124)* and *let-60(s1155)* mutant hermaphrodites are maternally rescued for larval lethality by a copy of *let-60(n1046)*, a dominant, hypermorphic mutation (Han and Sternberg 1991; Church *et al.* 1995). A maternal copy of *let-60(n1046)* does not, however, rescue the later defects caused by *let-60(lf)* mutations, and *let-60(s1124)* hermaphrodites derived from a parent of the genotype *let-60(n1046)/let-60(s1124)* are Vulvaless and sterile (Church *et al.* 1995). To examine whether a *lin-25* mutation could enhance the germ line defect caused by *let-60(s1155)*, exit of germ cells from the pachytene stage was examined in Unc-22 Unc-31 hermaphrodites segregating from a strain of the genotype *let-60(n1046)/let-60(s1155) unc-22(s7) unc-31(e169); lin-25(ar90)*. Exit of germ cells from the pachytene stage was assessed by examining the morphology of the germ cell chromosomes stained with 4′,6-diamidino-2-phenylindole (Church *et al.* 1995). In control experiments, exit of germ cells from the pachytene stage was examined in wild-type worms, in *let-60(s1155) unc-22(s7) unc-31(e169)* mutants segregating from a strain of the genotype *let-60(s1155) unc-22(s7) unc-31(e169)/let-60(n1046)*, and in *let-60(s1124) unc-22(s7) unc-31(e169)* hermaphrodites segregating from a strain of the genotype *let-60(s1124) unc-22(s7) unc-31(e169)/let-60(n1046)*.

One possible caveat to the results with *let-60(s1155); lin-25(ar90)* double mutants could have been that those animals with least Ras activity might have arrested at the L1 stage because of a defect in the specification of the duct cell fate. While we cannot entirely exclude this possibility, we note that hermaphrodites of the genotype *let-60(n1046)/let-60(s1155) unc-22(s7) unc-31(e169); lin-25(ar90)* segregate 17% Unc-22 Unc-31 animals, implying that the majority of the *let-60(s1155); lin-25(ar90)* double-mutant individuals are rescued for the duct cell defect by a maternal copy of *let-60(n1046)*. Furthermore, *let-60(s1155)* hermaphrodites segregating from a strain of the genotype *let-60(s1155) unc-22(s7) unc-31(e169)/let-60 (n1046)* all show defects in vulval development, suggesting that the *let-60(n1046)* gene product provided by the mother does not perdure to the late L2/early L3 stage when the fates of the VPCs are determined. Germ cells first begin to exit pachytene during the L4 stage.

To examine whether a *sur-2* mutation could enhance the germ line defect caused by *let-60(s1155)*, exit of germ cells from the pachytene stage was examined in Unc-22 Unc-31 hermaphrodites segregating from a strain of the genotype *sur-2(ku9); let-60(n1046)/let-60(s1155) unc-22(s7) unc-31(e169)*.

Body morphology: To determine whether or not *lin-25* or *sur-2* mutations could suppress the Clr phenotype caused by constitutive activation of the signaling pathway regulating body morphology, *clr-1(e1745*ts*); lin-25(ar90)* and *clr-1(e1745*ts*); sur-2(ku9)* double-mutant hermaphrodites raised at 15° were shifted to 25° (the restrictive temperature for *e1745*ts) during the L4 stage.

Preanal ganglion equivalence group: The lineages of P9.p, P10.p, and P11.p were followed from the early L3 stage until 1 hr after the completion of the molt to the L4 stage. *lin-15(n309)* mutant males examined were generated from a cross between N2 males and *rol-4(sc8); lin-15(n309)* mutant hermaphrodites. The genotype of *lin-25* mutant males examined was *him-8(e1489); lin-25(ar90)*, and the genotype of *lin-25;*

lin-15 mutant males was *him-8(e1489); lin-25(ar90); lin-15(n309)*. In five of eight *lin-15(n309)* mutant males examined, P10.p and P11.p adopted their wild-type fates (2° and 1°, respectively), but P9.p, instead of adopting the 3° fate, was induced. The lineage generated by P9.p had some characteristics of the 1° lineage; in particular, in most animals examined one of the Pn.xxx cells failed to divide and had the morphology of P11.ppp. It is known, however, that P9.p in *lin-15(n309)* males often produces a hook (P. Sternberg, personal communication), a structure that in wild-type worms is formed by P10.p (Sulston and Horvitz 1977). It is possible that the lineage generated by P9.p in *lin-15(n309)* is hybrid.

Besides directly affecting the fate of cells within the preanal ganglion equivalence group, *lin-15* mutations also affect which cells become part of the group. In *lin-15* mutant males, P11 sometimes adopts the P12 cell fate (Fixsen *et al.* 1985). In animals in which such a fate transformation occurs, P8.p can be recruited into the equivalence group. In 3/8 *lin-15(n309)* mutant males examined, P11 appeared to have adopted the P12 cell fate. In particular, a cell in the position normally occupied by P11.p failed to divide and instead displayed the morphology shown by P12.pa in wild-type males. In these three animals, P10.p adopted the 1° fate and P9.p adopted the 2° fate. In two of the three, P8.p was recruited into the equivalence group and was induced. *lin-25* mutations suppress both the ectoptic induction within the preanal ganglion equivalence group and the P11 to P12 fate transformation. In none of the 10 *lin-25; lin-15* mutant males examined had the P11 to P12 cell fate transformation occurred; in only 1/10 was P9.p induced.

Cloning of *C. briggsae lin-25* genomic DNA: A *C. briggsae* genomic DNA lambda phage library (a kind gift from T. Snutch and D. Baillie) was screened under low stringency conditions with a 800-bp DNA fragment spanning the *C. elegans* gene, W05B10.2 (which lies immediately downstream of *lin-25*). A single positive clone, VB#SG1, was isolated, and a 3.5-kb *Eco*RI fragment (that hybridized to the probe) was subcloned from VB#SG1 into pBluescriptII KS(+) to generate the plasmid pVB65SG. A 1.3-kb *Sac*I fragment (which contains part of a predicted gene whose sequence is similar to that of *C. elegans* W05B10.1) was purified from pVB65SG and used to screen a filter containing a gridded array of fosmid clones from *C. briggsae* (Genome Systems). We identified six independent fosmid clones that according to fingerprint data from the *C. briggsae* sequencing consortium formed a single contig. Low stringency Southern blot analysis suggested that one of these clones, G05D12, contained *C. briggsae lin-25* as well as sequences upstream of *lin-25*. To identify potential coding regions we analyzed the sequence generated by the *C. briggsae* sequencing consortium with the Genefinder program (L. Hillier and P. Green, unpublished results).

To determine whether or not *C. briggsae lin-25* could rescue the Egl defect of a *C. elegans lin-25* mutation, G05D12 fosmid DNA was injected at a concentration of 50 μg/ml into *lin-25(n545*ts*)* mutant hermaphrodites (raised at 15°) together with 50 μg/ml of pRF4 plasmid DNA. *lin-25(n545*ts*)* mutant hermaphrodites raised at 25° are 100% Egl (Ferguson and Horvitz 1985). pRF4 encodes *rol-6(su1006)*, which confers a Rol phenotype on transformed progeny (Mello *et al.* 1991). Injected animals were placed at 25° and F_1 Rol progeny were scored for the ability to lay eggs.

