Structural Requirements for the Tissue-Specific and Tissue-General Functions of the *Caenorhabditis elegans* Epidermal Growth Factor LIN-3

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ABSTRACT

Caenorhabditis elegans lin-3 encodes a homolog of the epidermal growth factor (EGF) family of growth factors. LIN-3 is the inductive signal for hermaphrodite vulval differentiation, and it is required for animal viability, hermaphrodite fertility, and the specification of anterior cell fates in the male B cell lineage. We describe the cloning of a *lin-3* homolog from *C. briggsae*, sequence comparison of *C. elegans lin-3* with *C. briggsae lin-3*, and the determination of molecular lesions in alleles of *C. elegans lin-3*, including three new alleles. We also analyzed the severity of phenotypes caused by the new and existing alleles of *lin-3*. Correlation of mutant phenotypes and their molecular lesions, as well as sequence comparison between two species, reveal that the EGF motif and the N-terminal portion of the cytoplasmic domain are important for the functions of LIN-3 in all tissues, while the C-terminal portion of the cytoplasmic domain is involved in the tissue-specific functions of *lin-3*. We discuss how the structure of *lin-3* contributes to its functions in multiple developmental processes.

THE epidermal growth factor (EGF) family comprises small peptide growth factors widely used in animal development. Members of this family function in cell fate specification, cell growth, division, and survival, and they can function in multiple tissues and time points in one organism. In vertebrates and Drosophila, different genes encode multiple members of the EGF family (reviewed by Groenen *et al.* 1994; Perrimon and Perkins 1997). In *Caenorhabditis elegans*, however, there is only one known member of the EGF family, encoded by *lin-3*. *lin-3* is involved in at least five distinct developmental processes, and one interesting question is how one intercellular signal mediates multiple developmental processes.

The function of LIN-3 was first studied in the induction of the hermaphrodite vulva (Horvitz and Sulston 1980; Sulston and Horvitz 1981; Ferguson and Horvitz 1985; Ferguson *et al.* 1987; Hill and Sternberg 1992; Katz *et al.* 1995). During midlarval stages, six multipotent vulval precursor cells (VPC) form an anterior-posterior array in the ventral epidermis (Sulston and Horvitz 1977; Sternberg and Horvitz 1986; Thomas *et al.* 1990). A VPC's default fate is to divide once and fuse with the hyp7 epidermis (Kimble 1981). The inductive signal, LIN-3, is expressed in the gonadal anchor cell (AC) immediately dorsal to the VPCs (Hill and Sternberg 1992). In wild-type animals, only the three VPCs that are closest to the AC generate vulval progeny, although all six have the potential to do so. One VPC adopts a primary (1°) vulval fate, and the two VPCs flanking it adopt a secondary (2°) vulval fate. In general, hyperactivation of the induction pathway, *e.g.*, animals bearing multiple copies of *lin-3* as transgene, have more than three VPCs that adopt vulval fates and, consequently, a multivulva (Muv) phenotype (Hill and Sternberg 1992); insufficient activation, *e.g.*, loss of *lin-3* function, causes fewer than three VPCs to adopt vulval fates and the animals are vulvaless (Vul; Horvitz and Sulston 1980; Ferguson and Horvitz 1985). In addition to vulval induction, the function of *lin-3* is required in several other aspects of *C. elegans* development. *lin-3* is also required for animal viability, hermaphrodite fertility, as well as cell fate specifications in the male B cell lineage, of the P12 neuroblast, and of the uterine uv1 cells (Sulston and Horvitz 1981; Ferguson and Horvitz 1985; Chamberlin and Sternberg 1994; Clandinin *et al.* 1998; Jiang and Sternberg 1998; Chang *et al.* 1999; J. McCarter, B. Bartlett, T. Dang, R. Hill and T. Schedl, unpublished results). We present genetic analysis of the phenotypes caused by nine alleles of *lin-3*, from which we find that some alleles affect different developmental processes preferentially. These results suggest that *lin-3* may function in different manners for different developmental processes.

We have also determined the molecular lesions of the nine *lin-3* alleles to infer how the structure of *lin-3* defines its function. Many EGF are synthesized as transmembrane precursors, which are cleaved to form solu-

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ble protein products (reviewed by Massagué and Pandielle 1993). The receptor-binding domain is the EGF motif, a short peptide of 50–60 residues. The genomic sequence of *lin-3* predicts that the LIN-3 protein has an extracellular domain with one EGF motif, a transmembrane domain, and a cytoplasmic domain. LIN-3 has no homology to other proteins except the EGF motif (Hill and Sternberg 1992; reviewed by Massagué and Pandielle 1993). LIN-3 is presumably expressed as a transmembrane precursor (Hill and Sternberg 1992). Although cleavage of the LIN-3 precursor has not been demonstrated directly, evidence from genetic and laser ablation studies, as well as structural comparison with other EGFs, suggest that the transmembrane LIN-3 protein can be cleaved to form a secreted product (Sternberg and Horvitz 1986; Thomas *et al.* 1990; Hill and Sternberg 1992; Katz *et al.* 1995). The EGF motif alone is sufficient to mediate some biological activities of LIN-3 (Katz *et al.* 1995). By correlating the mutant phenotypes and the molecular lesions, we demonstrate that the EGF motif is indeed required for LIN-3 function in all developmental processes that require LIN-3. We have also found that the LIN-3 cytoplasmic domain also plays important roles in LIN-3 functions: the N-terminal portion of the cytoplasmic domain is required for LIN-3 function in general, while the C-terminal portion contributes to tissue-specific functions. We also cloned a *lin-3* homolog from another nematode species, *C. briggsae*, which is thought to have diverged from *C. elegans* tens of millions of years ago (Butler *et al.* 1981). Sequence comparison and transgenic studies of *lin-3* in the two species show that the *lin-3* gene has limited functional divergence and high sequence conservation in the two species. The sequence conservation supports our findings in the study of *C. elegans lin-3* mutant alleles.

The EGFs bind to a subfamily of receptor tyrosine kinases, represented by the EGF receptor (EGFR). A major signal transduction pathway activated by the EGFR is the Ras/Raf/mitogen-activated protein kinase (MAPK) pathway. EGFs can also activate phospholipase C-γ (PLC-γ), which is involved in the regulation of intracellular Ca^{2+} and protein kinase C (reviewed by van der Geer *et al.* 1994). The tissue specificity of signaling can be generated by employing different receptors for different tissues. *lin-3* signaling indeed utilizes tissue-specific downstream signal transduction pathways, although there is only one known EGFR in *C. elegans*, encoded by *let-23*. A signaling pathway involving inositol trisphosphate is used downstream of LET-23 for LIN-3 function in hermaphrodite fertility (Lesa and Sternberg 1997; Clandinin *et al.* 1998). In vulval induction, LIN-3 activates LET-23, which is expressed on the surface of the VPCs (Aroian *et al.* 1990; Koga and Ohshima 1995; Simske and Kim 1995; Simske *et al.* 1996). In the VPCs, LET-23 activates LET-60 Ras, which then activates a Raf/MAPK signaling pathway. This signal transduction leads to the division of the VPCs and the morphogenesis of their progeny to form the vulva (reviewed by Sundaram and Han 1996). One nuclear factor downstream of the MAPK pathway is LIN-1, an ETS domain protein homologous to Drosophila Yan (Beitel *et al.* 1995). We present evidence that to maintain animal viability, LIN-3 may utilize yet another pathway or a different regulatory mechanism of one of the aforementioned pathways.