Reverse transcription-PCR: Total nematode RNA was isolated from a mixed-stage population of *C. briggsae* nematodes and from the transgenic *C. elegans* strain VB0231 by a guanidine thiocyanate procedure (Chirgwin *et al.* 1979). VB231 (genotype *lin-25(n545*ts*); svEx40[GO5D12; rol-6(su1006)]*) harbors multiple copies of GO5D12 and thus presumably overexpresses *Cb-lin-25* RNA. Poly(A)$^+$ RNA was enriched from total RNA preparations by selection on oligo(dT)-coated magnetic beads (Fast track; Promega, Madison, WI). Approximately 3–5 μg of poly(A)$^+$ RNA was used in reverse transcription reactions catalyzed by AMV reverse transcriptase (Promega). The 5′ end of the *Cb-lin-25* cDNA (comprising exons 1–6) was isolated by nested PCR of total *C. briggsae* cDNA using the SL1 *trans*-spliced leader oligonucleotide as the forward primer and two gene-specific, nested reverse primers, Cb2 and Cb12. The 3′ end of the *Cb-lin-25* cDNA (comprising exons 11–13) was amplified using a modified 3′ Race protocol (Innis *et al.* 1989; De Bono and Hodgkin 1996). Poly(A)$^+$ RNA was reverse transcribed using a hybrid primer consisting of oligo(dT) and an adapter sequence. The first strand DNA generated was amplified by nested PCR using a primer corresponding to the adapter sequence and two gene-specific, nested forward primers, Cb10 and Cb5. The midportion of the *Cb-lin-25* cDNA was amplified by the primer pairs Cb1 and Cb4 (exons 6–8) and Cb3 and Cb6 (exons 8–12). In all reactions the final PCR products were cloned into pBluescriptII KS(+) by blunt-end ligation and sequenced. For the most part, the pattern observed agreed with that predicted by Genefinder, but differences were observed at 5′ and 3′ ends. The complete nucleotide sequence of the *C. briggsae lin-25* cDNA (3555 bp in total) has been submitted to GenBank and has the accession no. AF263434.

Primers used are as follows:

SL-1: 5′-GGTTTAATTACCCAAGTTTGA-3′ (Krause and Hirsh 1987)
Adapter-Oligo(dT): 5′-TAGCTCTGCACCCGGATCCTCTTT TTTTTTTTTTTTTTTT-3′
Adapter: 5′-AGCTCTGCACCCGGATCCTCT-3′
Cb1: 5′-CGTCCGTAAGAATACTAGTT-3′
Cb2: 5′-CGCGTGTAGAAGATCAAATA-3′
Cb3: 5′-GGTCGATGTGGTATGTGAAA-3′
Cb4: 5′-CCTCTTCTATCCCAACTTCT-3′
Cb5: 5′-CGTGCAACGAAATGATTCAT-3′
Cb6: 5′-CCCACTATCTTGGTGATGTA-3′
Cb10: 5′-GCGCTTCCAAGTATGGGGAA-3′
Cb12: 5′-CTGTATAGTCTCCATTTCATCTTCGTCCC-3′.

Screens for suppressors of *lin-25*: *lin-25(ga67)* homozygous mutant hermaphrodites were treated with ethyl methanesulfonate (EMS), and their F_1 and F_2 progeny were screened for non-Egl individuals. A single suppressor mutation, *sv17*, was isolated from a screen of 102,000 haploid genomes. *sv17* was found to map within 0.5 map units of *lin-25* on chromosome V and to be a dominant suppressor of *ga67*. Sequencing of the *lin-25* allele on the *ga67sv17* double-mutant chromosome revealed that *sv17* is associated with a second mutation in the codon affected by *ga67* that restores the open reading frame. The TGA stop at codon 283 in *ga67* had been mutated to GGA, encoding a glycine. *sv17* therefore appears to be an intragenic revertant of *ga67*. However, the wild-type amino acid at this position is a conserved arginine and *ga67sv17* mutant worms are still partially Egl, indicating that *sv17* does not completely restore gene activity to wild type.

We also screened for suppressors of the Egl defect conferred by *lin-25(n545*ts*)* at 25°. Homozygous mutant hermaphrodites reared at 15° were treated with EMS and allowed to recover at 20° overnight. The following day, individuals were placed at 25° and allowed to lay eggs. Both the F_1 and F_2 generations were examined for non-Egl individuals. No suppressor mutations were isolated from a screen of 104,000 haploid genomes.

Determination of sequence changes associated with *lin-25* mutant alleles: The sequence changes associated with *lin-25(ar90*, *n545*ts, *ku70*, *ku77*, *n1063*, *sy29)* were determined by direct sequencing of PCR products. All coding regions and splice sites were sequenced for all six alleles. In all cases the

sequence changes were confirmed by sequencing two independently generated PCR products. For each allele, DNA was prepared from homozygous mutant strains and used as template in different PCR reactions using the primer pairs 5′-GAATATTGGGTTAATGTCGGTG-3′ and 5′-CATTTGCCA ATTTTGAACATA-3′ (exons 1–5), 5′-TATACTAATATTTGG GAACCAATAG-3′ and 5′-CTTCTGCATTCGCCAATCGC-3′ (exons 6, 7, and part of exon 8), 5′-GGGAAATCTGACGCCGA ACAGACG-3′ and 5′-TAGCAGTGTTAGCATGT-3′ (part of exon 8 and exon 9, part of exon 10), AGCACTGCTCGTA GATTCTG-3′ and 5′-CTTAATTTCACAATTGTGTG-3′ (part of exon 9 and exons 10–12), and 5′-CAACGCTCTAAACAT CATTCG-3′ and 5′-CTTCTGATGCAGTCAATGAGG-3′ (exon 13). Fractions of the products were used as templates for a second round of PCR in which only the first (forward) primer was included in the reaction mixture. The single-stranded PCR products were then used as templates in DNA sequencing reactions. The primers used for sequencing were 5′-CCA CAAAGCTGCTGAGT-3′, 5′-CGACAAGTTTGAGAAGATGG-3′, 5′-GCATTGCAAACATTTCG-3′, 5′-TTGGTAACCTTCAA CAT-3′, 5′-GCTGATTTGTCAGGTGAACG-3′, 5′-AAAATGCG AATGGAAGG-3′, 5′-GTTGTGGATTGCCATCGAAAGTCAG CACGATGTTGT-3′, 5′-AAATGATGCATTCTGCC-3′, 5′-GTG CTCTCTCCGTCGTAGAC-3′, 5′-CCAACTTCCATAAGTCG-3′, 5′-AGAATTATCAACACGAG-3′, 5′-AGAGCAATGTACTCTG-3′, 5′-TATCCATTTGCTCGACT-3′, 5′-CAAATTCG GATCAT CAG-3′, 5′-TGGCTCACAATCCAACCATTCGAGAAGGC-3′, 5′-GCTGAAAGCCGTACAGG-3′, 5′-TAGCAGTGT TAGCA TGT-3′, 5′-TTGTCCTGCATCCTCAC-3′, 5′-TCTTCTGGTTC AGGAGC-3′, 5′-GGTGAGACCCATAAATG-3′, 5′-CATATTG CTGTGATGAG-3′, and 5′-TTGGCATACATC GAACC-3′.

To identify sequence changes associated with *lin-25(e1446, ga67, ku78, n1722*ts, *ga67sv17)* we first used the method of RNAse cleavage mismatch detection (Ambion, Austin, TX) to determine in which region of the gene the lesion resided. Sets of nested primers were used to amplify genomic regions encompassing *lin-25* exons from both wild-type and *lin-25* mutant worms. The top and bottom strands of the resulting PCR products were converted into RNA in separate *in vitro* transcription reactions with SP6 and T7 polymerases. For a given region, these two separate RNA products were then mixed and reannealed. For each region three separate annealing reactions were set up, one in which both RNA products were derived from DNA amplified from wild-type worms, one in which both RNA products were derived from DNA amplified from a *lin-25* mutant, and one in which one product was generated from DNA amplified from wild type and the other from DNA from a mutant strain. The three different reactions were treated with RNAse and loaded onto an agarose gel. In cases where the product amplified from the mutant harbored a mutation, the *lin-25*/N2 RNA hybrid was cleaved by RNAse and gave rise to two bands on the agarose gel. The precise sequence change associated with each *lin-25* allele was determined by direct sequencing of the appropriate PCR product.

***In vitro* mutagenesis analysis:** All mutations introduced *in vitro* into *lin-25* were generated in the plasmid pVB43LN. pVB43LN harbors *lin-25* genomic DNA from which most (2.7 kb) of intron 5 has been deleted. This plasmid rescues the *lin-25* phenotype with high efficiency and serves as a wild-type control. Mutations were introduced into pVB43LN by PCR-based overlap extension (Ho *et al.* 1989). PCR-amplified fragments containing sequence changes were cloned into pVB43LN using appropriate restriction enzymes. Each construct was sequenced to confirm that the expected mutations had been created and that no extra mutations had been introduced. The ability of the mutated *lin-25* genes generated *in vitro* to function *in vivo* was tested by assaying for rescue of the Egl phenotype conferred by *lin-25(n545*ts*)* at 25°. *lin-25(n545*ts*)* mutant hermaphrodites raised at 15° were injected with 50 μg/ml of the relevant plasmid together with 50 μg/ml of the plasmid pRF4 (Mello *et al.* 1991). F_1 Rol progeny were examined for their ability to lay eggs.

RESULTS

The effect of *lin-25* mutations on cell specification events involving *let-60 ras*: If LIN-25 functioned as a cofactor for LIN-39 during VPC fate specification and not as part of the Ras/MAP kinase signaling machinery, then *lin-25* mutations might not affect cells outside the domain of the worm where LIN-39 is expressed and required (Clark *et al.* 1993; Wang *et al.* 1993). Conversely, if LIN-25 were itself a target of MPK-1 or required for the activity of a target, then *lin-25* mutations might affect cell fate specification events in other parts of the worm, known to require *let-60 ras*. Therefore, to understand better the role of LIN-25 in VPC fate specification we examined the effect of *lin-25* mutations on fate determination events outside the vulval equivalence group: events that require *let-60 ras*-mediated signaling but not *lin-39* activity.

In hermaphrodites, *let-60* and *let-23* are required for the specification of the P12 cell fate (Jiang and Sternberg 1998). The presumptive P12 cell and its left homologue (which normally becomes P11) constitute an equivalence group (Sulston and White 1980). In individuals homozygous for certain hypomorphic alleles of *let-23* or *let-60*, P12 appears to adopt the fate adopted by P11 in wild-type hermaphrodites (Jiang and Sternberg 1998). Conversely, mutations that constitutively activate the Ras pathway (such as null mutations at the *lin-15* locus) cause the opposite transformation: both P11 and P12 adopt the P12 cell fate. The data presented in Table 1 show that a null mutation in *lin-25, ar90*, causes P12 to adopt the P11 cell fate at a low penetrance and suppresses the P11 to P12 fate transformation caused by *lin-15(n309)*. Furthermore, a partial reduction of *lin-25* activity enhances the penetrance of the P12 fate determination defect caused by a hypomorphic allele of *let-23*. A representative individual in which both P12 and P11 appeared to have adopted the P11 fate is shown in Figure 1. We conclude that *lin-25* is required for the efficient induction of the P12 fate.

An early requirement for *let-60* is in a signaling event occurring in the embryo that is required for the specification of the excretory duct cell fate (Yochem *et al.* 1997). The excretory duct cell, an essential component of the osmoregulatory system, can be generated by either of two cells, ABplpaaaap or its homologue, ABprpaaaap (Sulston *et al.* 1983). In *let-60* null mutants neither cell gives rise to a duct cell, and, as a result, L1 mutant larvae contain no duct and are incapable of secreting excess fluid (Yochem *et al.* 1997). Such larvae become filled with liquid and arrest with a characteristic rod shape. Worms homozygous for a dominant hyper-

TABLE 1

***lin-25* is involved in P12 cell fate specification**

Genotype	Temperature	n^a	P11 → P12[b] (%)	P12 → P11[b] (%)
N2 (wild type)	20°	47	0	0
lin-25(ar90)	20°	88	0	8
*lin-25(n545*ts*)*	15°	76	0	0
*lin-25(n1722*ts*)*	20°	64	0	0
let-23(n1045)	15°	80	0	11
let-23(n1045)	20°	89	0	12
*let-23(n1045);lin-25(n545*ts*)*	15°	60	0	78
*let-23(n1045);lin-25(n1722*ts*)*	20°	94	0	35
lin-15(n309)	20°	115	32	0
lin-25(ar90);lin-15(n309)	20°	52	13[c]	6
sur-2(ku9)	20°	72	0	6

Alleles used are as follows: *lin-25(ar90)*, which strongly reduces or eliminates gene activity; *lin-25(n545*ts*)* and *lin-25(n1722*ts*)* appear to have reduced gene activity at 15° and 20°; 8% of *lin-25(n545*ts*)* mutant hermaphrodites are Egl at 15° (Ferguson and Horvitz 1985) and 2% of *lin-25(n1722*ts*)* mutant hermaphrodites are Egl at 20° (Tuck and Greenwald 1995); *lin-15(n309)* is most likely a null allele (Clark *et al.* 1994).

[a] Number of animals examined.

[b] The fates of P11 and P12 were assessed using the criteria of Jiang and Sternberg (1998).

[c] Four animals of this genotype had two cells with the morphology of P12.pa and one of P11.p. *lin-25* mutations sometimes cause Pn.p cells that later become part of the vulva equivalence group to divide during the L1 stage to give rise to two cells that both become VPCs. One possibility is that in the four animals with two P12.pa cells and one P11.p cell seen here, P11 or P12 divided to give rise to two cells that subsequently both became part of the P11/P12 equivalence group.

morphic mutation in *let-60 ras*, *n1046*, often contain two duct cells (Yochem *et al.* 1997). The majority of hermaphrodites homozygous for a *lin-25* null mutation do not arrest as L1 larvae but instead grow to become fertile adults, all of which contain a duct cell. To investigate whether *lin-25* mutations could affect the specification of the duct cell fate we first examined whether a *lin-25* null mutation could affect the two-duct cell