MATERIALS AND METHODS

General methods: Methods for culturing and handling worms, as well as mutagenesis, are described by Brenner (1974). All experiments were performed at 20° unless otherwise noted. Cell and tissue anatomy was observed with Nomarski DIC optics, as described by Sulston and Horvitz (1977). Standard cellular and genetic nomenclature is as defined by Sulston and Horvitz (1977) and Horvitz *et al.* (1979). Transgenic animals were generated by microinjection of DNA (Mello *et al.* 1991; Mello and Fire 1995).

Statistical analysis: Fisher's exact test on a two-by-two contingency table was performed using Instat (GraphPad Software). The null hypothesis that two sets of data are not significantly different is rejected if $P < 0.05$.

Strains: Wild-type *C. elegans* N2 (var. Bristol) is described by Brenner (1974). Wild-type *C. briggsae* was obtained from the *Caenorhabditis* Genetics Center.

Mutant strains of *lin-3* are *lin-3(n1058, n1059, n378)* (Ferguson and Horvitz 1985), *lin-3(s751, s1263)* (Clark *et al.* 1988), and *lin-3(e1417)* (Horvitz and Sulston 1980; Sulston and Horvitz 1981).

Other strains are as follows [strains are described by Brenner (1974) unless otherwise noted]. LGII: *let-23(sa62)* (Katz *et al.* 1996). LGIV: *unc-24(e138), dpy-20(e1282), mec-3(e1338), unc-31(e169), unc-22(s7)* (Moerman and Baillie 1979), *lin-1(e1777)* (Horvitz and Sulston 1980), *let-60(sy130), let-60(sy100)* (Han *et al.* 1990), and *lfe-1(sy290)* (Clandinin *et al.* 1998). LGV: *him-5(e1490)* (Hodgkin *et al.* 1979). Balancer: *DnT1 = nT1[unc(n754dm) let] (IV; V)* (Ferguson and Horvitz 1985).

Characterization of *lin-3* phenotypes: Vulval induction defects were scored by observing how many VPCs generate vulval progeny at the L4 stage. In wild-type animals, three VPCs are always induced (100% induction). In mutant animals, from zero to six VPCs can be induced. Sometimes only one of the two daughters of a VPC adopts vulval fate, and it is counted as 0.5.

Spicule defects were observed in adult males. Wild-type adult males have two spicules that are long, straight, and symmetrically placed on the left and right sides of the animal. The defects in the B lineage caused by *lin-3*/*let-23* pathway mutations make one or both of the spicules short and crumpled (Chamberlin and Sternberg 1993, 1994).

The penetrance of lethality caused by the *lin-3* alleles was determined using the following strains: *unc-24 lin-3(n1059) dpy-20/DnT1, lin-3(sy51) dpy-20/DnT1, mec-3 lin-3(sy52) dpy-20/DnT1, unc-24 mec-3 lin-3(sy53) dpy-20/DnT1, lin-3(s751) unc-22 unc-31/DnT1, lin-3(s1263) unc-22 unc-31/DnT1,* and *lin-3(n1058) dpy-20/DnT1. DnT1* is a translocation between LGIV and LGV, and the only viable animals from +/*DnT1* are +/+ and +/*DnT1*, with a ratio of 1:4 (Ferguson and Horvitz 1985). We therefore multiplied by four the ratio of animals homozygous for *lin-3* to +/*DnT1* animals to infer the percentage of *lin-3* animals that are viable.

The strains that are heterozygous for *lin-3(n1058)* and one *lin-3* lethal allele were constructed in the following manner [using *lin-3(n1059)* as an example]: *unc-24 mec-3 dpy-20*/+ males were crossed with *n1058*/*DnT1* hermaphrodites. Individual male non-Unc progeny were mated with *unc-24 n1059 dpy-20*/*DnT1* hermaphrodites, and on the plates that segregated Unc-Dpy hermaphrodites, the non-Unc, non-Dpy hermaphrodites should be *unc-24 n1059 dpy-20*/*n1058*. The viability was then determined by comparing the number of *unc-24 n1059 dpy-20*/*n1058* animals with that of *unc-24 n1059 dpy-20*/*unc-24 mec-3 dpy-20*, which should be 1:1 if the viability is 100%.

To determine the effects of combinations of *lin-3* alleles on fertility, as well as vulval and male spicule development, we built strains that carried one *lin-3* lethal allele and either *n378* or *n1058* as follows (again using *n1059* as an example): (1) *n378; him-5* males were crossed with *unc-24 n1059 dpy-20*/*DnT1* hermaphrodites. F_1 non-Unc animals were *unc-24 n1059 dpy-20*/*n378* and were used to observe phenotypes. (2) *n1058 dpy-20*/*DnT1* hermaphrodites were crossed with N2 males, and F_1 non-Unc males were then crossed with *unc-24 n1059 dpy-20*/*DnT1*. Dpy hermaphrodites were used to determine their fertility and vulval differentiation; Dpy males were used to observe spicule development. In all strains, vulval differentiation was quantified as the percentage of induction relative to wild type. Male spicule defects were represented by the percentage of crumpled spicules in all spicules observed. Brood size was determined by counting the number of larvae generated by a hermaphrodite.

Suppression of *lin-3* lethality: To determine the effect of *lin-1(e1777)* on *lin-3* lethality, we crossed *lin-1*/+ males with *unc-24 lin-3(n1059) dpy-20*/*DnT1* hermaphrodites. We individually picked the Muv progeny of *unc-24 lin-3(n1059) dpy-20*/*lin-1* animals and observed whether some of them segregated Unc Dpy Muv (*lin-1 unc-24 lin-3 dpy-20*) animals. We confirmed it by taking advantage of the fact that *lin-1* does not suppress the sterility caused by *lin-3* (Clandinin *et al.* 1998); therefore, *lin-1unc-24 lin-3 dpy-20* animals should be sterile. To test the effect of *let-60(sy130)* on *lin-3* lethality, we crossed *lin-3(n1059) let-60(sy130) dpy-20*/*unc-24 let-60(sy100) dpy-20* hermaphrodites with *lfe-1 unc-24*/+ males. We then looked for Dpy animals, which should be *lin-3(n1059) let-60(sy130) dpy-20*, in the progeny of *lin-3(n1059) let-60 dpy-20*/*lfe-1 unc-24*. To test whether the gain-of-function *let-23* mutation [*let-23*(*gf*)] suppresses the lethality of a non-null *lin-3* allele, *let-23(gf) unc-4*/+ males were crossed with *lin-3(sy51) dpy-20*/*DnT1* hermaphrodites. We observed whether there were Unc Dpy animals that should be *let-23(gf) unc-4; lin-3(sy51) dpy-20* in the progeny of *let-23(gf) unc-4; lin-3(sy51) dpy-20*/+ hermaphrodites.