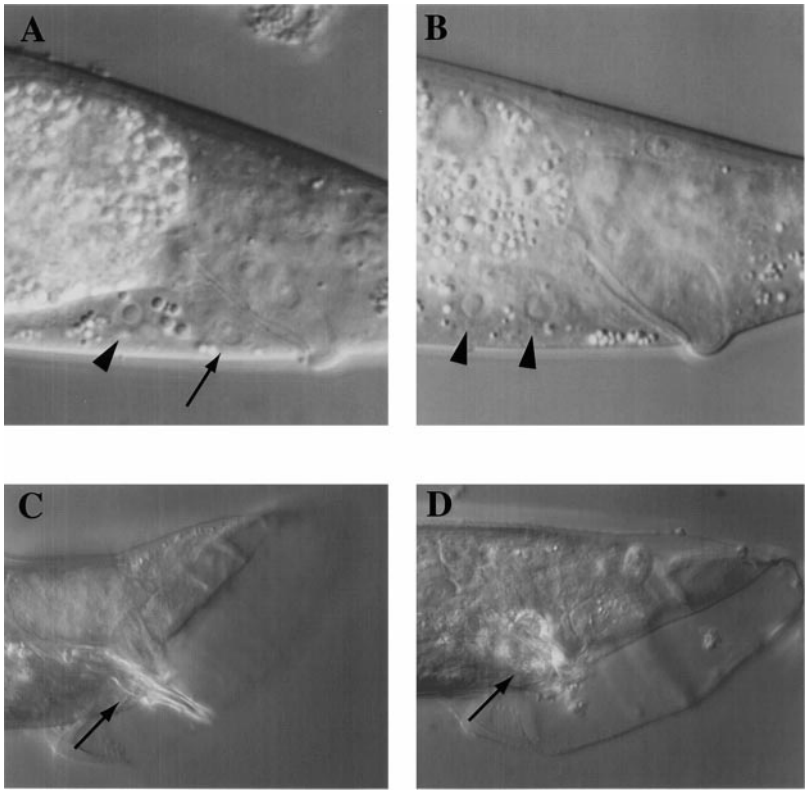

Figure 1.—*lin-25* mutations cause defects similar to those seen in animals with reduced Ras function. Anterior is to the left; ventral is down. (A and B) Nomarski photomicrographs of wild-type (A) and *lin-25(ar90)* mutant (B) hermaphrodites showing the posterior ventral cord region. In A the arrowhead denotes P11.p and the arrow denotes P12.pa. In wild type, both the nucleus itself of P12.pa and the nucleolus are smaller than in P11.p. In addition, the P12.pa nucleus is oval shaped and has a granular morphology. The P11.p nucleus is large, round, and less granular. The arrowheads in B denote P11.p and a cell, presumed to be P12.p, that shows the typical P11.p nuclear morphology. (C and D) Nomarski photomicrographs of wild-type (C) and *lin-25(ar90)* mutant (D) males showing the tail region. The copulatory spicules are indicated by arrows. The spicules are long and thin in wild-type males but short and "crumpled" in *lin-25* mutants. The complete genotype of *lin-25* mutant males examined was *him-8(e1489); lin-25(ar90)*.

phenotype caused by *let-60(n1046)*. Whereas 58% (30/52) of *let-60(n1046)* mutant hermaphrodites contained two duct cells, 13% (6/46) of *let-60(n1046); lin-25(ar90)* did so. Thus *lin-25* is required for the ectopic induction of the duct cell fate by *let-60(gf)*. To investigate further whether *lin-25* is required for the specification of the duct cell fate we examined the ability of *lin-25* mutations to enhance the lethal phenotype caused by reduction-of-function mutations in *let-60* or *let-23*. *let-60(n2021)* reduces but does not eliminate Ras activity (Beitel *et al.* 1990). A total of 55% (116/211) of *let-60(2021)* mutant hermaphrodites died as larvae. In contrast, 100% (n = 156) of hermaphrodites homozygous for *let-60(n2021)* that also lacked *lin-25* activity died as rod-shaped larvae. All double-mutant animals examined had a fluid-filled morphology, suggesting that the excretory system was not functioning properly (see materials and methods). Similar results were obtained with *let-23(n1045)*. Whereas 51% (93/183) of *let-23(n1045)* hermaphrodites died as larvae at 20°, 100% (n = 107) of *let-23(n1045); lin-25(ar90)* double-mutant hermaphrodites did so. In contrast, a null mutation in *lin-39* did not significantly enhance the growth arrest phenotype caused by *let-60(n2021)* or *let-23(n1045)*. A total of 56% (65/117) of *lin-39(n1760); let-60(n2021)* double-mutant hermaphrodites and 63% (115/183) of *let-23(n1045); lin-39(n1760)* double mutants arrested as L1 larvae.

Besides mediating a number of signaling events occurring in the soma, *let-60 ras*, *mek-2*, and *mpk-1* are also required within the germ line (Church *et al.* 1995; Yochem *et al.* 1997). In wild-type hermaphrodites, germ cells at the dorsoventral flexure exit the pachytene stage of the first meiotic prophase and proceed to diakinesis. Germ cells that lack *let-60* function invariably fail to exit the pachytene stage. Germ cells that are mutant for the nonnull allele of *let-60*, *s1155*, are delayed in their exit from the pachytene stage but are not blocked. This observation suggests that the signaling event inducing exit from pachytene occurs in these animals but is compromised. To determine whether *lin-25* mutations could enhance the *let-60* germ line defect, we examined whether exit from pachytene was blocked in *lin-25(ar90)* hermaphrodites mutant for *let-60(s1155)* in the germ line (see materials and methods). In no case was such a block observed, suggesting that *lin-25* does not function in the signaling event promoting exit from pachytene.

Hermaphrodites with strongly reduced *let-60 ras* function are Scrawny, a phenotype characterized by thinness and slow growth (Church *et al.* 1995). Wild-type (non-Scrawny) growth may require a signaling event in which LET-60 is activated by the FGF receptor-like protein, EGL-15. First, reduction-of-function mutations in *egl-15* cause a Scrawny phenotype similar to that caused by *let-60* mutations. Second, mutations in a gene encoding a Ras-binding protein, SOC-2/SUR-8, suppress phenotypes associated with overexpression of EGL-15. Constitutive activation of EGL-15, caused, for example, by mutations in *clr-1* (which encodes a phosphatase thought to negatively regulate EGL-15; Kokel *et al.* 1998), gives rise to a Clear (Clr) phenotype characterized by a marked increase in transparency (Hedgecock *et al.* 1990). Mutations in *soc-2/sur-8* suppress this Clr phenotype to wild type (Selfors *et al.* 1998; Sieburth *et al.* 1998). A mutation in *lin-25* had no effect on the Clr phenotype caused by *clr-1(e1745*ts*)*. A total of 100% (n = 176) of *clr-1; lin-25* double-mutant animals were Clr. These results suggest that *lin-25* may not function downstream of *egl-15* in the signaling event required for wild-type morphology.

In males, three cells in the preanal ganglion, P9.p, P10.p, and P11.p, form an equivalence group in which Ras appears to function (Sulston and White 1980; P. Sternberg, personal communication). In wild-type males these cells adopt 3°, 2°, and 1° fates, respectively (these fates are different from those of the VPCs in hermaphrodites). Mutations that constitutively activate *let-60 ras* cause P9.p to be induced (adopt either the 1° or 2° cell fate; P. Sternberg, personal communication; our own unpublished observations). We followed the lineages of P9.p, P10.p, and P11.p in 10 *lin-25(ar90)* mutant males, but in no case did P10.p or P11.p fail to be induced (adopt the 1° or 2° fate). *lin-25(ar90)* did, however, suppress the ectopic induction caused by *lin-15(n309)*. P9.p was induced in all 8 *lin-15(n309)* males examined but in only 1 of 10 *lin-25(ar90); lin-15(n309)* double-mutant males.

Mutations in genes in the Ras/MAP kinase pathway cause fate transformations in a group of cells in the tail region of the male, Bα, Bγ, B.alap, and B.arap (Chamberlin and Sternberg 1994). In individuals with reduced *let-60 ras* activity, each of these cells adopts the fate of a cell lying more posteriorly. Bα, Bγ, B.alap, and B.arap each give rise to cells forming part of the copulatory spicules (Sulston and Horvitz 1977), and in mutants in which Ras/MAP kinase signaling is compromised, the spicules have a crumpled morphology (Chamberlin and Sternberg 1994). *lin-25* mutations also cause defects in the specification of the anterior fates, and *lin-25* mutant males sometimes have crumpled spicules (H. Chamberlin and P. Sternberg, personal communication). We examined the spicules in males homozygous for the null allele *lin-25(ar90)*. In 74% (52/70) of the individuals examined, the spicules were obviously crumpled. A representative individual is shown in Figure 1. Lineage analysis of Bα and Bγ revealed that the lineages were often abnormal and were consistent with a partial transformation toward the fate adopted by a more posterior cell in wild-type males. Bα, for example, generated a lineage intermediate between those generated by Bα and Bβ in wild-type males, and Bγ generated a lineage intermediate between those generated by Bγ and Bδ (data not shown). Such partial transformations are also observed in males carrying mu-

tations that partially reduce Ras pathway signaling activity (Chamberlin and Sternberg 1994).