Determination of molecular lesions of *lin-3* alleles: Mutant *lin-3* genomic DNA was amplified by the single-worm PCR method (Williams *et al.* 1992). Two to three L2 or L3 larvae were used as a template for *n378*, *e1417*, and *n1058*. For alleles that cause a completely penetrant lethal phenotype, 10 dead larvae were used as the source of DNA template. The exons D to K were sequenced first, since this region is sufficient for wild-type function in vulval induction (Figure 2; Hill and Sternberg 1992). Only when no mutation was identified in this region were other exons sequenced. The exons and exon-intron junctions were PCR-amplified as several fragments. Each fragment was cloned into pBluescript (SK+) (Stratagene, La Jolla, CA). For each region, the products of at least two different PCR reactions were sequenced. An ABI automated sequencer was typically used for sequencing, with the Sanger method of dideoxy-mediated chain termination used for a few samples (Sambrook *et al.* 1989). The sequences were compared with the wild-type sequence. To avoid PCR and sequencing errors, a mutation was confirmed only if sequences from all PCR reactions show the same nucleotide change.

The DNA oligos for the PCR and sequencing reactions are as follows (from 5′ to 3′): CTCTGATTATTTTCCAGTTTTCC, CGACAATATTTCCTTATGTTTCTC, CGATTTTCAAAATT TGGAGACATG, and CGATCCGTTCAATATGTTTTAAG for exons A, B, and C; CGTCTGGCGAATAGCCGTATTTTG and CGAAGGGAGACACGATTCTGAAAC for exon C2; ATGTT CGGTAAATCGATTCTGAAC, GTTCGGTAAATCGATTCC, GGAACGAAAACTCAAAAGG, CTCAGAAGTCAAGGTACCA TTCC, CGAAAACCGAGAAATCTG, and ACCGAGAAATCT GAAAAATGGAACG for exons D and E; GCTTGTTGAAA TAATTAAAAACGGG, CCGAAAATCGACACCCTTG, CATG CAACTTAATTAGGG and ATGCTACATGCAACTTAATT AGGG for exons F and G; TGGGCTTTATGAGAGAA TTGTGG, GAGAGAATTGTGGTGAG, and CAAATTTATCG GTCATTTTTCTCC for exon H; ATTGTTTTCTAATCAACA CACAGC, GTTTTCTAATCAACACACAG, CTCAGAAGTC AAGGTACCATTCC, CTTGTAGTGCTTCGGCGTGTCG, GT AATATCACCTCGATTC, and GCATTTGAGTAATATCAC CTCG for exons I, J, and K; and GACGCAGTTCAACCTGG TATCG, GAGAACTATAGAACATTTGGGTGG, CTCTGTG CTATAATTGTGATTTAC, and CTCGACATCAAGGTTCAC GGAG for exon L.

F_1 noncomplementation screen of *lin-3* mutants: To screen for new *lin-3* alleles, *unc-24 mec-3 dpy-20* hermaphrodites were treated with ethyl methanesulfonate (EMS) and then mated with *lin-3(e1417); him-5* males. A total of 8000–12,000 F_1 cross-progeny were observed, and egg-laying-defective animals were picked. Three alleles of *lin-3*, *sy51*, *sy52*, and *sy53* were obtained from the screen. They caused completely penetrant lethality at the L1–L3 stages.

Cloning of *lin-3* from *C. briggsae* and test of functional equivalence of *C. elegans lin-3* and *C. briggsae lin-3*: *C. briggsae lin-3* was isolated from a *C. briggsae* genomic library (provided by D. Baillie) by hybridization with a *C. elegans lin-3* cDNA clone, pRH40 (Hill and Sternberg 1992). Low-stringency conditions were used for hybridization: low-stringency buffer (Sambrook *et al.* 1989), 50° for primary screening, 65° for secondary and tertiary screenings for 8 hr or more, washed at 50° (2× SSC, 1% SDS for 10 min; 0.2× SSC, 1% SDS for 30 min). A total of 3 different clones were selected from 11 positive clones that were identified from >250,000 plaques. From one of the three clones, λPT-4b, 6.5 kb was sequenced and found to contain a *C. briggsae* homolog of *lin-3*. For functional tests, λPT-4b was injected into wild-type *C. elegans* and *C. briggsae* with pRF4 (Mello *et al.* 1991) as a transformation marker. For injections into *C. briggsae*, 0 of 4 lines of 25 ng/μl λPT-4b (plus 25 ng/μl pRF4) segregated Muv animals; 1 of 1 line of 50 ng/ml λPT-4b (plus 50 ng/μl pRF4) segregated Muv animals; and 6 of 14 lines of 75 ng/μl λPT-4b (plus 50 ng/μl pRF4) segregated Muv animals. For injections into *C. elegans*, 2 of 8 lines of 50 ng/μl λPT-4b (plus 50 ng/μl pRF4) segregated Muv animals, and 8 of 10 lines of 100 ng/μl λPT-4b (plus 100 ng/μl pRF4) segregated Muv animals. pRH36, a construct containing *C. elegans lin-3* (Hill and Sternberg 1992), was injected at 50 ng/μl (plus 50 ng/μl pRF4) into *C. briggsae* for the reciprocal experiment; three of eight stable lines segregated animals with a Muv phenotype. In addition, PS1253 carrying transgene *syEx30* was derived from an injection of 65 ng/μl pRH36 plus 50 ng/μl pRF4. The *C. briggsae* strain PS1252, which bears a transgene of *C. briggsae lin-3*, *syEx29* (derived from a 50 ng/μl injection), was used to observe the vulval phenotype under Nomarksi optics.

Detection of exon C2: To determine whether the predicted exon C2 is utilized in wild-type animals to provide an alternative signal sequence, we performed reverse-transcriptase PCR (RT-PCR) on total RNA extracted from N2 worms. Worms of mixed stages were grown on three 15-cm special nematode growth medium (NGM) plates [modified from Brenner

(1974) by increasing peptone to 2% (w/v) and cholesterol to 20 mg/liter] until confluence. Total RNA was extracted using the method developed by R. Burdine and M. Stern (personal communication). Before RT-PCR reactions, the RNA template was treated with RNase-free DNase I (Promega, Madison, WI) for 30 min. RT-PCR was performed using the GeneAmp EZ r*Tth* RNA PCR kit (Perkin Elmer, Norwalk, CT). The primers used are 5′ CCTGAACGACTTCTAGTCGC 3′, which is within the predicted exon C2, and 5′ GCGAAGAAGATGAAGAAG TAGTTGG 3′, which is in exon D. A band of 350 base pairs should be amplified if exon C2 is indeed utilized. A primer spanning part of exon C, 5′ TCGTGTCTCCCTTCGT GGTTTCGTC 3′, was used in conjunction with the primer in exon D to confirm that exon C is used. The negative control was performed using random *Escherichia coli* primers.