Mutations in *sur-2* cause similar defects to those caused by *lin-25*: We have previously reported that LIN-25 likely functions with SUR-2 in VPC fate specification. Therefore, in light of the results presented above on the effects of *lin-25* mutations on cell fate specification events outside the vulval equivalence group, we investigated whether *sur-2* mutations also affected these other fate specification events involving *let-60 ras*. Hermaphrodites homozygous for a *sur-2* null mutation, *ku9*, were found to display defects in the specification of the P12 cell fate at a low penetrance (Table 1). Furthermore, *sur-2(ku9)* also suppressed the generation of the supernumary duct cells caused by *let-60(n1046gf)*. Only 14% (7/42) of *sur-2(ku9); let-60(n1046)* double-mutant hermaphrodites contained two duct cells compared to 58% (30/52) of *let-60(n1046)* single mutants. A total of 53% (31/59) of *sur-2(ku9)* mutant hermaphrodites were found to have crumpled spicules. It is known that *sur-2* mutations cause partial fate transformations of cells in the tail region of the male that give rise to part of the spicules (H. Chamberlin and P. Sternberg, unpublished results, cited in Singh and Han 1995).

Like mutations in *lin-25*, *sur-2(ku9)* did not suppress the Clr phenotype caused by a mutation in *clr-1*. Similarly, *sur-2(ku9)* did not enhance the *let-60* germ line defect: exit from pachytene was not blocked in *sur-2(ku9)* hermaphrodites mutant for *let-60(s1155)* in the germ line. Thus *sur-2* mutations appear to affect the same spectrum of events as *lin-25* mutations. These observations further strengthen the idea that LIN-25 and SUR-2 may function together in *C. elegans* development. In an effort to identify other genes encoding proteins that act together with LIN-25, we have carried out extensive screens for suppressors of the Egl defects caused by strong *lin-25* mutations. Only one suppressor mutation was isolated, however, and this was found to be intragenic. Suppressors of strong *lin-25* mutations, therefore, appear to be rare.

Cloning of *C. briggsae lin-25* by synteny: The effects that *lin-25* mutations have on cell fate specification events involving *let-60* suggested that, at least in some cells, *lin-25* might be part of the Ras signal transduction pathway. To gain insights into how LIN-25 might function at a biochemical level, we sought to identify regions of the protein that have been most conserved during evolution. Southern blot analysis at low stringencies with *lin-25* probes suggested that *lin-25* genes have diverged significantly during evolution (data not shown). Attempts to clone *C. briggsae lin-25* (*Cb-lin-25*) by DNA hybridization with *C. elegans lin-25* (*Ce-lin-25*) DNA probes were not successful. To isolate *C. briggsae lin-25*, therefore, we screened libraries of *C. briggsae* genomic DNA with probes from genes lying close to *lin-25* in the *C. elegans* genome (see materials and methods). A fosmid clone, G05D12, was identified that hybridized both to DNA spanning the gene 5′ of *lin-25* in the *C. elegans* genome and to DNA spanning WO5B10.1, which lies 3′. G05D12 was sequenced by the *C. briggsae* sequencing consortium (St. Louis), and analysis of the sequence with the Genefinder program revealed that G05D12 apparently contained a gene with overall similarity to *C. elegans lin-25*. We determined the splicing pattern of this predicted gene by sequencing cDNAs generated by reverse transcription (RT)-PCR. The results of this analysis are shown in Figure 2.

The gene has a pattern of exons and introns similar to that of the *C. elegans lin-25* and is predicted to encode a protein similar in size to Ce-LIN-25. The position of all but one of the splice sites has been conserved between *C. elegans* and *C. briggsae*. The one exception is splicing between exons 9 and 10: codon 878 in *C. elegans* is part of exon 9 whereas in *C. briggsae* the corresponding

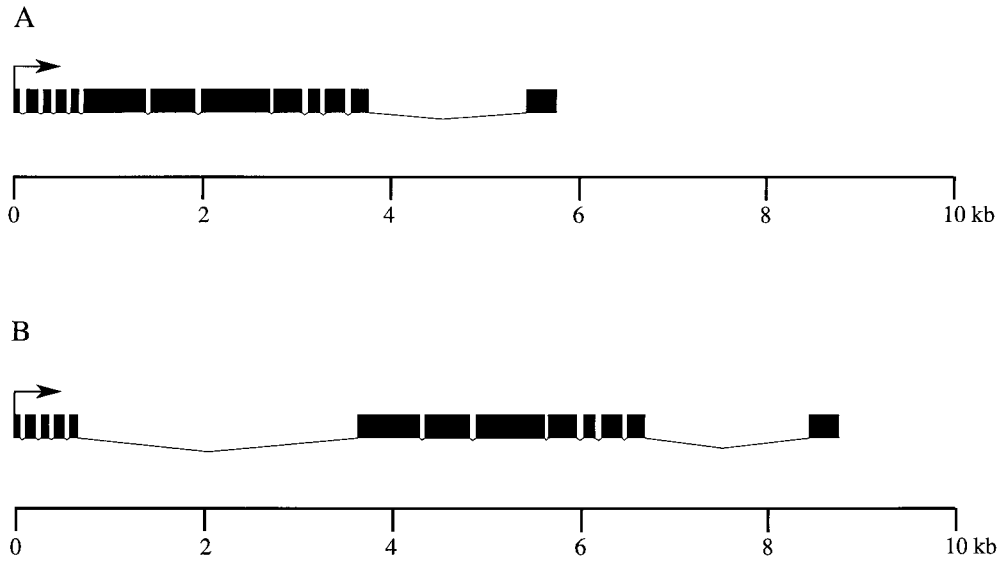

Figure 2.—(A) The arrangement of exons in *C. briggsae lin-25*. Solid boxes indicate exons. The horizontal line beneath denotes genomic DNA. Transcription is from left to right. "0" indicates the position of the splice site, 5′ of the gene, used in the splicing of the SL1 *trans*-spliced leader sequence to *lin-25*. The position of all splice sites was confirmed by sequencing cDNAs generated by RT-PCR. (B) The arrangement of exons in *C. elegans lin-25* (Tuck and Greenwald 1995) is shown for comparison.

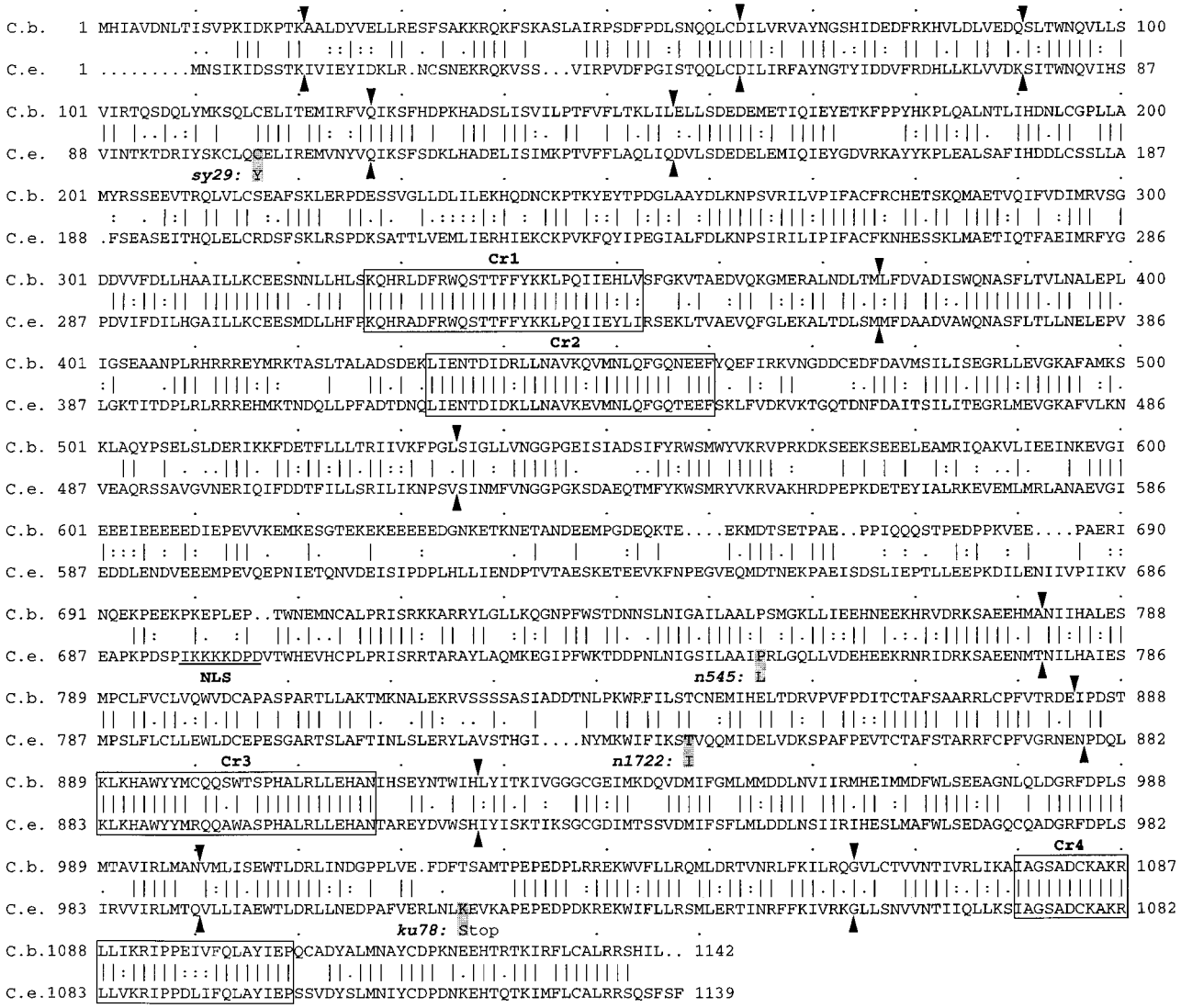

FIGURE 3.—Sequence alignment of *C. briggsae* and *C. elegans* predicted *lin-25* protein sequences. C.b., the predicted sequence of *C. briggsae* LIN-25. C.e., the predicted sequence of *C. elegans* LIN-25 (TUCK and GREENWALD 1995). Vertical lines denote positions at which the same amino acid is found in Cb- and Ce-LIN-25. Two dots indicate positions at which nonidentical but closely similar amino acids are found. Arrowheads indicate the positions of introns. Conserved regions 1, 2, 3, and 4 (Cr1, 2, 3, and 4) are boxed. The predicted amino acid sequence changes in proteins encoded by the alleles, *sy29*, *n545*ts, and *n1722*ts, are indicated. The position of the stop codon in *ku78* is also shown.

codon, 884, is part of exon 10. The principal difference in structure between the two genes is in the size of intron 5, which is 2974 bp in length in *C. elegans* but only 46 bp in *C. briggsae*. Results presented in Figure 4 demonstrate that deletion of most of intron 5 in *C. elegans lin-25* has no effect on the efficiency with which the gene rescues the *lin-25* mutant phenotype.

A comparison of the predicted sequences of Ce-LIN-25 and of the predicted *C. briggsae* protein is presented in Figure 3. Although the *C. elegans* and *C. briggsae* genes have similar structures, the proteins they are predicted to encode are significantly different in sequence. At only 56% of the amino acid positions in the predicted *C. briggsae* protein is the same amino acid found in Ce-LIN-25. Despite this degree of difference, however, the *C. briggsae* gene efficiently rescues *C. elegans lin-25* mutant phenotypes. A total of 65% (28/43) of the F_1 Rol animals were non-Egl. In 10 stable transgenic lines, at least 50% of the individuals carrying the transgene were rescued for the Egl defect, indicating that *Cb-lin-25* efficiently rescues the *Ce-lin-25* mutant phenotype. We therefore conclude that the product of the *C. briggsae* gene is a functional orthologue of Ce-LIN-25 and refer hereafter to the *C. briggsae* gene as *Cb-lin-25*.

***In vitro* mutagenesis of *lin-25*:** The amino acid identities between *C. elegans* and *C. briggsae* LIN-25 are fairly evenly distributed throughout the entire lengths of the two proteins but several regions can be discerned within which the degree of sequence conservation is higher. These regions [which we have designated conserved