RESULTS

Comparison of *C. elegans lin-3* and *C. briggsae lin-3*: Outside of the EGF motif, *C. elegans* LIN-3 has no sequence similarity to any other known protein. To infer functional importance of other domains, we cloned the *C. briggsae lin-3* by low-stringency hybridization using a *C. elegans lin-3* genomic probe. The genomic structure of *C. briggsae lin-3*, therefore, is inferred from the comparison to *lin-3* structure in *C. elegans*.

We first tested whether *C. elegans lin-3* and *C. briggsae lin-3* have interchangeable functions. Most *C. elegans* animals bearing multiple copies of *lin-3* as a transgene exhibit a Muv phenotype ($n > 50$; also see Hill and Sternberg 1992; Chang *et al.* 1999). Likewise, we found that *C. elegans* animals become Muv when bearing multiple copies of *C. briggsae lin-3* as a transgene ($n > 50$). When *C. briggsae* animals bear a transgene with multiple copies of *C. briggsae lin-3*, the animals have vulval defects. Similarly, the expression of multiple copies of *C. elegans lin-3* from a transgene induces similar defects in *C. briggsae* (Figure 1). However, instead of the pseudovulvae seen in *C. elegans*, many *C. briggsae* animals have one big, protruding vulva. Closer examination revealed that this is because some *C. briggsae* transgenic animals have adjacent 1° vulval cells (four of the five animals we observed had more than three VPCs induced; among the four animals, three had anatomy consistent with the presence of adjacent 1° vulval precursor cells), which can cause the formation of protruding vulvae (P. Sternberg and R. Palmer, unpublished observations). The phenotypic difference caused by the expression of multiple copies of *lin-3* in the two species might reflect subtle differences in the signaling mechanism of *lin-3* or in the responses of VPCs to *lin-3*. However, it is clear that *lin-3* in both species plays a role in vulval formation. Other evidence supporting the functional conservation of *lin-3* in the two species is that a *C. elegans lin-3::lacZ* construct was expressed in the AC of *C. briggsae* when introduced as a transgene (data not shown).

We then compared the number of exons, intron locations, nucleotide sequences, and the inferred protein sequences of *C. briggsae* and *C. elegans lin-3*. The number of exons and intron locations of *lin-3* are almost identical in the two species (Figure 2). The most notable difference is that the EGF motif spans three exons in *C. elegans lin-3*, but only two in *C. briggsae lin-3*. The exons have identity at the nucleotide level ranging from 65 to 91%. For introns, 39% of the *C. elegans* sequence are identical to that of *C. briggsae*, and 53% of the *C. briggsae* intron sequences are identical to that of *C. elegans*. The difference arises because the introns in the two species are of different total lengths. The identity between individual introns ranges from 38 to 63%. This is an overestimate, since we always use the shorter sequence as the reference when two corresponding introns have different sizes in the two species. The overall amino acid sequence identity in the coding region is 76% (Figure 3). Previous sequence comparisons between *C. elegans* genes and their homologs in *C. briggsae* have demonstrated that exon and intron structures and protein sequences are highly conserved in the two species, but intronic and flanking sequences are not. The nucleotide identity for the coding region is generally >60%, and an even higher degree of identity is present for protein sequences (for examples, see Kennedy *et al.* 1993; Maduro and Pilgrim 1996). Results from our comparison of *lin-3* in the two species are consistent with the previous studies.

LIN-3 protein can be divided into several domains: the signal sequence (which will be described later), the region between the signal sequence and the EGF motif, EGF motif, the region between the EGF motif and the transmembrane domain, the transmembrane domain, and the cytoplasmic domain. However, we will not attempt to determine which domain is functionally important using sequence identity as the sole criterion, since the overall degree of identity between LIN-3 from the two species is very high. The EGF motif is the most conserved region with 96% amino acid identity. This is consistent with the functional importance of this motif. The region between the signal sequence and the EGF motif is less conserved, with 65% amino acid identity. The 16 amino acids preceding the EGF motif, however, are completely conserved and contain several lysines and arginines. A region rich in lysines and arginines N-terminal to the EGF motif is required for the heparin binding of heparin-binding EGF (Thompson *et al.* 1994), and it is possible that this domain of LIN-3 also interacts with extracellular matrix proteins. Most precursors of EGF family members are cleaved at both the N and C terminus of the EGF motif, and the lysines and arginines N-terminal to the EGF motif are required for the cleavage of amphiregulin precursor (Thorne and Plowman 1994). This region of LIN-3 might also have functions in the secretion of a soluble LIN-3. The region between the EGF motif and the transmembrane domain should also be involved in the cleavage of the LIN-3 precursor, and 89% of the residues in this region are conserved. The transmembrane domain in the two spe-