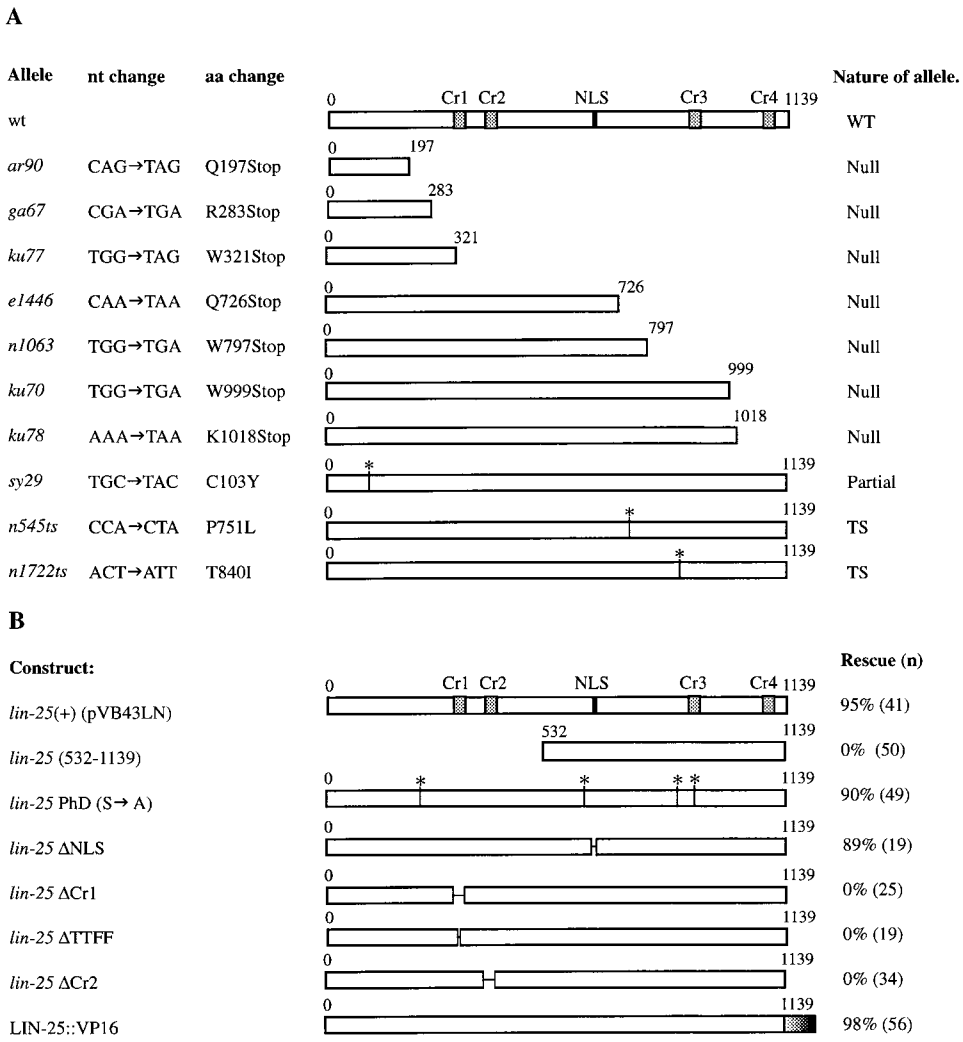

FIGURE 4.—Molecular characterization of *C. elegans lin-25* alleles (A) and the activity of *lin-25* mutant transgenes generated *in vitro* (B). (A) The nucleotide sequence changes and presumed amino acid sequence changes for 10 *lin-25* alleles are shown on the left. The predicted wild-type and mutant proteins are represented schematically by boxes to the right. The lengths of the predicted proteins encoded by each allele are indicated to the right of each box. The positions of the amino acid sequence changes in the proteins encoded by *sy29*, *n545*ts, and *n1722*ts are indicated by vertical lines with an asterisk above. All alleles were generated by ethyl methanesulfonate (EMS). *ar90*, *ga67*, *ku77*, *e1446*, *n1063*, *ku70*, and *ku78* behave as null alleles by genetic criteria (TUCK and GREENWALD 1995). *sy29* is a partial reduction-of-function allele and *n545*ts and *n1722*ts are temperature-sensitive alleles. (B) The names of mutant *lin-25* transgenes generated *in vitro* are given on the left. The proteins they are predicted to encode are represented by boxes to the right. *lin-25* ΔNLS is predicted to encode a protein lacking amino acids 695–701. *lin-25* ΔCr1, *lin-25* ΔTTFF, and *lin-25* ΔCr2 are predicted to encode proteins lacking, respectively, amino acids 313–339, 324–327, and 419–446. LIN-25::VP16 encodes full-length wild-type LIN-25 fused to the transcriptional *trans*-activation domain of Herpes Simplex virus VP16. LIN-25::VP16 rescues the Egl defect of *lin-25* mutant hermaphrodites but does not cause a Multivulva phenotype.

regions 1, 2, 3, and 4 (Cr1, 2, 3, and 4)] are shown boxed in Figures 3 and 4. To determine whether or not Cr1 or Cr2 is important for LIN-25 function we generated mutant versions of *Ce-lin-25 in vitro* that encode proteins that lack Cr1 or Cr2 (*lin-25* ΔCr1 and *lin-25* ΔCr2, respectively) and assayed their ability to rescue *lin-25* mutant defects *in vivo*. The results of these experiments are shown in Figure 4. Neither mutant protein was able to rescue, indicating that these regions are important for function or stability of LIN-25. Likewise a mutant generated *in vitro* (*lin-25* ΔTTFF), predicted to encode a protein lacking four amino acids in the center of Cr1, failed to rescue *lin-25(n545*ts*)* for the Egl defect. Analysis of the *lin-25* allele, *ku78*, suggests that Cr4 is important for protein stability or function (see below).

lin-25 lies downstream of *mpk-1* MAP kinase in the genetic pathway for the induction of the primary fate, and *C. elegans* LIN-25 is predicted to contain four potential target sites for MAP kinase (S/T-P; TUCK and GREENWALD 1995). We have therefore previously speculated that LIN-25 might be a direct target of MPK-1 and that phosphorylation of LIN-25 might be important for its function or regulation. To test this possibility we generated a *lin-25* mutant *in vitro* that is predicted to encode a protein lacking all four potential MAP kinase phosphorylation sites. Data presented in Figure 4 show that the mutant protein, LIN-25 PhD (Phosphorylation Deficient), rescues the *lin-25* phenotype with almost wild-type efficiency.

The predicted LIN-25 amino acid sequence contains a stretch of seven amino acids that shows similarity to SV40 large T-type nuclear localization signal sequences (TUCK and GREENWALD 1995). In worms containing multiple copies of a wild-type *lin-25* gene, LIN-25 protein is found predominantly in the nucleus although appreciable amounts are also found in the cytoplasm (NILSSON *et al.* 1998). To determine whether the putative nuclear localization signal (NLS) is required for translocation of LIN-25 to the nucleus, we generated a mutant

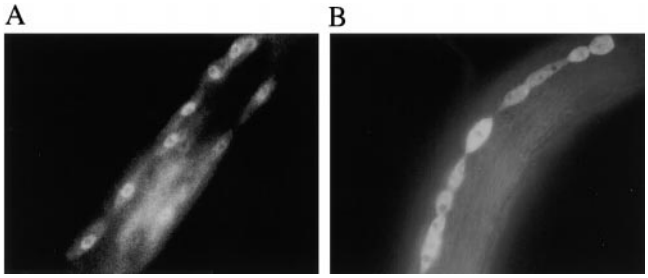

FIGURE 5.—Nuclear translocation of LIN-25 is mediated in part by an SV40 large T-type NLS. (A) Wild-type hermaphrodites containing multiple copies of a wild-type *lin-25* gene on an integrated array (*svIs1*) stained with an anti-LIN25 antibody, 4478 (NILSSON *et al.* 1998). To the left are the LIN-25 positive seam cells that lie in a lateral row. (B) LIN-25 positive seam cells, shown in wild-type hermaphrodites containing multiple copies of *lin-25* ΔNLS on an extrachromosomal array (*svEx70*) stained with the 4478 antisera.

in vitro, *lin-25* ΔNLS, that encodes a protein lacking this sequence and examined the subcellular localization of the mutant protein *in vivo*. *lin-25* ΔNLS contains an in-frame deletion that removes the nucleotides that encode the stretch of amino acids, IKKKKDP, between codons 695 and 701. The results of this analysis are shown in Figures 4 and 5. In transgenic worms harboring *lin-25* ΔNLS the distribution of the LIN-25 was significantly different from that of the wild-type protein, and a large fraction of the mutant protein was present in the cytoplasm. Despite the absence of the NLS, however, some LIN-25 protein was still able to enter the nucleus. *lin-25* ΔNLS was able to rescue the *lin-25* mutant phenotype *in vivo* with almost wild-type efficiency.

Molecular characterization of *lin-25* mutant alleles: Ten *lin-25* mutant alleles have been described previously (FERGUSON and HORVITZ 1985; TUCK and GREENWALD 1995). Seven of these, *ar90*, *e1446*, *ga67*, *ku70*, *ku77*, *ku78*, and *n1063*, behave as null alleles by genetic criteria (TUCK and GREENWALD 1995), and the remaining three alleles, *n545*ts, *n1722*ts, and *sy29*, appear to reduce gene activity (FERGUSON and HORVITZ 1985; TUCK and GREENWALD 1995). To help identify regions of LIN-25 important for function we determined the sequence changes associated with each allele. The results of this analysis are summarized in Figure 4. All alleles were found to harbor changes within the coding region. The reduction-of-function allele, *sy29*, as well as the two temperature-sensitive alleles, *n545*ts and *n1722*ts, encode proteins with amino acid substitutions. All three amino acids affected have been conserved between *C. elegans* and *C. briggsae*. All seven null alleles were found to contain mutations that lead to the generation of premature stop codons in the *lin-25* coding region and are therefore predicted to encode truncated proteins. The *ga67* mutation has previously been incorrectly reported to be an amino acid substitution F284Y (TUCK and GREENWALD 1995). We determined in the present analysis that the *ga67* mutation in fact introduces a stop codon at position 283.