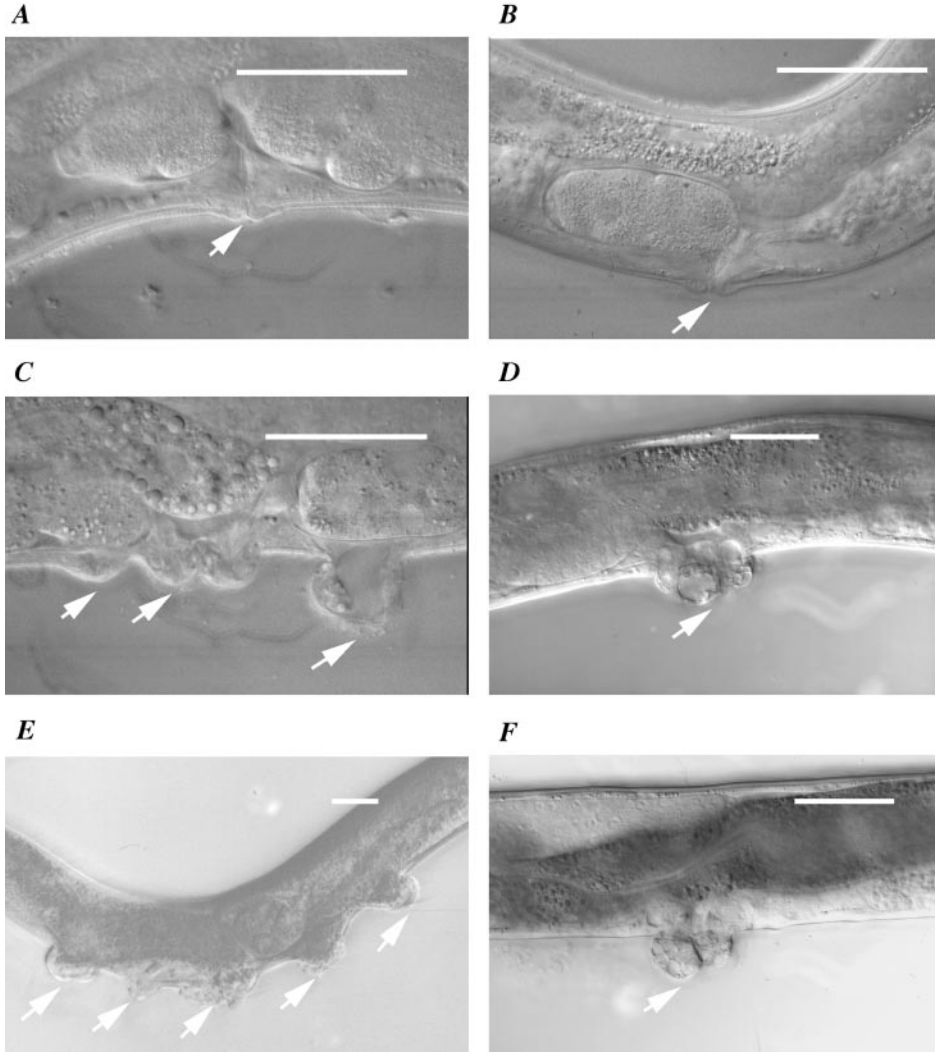

Figure 1.—*lin-3* and its *C. briggsae* homolog are functionally interchangeable in vulval induction. Adult animals are shown in all panels. The anterior of the animal is to the left and the ventral side is down. Wild-type vulvae in (A) *C. elegans* and (B) adult *C. briggsae*, respectively. (C) Overexpression of *C. elegans lin-3* causes a Muv phenotype in *C. elegans*. (D) Overexpression of *C. briggsae lin-3* causes the formation of a big, protruding vulva in *C. briggsae*. (E) Overexpression of *C. briggsae lin-3* causes a Muv phenotype in *C. elegans*. (F) Overexpression of *C. elegans lin-3* causes the formation of a big, protruding vulva in *C. briggsae*. Scale bars represent 0.04 mm in A–C and ~0.04 mm in D–F. The arrows indicate the positions of vulvae or pseudovulvae.

cies has 88% of the amino acids conserved. Although none of the *lin-3* alleles has a mutation in this domain (see below), it should nonetheless be important for membrane localization of the LIN-3 precursor. The cytoplasmic domain has an overall amino acid identity of 79%. The N-terminal portion of the cytoplasmic domain is less conserved compared to the more C-terminal region. The cytoplasmic domains of various EGF growth factors are quite dissimilar and usually very short (Massagué and Pandiella 1993). However, the cytoplasmic domain of LIN-3 is unusually long, and our findings from the study of *lin-3* phenotypes and molecular lesions suggests that the cytoplasmic domain is functionally important (see below).

There are two alternative splice sites in *C. elegans lin-3*, and both are conserved in the *lin-3* homolog of *C. briggsae* (Figures 2 and 3). One alternative splice site lies between the EGF motif and the transmembrane domain (Hill and Sternberg 1992). The two putative protein products in *C. elegans* may differ in the range of their signaling (J. Liu and P. W. Sternberg, unpublished results). The alternatively spliced region consists of 15 amino acids and is 87% identical in the two species. This is consistent with the possibility that both proteins are functionally important. LIN-3 may also have two alternative N termini: one encoded by exons A, B, and C, and the other encoded by exon C2. Either exon C or C2 could provide a signal sequence for the membrane localization of the LIN-3 precursor (Hill and Sternberg 1992; Figure 2). Although all known cDNAs of *C. elegans lin-3* contain exon C but not C2, exon C2 appears to be functional since transgenes containing exon C2, but not C, can induce vulval differentiation (Hill and Sternberg 1992; J. Liu and R. Hill, unpublished data). It is possible that in a genomic sequence lacking exon C, C2 can substitute for its function. When we performed RT-PCR on RNA extracted from *C. elegans* of mixed developmental stages, we were indeed able to amplify sequences encoded by exon C2, although with very low efficiency (data not shown). A total of 64% of the amino acids of the signal sequence encoded by exon C and 63% of those encoded by exon C2 are conserved between the two species (Figure 3).

Exons A, B, and part of C constitute the 5′ untrans-

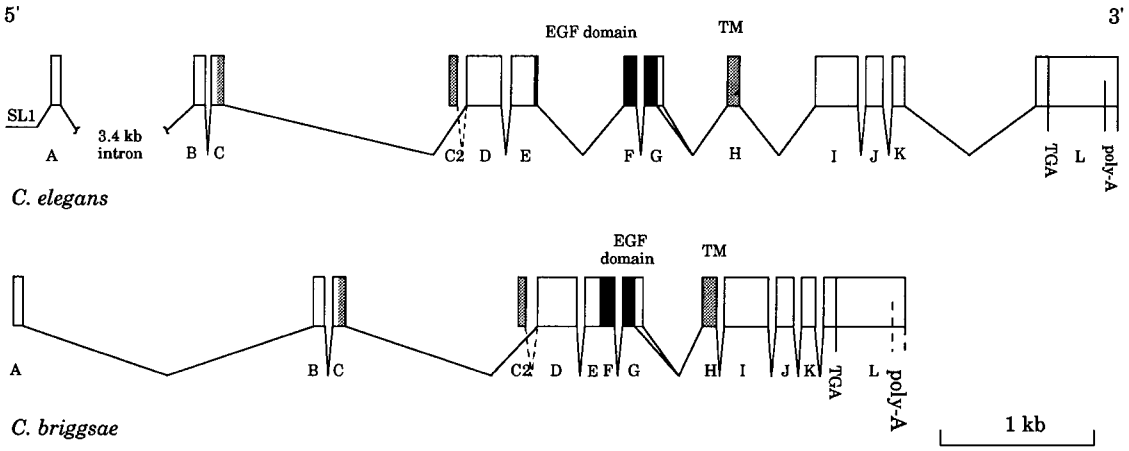

Figure 2.—Genomic structures of *C. elegans* and *C. briggsae lin-3*. Exons are represented as boxes. Three exons code for the EGF motif in *C. elegans*, E, F, and G, but only two in *C. briggsae*. We named these two exons EF and G to facilitate comparison. The black region is the EGF motif. The shaded region 3′ to the EGF motif is the transmembrane domain. The shaded regions 5′ to the EGF motif are signal sequences. Exon C is present in *C. elegans lin-3* cDNA, but not the original genomic clone, which nonetheless provided vulval-inducing function (Hill and Sternberg 1992). Exon C2 may provide an alternative signal sequence.

lated region (UTR) in *C. elegans* cDNA. The 5′ UTR is 71% identical (out of 152 nucleotides) with a region in the *C. briggsae* genomic DNA that is 5′ to the open reading frame. The degree of identity is higher than that between the introns. This region may have some regulatory functions.

***lin-3* alleles can cause either tissue-specific or tissue-general defects:** Seven *lin-3* mutant alleles have been obtained previously in various genetic screens: *e1417* and *n378* in a screen for recessive mutations with vulval defects (Horvitz and Sulston 1980; Ferguson and Horvitz 1985), *n1058* and *n1059* in a screen for mutations that fail to complement *lin-3(e1417)* (Ferguson and Horvitz 1985), *sy91* as a transposon-induced allele in a screen for mutations that fail to complement *e1417* (Hill and Sternberg 1992), and *s751* and *s1263* in a screen for recessive lethal mutations (Clark *et al.* 1988). We carried out an additional noncomplementation screen against *e1417*, and we obtained *sy51*, *sy52*, and *sy53*. All three are recessive alleles (data not shown) and cause completely penetrant lethality when homozygous. All 10 alleles of *lin-3* are recessive, loss-, or reduction-of-function alleles. *sy91* has a transposon insertion in one of the introns (Hill and Sternberg 1992), and it is not clear what effect such an insertion has on LIN-3 protein. We therefore focused our study on the 9 EMS-induced alleles.

Mutations in *lin-3* affect vulval induction as well as hermaphrodite fertility, male spicule development, and viability of both hermaphrodites and males. Ferguson *et al.* (1985) demonstrated that the lethality and Vul phenotype caused by *lin-3* are loss-of-function phenotypes. The loss-of-function phenotype of *lin-3* in male spicule development is an anterior-to-posterior fate transformation in the B lineage (Chamberlin and Sternberg 1993). Some *lin-3* alleles cause hermaphrodite sterility. A partial description of this phenotype can be found elsewhere (Clandinin *et al.* 1998). *lin-3* is also involved in the specification of P12 neuroectoblast in the ventral posterior epidermis. Since recessive *lin-3* alleles do not alter P12 fate specification unless combined with other mutations, we will not further discuss this phenotype (Jiang and Sternberg 1998).

Six alleles cause completely penetrant larval lethality: *n1059*, *s751*, *s1263*, *sy51*, *sy52*, and *sy53* (Table 1). *n1059* is a genetic null allele and causes animals to arrest at the first larval stage (L1; Ferguson and Horvitz 1985). *s1263* and *s751* animals arrest at L2 or L3 (Clark *et al.* 1988). *sy51* and *sy52* cause L2 to L3 lethality, and *sy53* causes L1 lethality, as judged by the size of dead worms. Animals homozygous for these alleles die without undergoing the other developmental events mediated by *lin-3* activity. We therefore studied the functions of these alleles in fertility and vulval and male spicule development by examining animals heterozygous for one lethal allele and a viable *lin-3* allele. These animals survive but show defects in vulval development, male spicule formation, and hermaphrodite fertility, indicating that the lethal alleles also disrupt these other developmental processes (Table 1). However, there is inhomogeneity between different lethal alleles. For example, animals heterozygous for *n1059* and *n378* display stronger defects in fertility and vulval and spicule development, compared to animals heterozygous for *n378* and any other lethal allele. This suggests that the other lethal alleles are not null. *sy53* retains some activity in hermaphrodite fertility when in *trans* to *n1058*, a feature unique among the lethal alleles.

Three other alleles, *e1417*, *n378*, and *n1058*, cause little or no lethality. Since they disrupt only some of the developmental processes mediated by *lin-3*, we refer to them as having tissue-specific defects. *e1417* causes de-

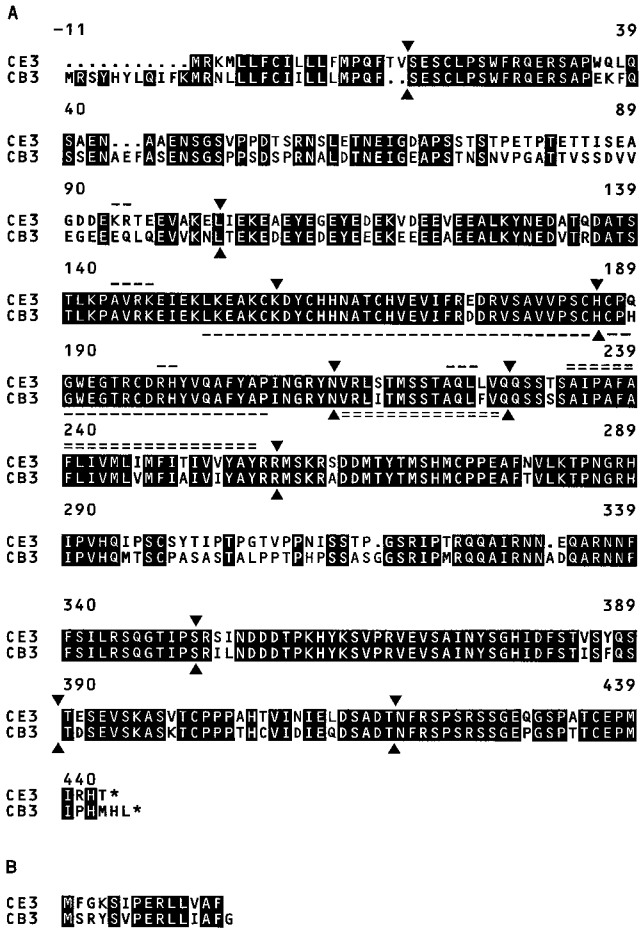

Figure 3.—(A). Comparison of protein sequences of *C. elegans* (CE3) and *C. briggsae* (CB3) *lin-3*. Letters in black boxes represent conserved amino acids. The triangles indicate intron positions. The EGF motif is underlined. The alternatively spliced region is doubly underlined. Overlined sequences are the putative cleavage sites of the transmembrane LIN-3. The transmembrane region is indicated with double overline. (B) The comparison of the alternative exon C2 in the two species. A total of 63% of the amino acids are conserved.

fects only in vulval development, both when homozygous and when *in trans* to the null allele *n1059* (Sulston and Horvitz 1981; Ferguson and Horvitz 1985; Table 1). Thus, *e1417* appears to retain wild-type *lin-3* activity in all other developmental processes. *n378* is defective only in the development of vulva and male spicules, causing a severe Vul phenotype and a weak spicule defect (Ferguson and Horvitz 1985; Table 1). When *in trans* to *n1059*, the defects become more severe, but there is still no defect in viability and fertility. *n1058* animals have a weak Vul phenotype and weak defects on viability, but hermaphrodites are completely sterile and males have strong spicule defects. *In trans* to *n1059*, the Vul defect and the lethality are enhanced (Ferguson and Horvitz 1985; Table 1). *n1058* also exhibits intragenic complementation on vulval induction with *n378* (Table 1) and *e1417* (Ferguson and Horvitz 1985).