DISCUSSION

The effects of *lin-25* mutations on cell fate specification events involving *let-60 ras*: We provide evidence here that, besides its role in vulval development, *lin-25* functions in a number of other cell fate specification events in *C. elegans* that are known to require Ras-mediated signaling. These include the specification of the excretory duct and P12 cell fates and, in the male, the specification of anterior fates in the tail region and of induced fates in the preanal ganglion equivalence group. The fact that *lin-25* mutations do not affect VPC fate determination exclusively argues against a role for *lin-25* specifically in determining VPC identity. Our results rule out, for example, a model in which the sole function of LIN-25 is to act as a cofactor for LIN-39 to allow the expression of targets of MPK-1 in the VPCs. The possibility that LIN-25 functions together with LIN-39 (or another protein) to specify the identity of VPCs and together with other transcription factors to specify the identity of other cells requiring *let-60 ras* cannot be excluded. However, the fact that *lin-25* is required specifically for cell fate determination events requiring *let-60* suggests that *lin-25* is involved more intimately in signaling. It is worth noting in this regard that the cells affected by *lin-25* mutations are not related to one another either with respect to their position within the worm or with respect to their lineage histories. Furthermore, *lin-25* mutations do not cause homeotic transformations in cells other than those requiring Ras for correct cell fate specification.

For all cases examined, the effects of *lin-25* null mutations on cell fate specification events involving Ras were weaker than those caused by strong mutations in *let-60 ras* itself both in the VPCs and other cells. Perhaps, in each signaling event, the signaling pathway diverges downstream of *mpk-1* MAP kinase. *lin-25* and *sur-2* might function on one branch of the pathway and removing the activity of this branch only partially reduces induction.

lin-25 may not be involved in all signaling events requiring Ras activity. We found no evidence, for example, that *lin-25* mutations affect exit of germ cells from the pachytene stage. It is possible, however, that LIN-25 does play a role in this signaling event but that the effects of *lin-25* mutations on it are not sufficiently strong to be detected in our assays. We also failed to detect any effect of *lin-25* mutations on the signaling event activated by EGL-15 required for wild-type body morphology. This event is known to require the Ras-binding protein *soc-2/sur-8*, which functions in Ras-mediated signaling during vulval development. It is striking that the spectrum of events affected by *lin-25* mutations is the same as that affected by mutations in *lin-1* (BEITEL *et*

al. 1995; T. Tiensuu and S. Tuck, unpublished observations). It will be interesting to determine whether LIN-1 and LIN-25 function together biochemically.

Sequence comparison of *C.elegans* and *C. briggsae lin-25*: The *C. briggsae* gene we have cloned shows a very similar exon-intron structure to that of *C. elegans lin-25*. The *C. briggsae* gene is flanked by genes that are similar in sequence to those to the right and left of *lin-25* in the *C. elegans* genome. Furthermore, the *C. briggsae* gene can rescue the *C. elegans lin-25* mutant phenotype. There is little doubt, therefore, that we have cloned an orthologue of the *C. elegans* gene. While we cannot exclude the possibility that the *C. briggsae* genome contains other *lin-25* genes, Southern blot analysis suggests that, if they exist, such genes are less similar to *Ce-lin-25* than is *Cb-lin-25*. No genes exist in the *C. elegans* genome that encode proteins significantly similar in sequence to Ce-LIN-25.

The predicted *C. elegans* and *C. briggsae* LIN-25 proteins have diverged significantly in sequence: the amino acid identity is 56%. *C. elegans* and *C. briggsae* are thought to have diverged from a common ancestor between 20 and 50 million years ago, and *C. briggsae* is thought to be the closest living relative of *C. elegans* (Emmons *et al.* 1979; Heschl and Baillie 1990). The overall amino acid sequence identity between Ce-LIN-25 and Cb-LIN-25 is lower than that seen between the predicted products of other genes that have been isolated from both *C. elegans* and *C. briggsae*. For example, the HSP-3 homologs Ce-HSP-3 and Cb-HSP-3 share 98% identity (Heschl and Baillie 1990), the *C. elegans* and *C. briggsae* ACE-1 acetylcholinesterases are predicted to share 95% identity (Grausco *et al.* 1996), and the gut esterases Ce-GES-1 and Cb-GES-1 are predicted to share 83% identity (Kennedy *et al.* 1993). Likewise, the novel proteins encoded by the *unc-119* genes in the two species are predicted to share 90% identity (Maduro and Pilgrim 1996). The relatively low level of conservation between Ce-LIN-25 and Cb-LIN-25 does not, however, preclude a role for the protein in cell signaling. TRA-2 appears to function as a cell surface receptor for HER-1 in the pathway for sex determination in *C. elegans* (Kuwabara *et al.* 1992). However, TRA-2 proteins from *C. elegans* and *C. briggsae* show only 43% identity, and the comparable figure for HER-1 is 57% (Kuwabara 1996; Streit *et al.* 1999).

Four potential MAP kinase phosphorylation sites present in *C. elegans lin-25* are not conserved in Cb-LIN-25. Consistent with this observation is the fact that a Ce-LIN-25 mutant lacking all four potential MAP kinase phosphorylation sites in Ce-LIN-25 rescues *lin-25* mutant defects with almost wild-type efficiency. These observations suggest that LIN-25 may not be a direct target of MPK-1 MAP kinase in the cell fate specification events in which both proteins are required. It is possible, however, either that LIN-25 functions together with a target of MPK-1 or that LIN-25 is phosphorylated by a kinase with a different substrate recognition sequence that is itself regulated by MPK-1. Another possibility is that MPK-1 functions to stabilize LIN-25. If this is the case, then overexpression of the mutant protein from a transgene might abrogate the need for phosphorylation by MPK-1.

An SV40 large T-type nuclear localization signal sequence, which we have shown is required for efficient translocation of Ce-LIN-25, is not present in the *C. briggsae* protein. Perhaps in *C. briggsae*, one or more non-SV40 large T-type NLSs function to allow translocation of Cb-LIN-25 to the nucleus. It is noteworthy in this respect that deletion of the NLS from Ce-LIN-25 does not completely abolish translocation, suggesting that one or more non-SV40 large T-type NLSs contribute to the nuclear translocation of Ce-LIN-25.

LIN-25, SUR-2, and transcriptional regulation: We have previously shown that LIN-25 protein levels are strongly reduced in the absence of *sur-2* activity (Nilsson *et al.* 1998). We report here that *sur-2* mutations cause a spectrum of defects very similar to that caused by *lin-25* mutations. Given that human SUR-2 binds Mediator, one possible model for the function of LIN-25 and SUR-2 in *C. elegans* is that they are required for transcriptional regulation in response to Ras-mediated signaling. LIN-25 from *C. elegans* nuclear extracts does not have DNA-binding activity *in vitro* (L. Nilsson and S. Tuck, unpublished observations), but we cannot exclude the possibility that LIN-25 binds DNA transiently in cells in which *let-60 ras* is activated. Arguing against a role for LIN-25 as a transcription factor binding directly to DNA, however, is the result (shown in Figure 4) that fusing the transcriptional transactivation domain from Herpes Simplex virus VP16 to LIN-25 does not confer on the protein the ability to cause phenotypes associated with activated *let-60 ras* alleles. The DNA-binding proteins UNC-86 and LIN-31 fused to the VP16 transactivation domain both cause dominant phenotypes that are thought to result from the recruitment of the transcription machinery to promoters regulated by the respective proteins (Sze *et al.* 1997; Tan *et al.* 1998). The failure to find evidence for binding of LIN-25 to DNA does not preclude a role for the protein in transcriptional regulation. LIN-25 and SUR-2 might, for example, function to link a transcription factor regulated by Ras to Mediator. We have found that LIN-25 is not itself a component of *C. elegans* Mediator (J.-Y. Kwon, L. Nilsson, S. Tuck and Y.-J. Kim, unpublished results). It will be interesting to see if LIN-25 or SUR-2 bind to Mediator in *C. elegans* and, if so, how the proteins function to recruit Mediator specifically in response to Ras-mediated signaling.

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