We examined the severity of the phenotypes caused by each *lin-3* allele for four separate developmental processes. For each process, we constructed an allelic series by ranking the alleles according to the severity of their effects on that process (Figure 4). We find that the allelic series is different for different processes. For example, *n1058* confers a much more severe defect in fertility than *n378*, but has a less severe effect in vulval induction. This suggests that not only do some alleles of *lin-3* cause tissue-specific defects, but also that their functions in different developmental processes are independently mutable.

To understand how various domains of LIN-3 may affect its function, we sequenced nine EMS-induced *lin-3* alleles to determine their molecular lesions. All except *e1417* have mutations within the coding region. *e1417* has mutations in neither the exons, nor the exon-intron junctions, nor the 5′ UTR, and the mutation may thus lie in a noncoding regulatory region.

Mutations in an extracellular region other than the EGF motif: *n378* changes Glu21 to Lys, which is the first amino acid after the signal sequence in the extracellular domain (Figure 5), regardless of which signal sequence is used. In many other EGFs, the region surrounding the signal sequence is removed to form the mature protein product (reviewed by Massagué and Pandiella 1993). Therefore, it is unlikely that the mutation in *n378* affects the properties of the final product of LIN-3. The effect of *n378* is thus more likely to be on the LIN-3 precursor. Since a signal sequence is critical in the translocation of proteins from the cytoplasm to the plasma membrane or extracellular space, the mutation in *n378* may result in a lower concentration of LIN-3 on the cell surface by affecting the structure and stability of the LIN-3 precursor.

EGF motif is essential for the function of *lin-3*: Four *lin-3* lethal alleles have mutations in the EGF motif (Figure 5). *n1059* changes Trp188 to a stop codon, which would result in a protein missing the last 17 residues of the EGF motif, including the sixth cysteine. This constitutes the molecular evidence that *n1059* is a null allele. *s1263*, another lethal allele, changes the first cysteine in the EGF motif, Cys154, to a tyrosine. *sy53* changes the last nucleotide in exon D from G to A. This nucleotide alteration could have two effects on the *lin-3* protein product. First, Asp156, between the first and second cysteine, would be changed to Asn. Second, it alters a partially conserved G in front of a splice donor into an A and could make RNA splicing less efficient (Blumenthal and Steward 1997). A failure to splice out the downstream intron would result in a message encoding a protein product with an incomplete EGF motif having only the first cysteine. However, we do not know which effect is the principal cause of the phenotypes associated with this mutation. *sy51* changes a conserved splice donor from *gt* to *at*. The mutation could result in a protein with a truncated EGF motif having

TABLE 1

lin-3 mutant phenotypes

lin-3 genotype	Viable (%)	Vulval induction (%)	Wild-type spicule (%)	Brood size
+	100 (>50)	100 (>50)	100 (90)	+[c] (>50)
n1059	0, Early larval[a] (>100)	NA	NA	NA
sy51	0, Midlarval (>100)	NA	NA	NA
sy52	0, Midlarval (>100)	NA	NA	NA
s751	0, Midlarval[b] (>100)	NA	NA	NA
s1263	0, Midlarval[b] (>100)	NA	NA	NA
sy53	0, Early larval (>100)	NA	NA	NA
e1417	100 (>100)	22 (102)	100 (70)	+[c] (>20)
n378	100 (>100)	19 (24)	94 (98)	+[c] (>20)
n1058	68 (708)	50 (15)	10 (40)	0 (>20)
e1417/n1059	100[a] (>100)	0.8 (23)	99 (70)	+[a,c] (>20)
n378/n1059	100[a] (100)	3 (30)	26 (84)	+[a,c] (>20)
n1058/n378		88 (31)	52 (50)	
sy51/n378		0 (36)	64 (86)	
sy52/n378		3 (34)	82 (74)	
s1263/n378		26 (38)	56 (68)	
sy53/n378		23 (35)	83 (64)	
n1058/n1059	20 (38)	1.3 (25)	12 (68)	0 (12)
sy51/n1058				0 (20)
sy52/n1058				0 (17)
s1263/n1058				0 (10)
sy53/n1058	47 (376)	43 (26)	2.5 (40)	5.1 (24)

We quantified the defects of *lin-3* alleles in viability, fertility, and vulva and male spicule development. See materials and methods for detailed descriptions. *sy52* and *s751* are two alleles with an identical molecular lesion; therefore, we characterized only the phenotypes of *sy52*. The numbers in the parentheses indicate the sample size. For viability and vulval induction, the sample size is the number of animals examined; for male spicule, the sample size is the number of spicules examined, which is twice the number of animals. For fertility, the sample size is the number of animals whose progeny were counted. We also indicate the stages of arrest for the lethal alleles. We calculated the *P* values for the severity of phenotypes caused by various alleles if the difference was not obvious. For viability, compared to +, $P_{ln1058} < 0.0001$. For vulval induction, $P_{n378/n1059\text{-}e1417/n1059} = 0.63$, $P_{n1058\text{-}n378} = 0.001$, and $P_{e1417\text{-}n1058} = 0.0003$. For male spicule development, $P_{n378\text{-}sy53/n378} = 0.01$, $P_{sy53/n378\text{-}sy51/n378} = 0.016$, $P_{sy51/n378\text{-}s1263/n378} = 0.32$, and $P_{n1058\text{-}n1058/sy53} = 0.36$. For fertility, assuming a brood size of 50 if the Vul animals are fertile, $P_{sy53/n1058\text{-}n1058} < 0.0001$. +, wild type; NA, not applicable.

[a] Also from Ferguson and Horvitz (1985).

[b] Also from Clark *et al.* (1988).

[c] Wild-type animals can have >300 progeny. Although not sterile, the brood sizes of Vul animals are generally much lower than 300 because the eggs cannot be laid in Vul animals; instead, they occupy the ovary and prevent late oocytes from being fertilized.

only the first cysteine. That all four alleles cause tissue-general defects demonstrates that the EGF motif is crucial for *lin-3* function in all developmental processes that require *lin-3*.

Why, if the EGF motif is crucial, is only one of the four alleles a null allele? There are two possible explanations. First, the position of the stop codon in *n1059* is 3′ to the altered splice donor in *sy51*. Therefore, *sy51* should produce a protein shorter than that in *n1059*. However, *sy51* causes a less severe defect than *n1059*. It is possible that some splicing still occurs in *sy51*, resulting in a small amount of full-length protein. In fact, it has been shown that altering the conserved nucleotides of splice donors diminishes, but does not abolish, splicing in *C. elegans* (Rushforth and Anderson 1996; Zhang and Blumenthal 1996). Second, the alteration of Cys154 in *s1263* results in severe reduction-of-function, but non-null, phenotypes (Table 1). It indicates that a full-length EGF motif containing a mutation in one of the conserved cysteines can have residual activity. This is consistent with previous findings that changing Cys164 to serine, or changing Cys184 to serine, does not completely abolish vulval induction (Hill and Sternberg 1992).

sy53 causes a lethal phenotype as severe as that caused by *n1059*, but retains some activity in fertility, vulval induction, and male spicule development. It is not clear why a change in the EGF motif would cause total loss of activity in one developmental process but partial loss in others.

The cytoplasmic domain is important for both tissue-general and tissue-specific functions of *lin-3*: The cytoplasmic domain of LIN-3 (185 residues) is longer than

A

	Viability	Vulval induction	Male spicule	Fertility
+	+	+	+	+
e1417	+	(-)	+	+
n378	+	(-)	(+)	+
n1058	(+)	(+/-)	-	-
n1059	-	-	-	-
s1263	(-)	(-)	(-)	-
sy51	(-)	-	(-)	-
sy52	(-)	-	(+/-)	-
sy53	-	(-)	(+/-)	(-)

B

Viability:
e1417, n378 > n1058 > sy51, sy52, s1263 > sy53, n1059

Vulval induction:
n1058 > e1417, n378, s1263, sy53 > sy51, sy52, n1059

Male spicule development:
e1417 > n378 > sy52, sy53 > sy51, s1263 > n1058, n1059

Hermaphrodite fertility:
e1417, n378 > sy53 > sy51, sy52, s1263, n1058, n1059

Figure 4.—(A) A summary of phenotypes caused by *lin-3* alleles. +, wild-type; (+), (+/−), (−), and −, four levels of activity, each level lower than the preceding one, with (+) closest to wild-type activity. (B) Phenotypic series of *lin-3* alleles. The four series were established on the basis of the data in Table 1. Alleles to the left have more wild-type activity than the ones to the right of each row. In the series of viability, *n1059* and *sy53* were determined to have less activity than the rest of the lethal alleles, since they both cause lethality at L1 stage, while the rest of the lethal alleles cause worms to die at the L2-L3 stages. In the series for vulval induction and spicule development, defects caused by the lethal alleles were assessed when the lethal alleles were *in trans* to *n378*. Since *sy52* and *s751* have the same molecular lesion, only the phenotypes of *sy52* were analyzed.

that of most other growth factors that have transmembrane precursors (reviewed by Massagué and Pandiel le 1993). Three alleles, *n1058*, *sy52*, and *s751*, have mutations in this domain.

n1058 disrupts a conserved splice donor at the 3′ end of exon I (Figures 2 and 5). It could make a protein that has only the N-terminal 94 residues of the cytoplasmic domain, but could also make a small amount of full-length protein if, as suggested for *sy51*, splicing is only diminished, but not abolished. Both *sy52* and *s751*, isolated in two laboratories, disrupt the splice acceptor of exon I (Figures 2 and 5). It may make a protein that lacks 92 residues at the N terminus of the cytoplasmic domain. It is also possible that other mutant protein products are made by mRNAs spliced at cryptic splice sites close to the mutated one (Aroian *et al.* 1993). *sy52* and *s751* cause tissue-general defects.

The role of *lin-3* in animal viability: We tested whether the *let-23*/*let-60* pathway, which mediates vulval induction, also mediates *lin-3* function in viability. A gain-of-function (*gf*) *let-60* mutation can rescue the lethality caused by *let-60* dominant-negative (*dn*) mutations and a *let-23* null mutation (Aroian *et al.* 1990; Beitel *et al.* 1990; Han *et al.* 1990). Similarly, a *let-23(gf)* mutation rescues the lethality caused by a *let-23* null allele (Katz *et al.* 1996). However, *let-23(gf)* and *let-60(gf)* failed to rescue the lethality caused by *lin-3(lf)*. In the progeny of *let-23(gf) unc-4; lin-3(sy51) dpy-20*/+ hermaphrodites, we have almost never seen adult Unc Dpy animals, which should be *let-23(gf) unc-4; lin-3(sy51) dpy-20* ($n > 500$). Instead, we have seen dead larvae. Similar results were obtained when we used *lin-3(n1059)* in the experiment. We only occasionally saw Dpy animals in the progeny of *lin-3(n1059) let-60(gf) dpy-20*/*lfe-1 unc-24* ($n > 500$). This failure to suppress *lin-3* lethality is unlikely to result from *let-23(gf)* and *let-60(gf)* only weakly activating the *let-23*/*let-60* pathway, since both are able to rescue the lethality caused by a *let-23* null allele. It is possible that the *let-23*/*let-60* pathway is not sufficient to mediate *lin-3* signaling in viability, and a different downstream pathway may be required. We then tested whether *lin-1(rf)* is able to suppress the lethality caused by *lin-3(n1059)*. A total of 10% of *unc-24 lin-3 dpy-20*/*lin-1* animals segregated recombinant *lin-1 unc-24 lin-3 dpy-20* ($n = 120$), which grew to sterile adults. If *lin-1* does not suppress *lin-3* lethality, no *lin-1 lin-3* animals should grow to adulthood. If *lin-1* completely suppresses *lin-3* lethality, all *lin-1 lin-3* animals should grow to reach adulthood. The

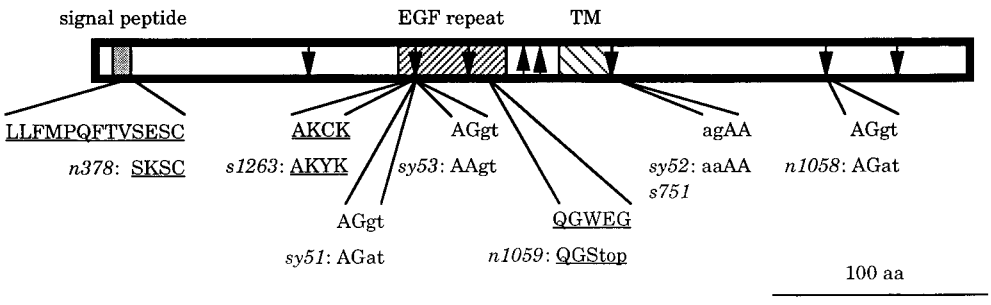

Figure 5.—Molecular lesions of *lin-3*. Codon changes and changes in the conserved splice sites are listed for all *lin-3* alleles except *e1417* and *sy91*, which have no change in the coding sequences and exon-intron junctions. Only exons C to K are shown, since there are no mutations in exons A, B, and L. *sy91* has a Tc1 insertion in the intron after exon C2 (see Figures 2 and 3). The downward arrows indicate intron positions, and the upward arrows indicate alternative splice sites. The underlined sequences are amino acids, and the nonunderlined sequences are nucleotides. Uppercase letters represent exon sequences, and lowercase letters represent intron sequences.

number of adult *lin-1 lin-3* recombinants is consistent with the null hypothesis that *lin-1 lin-3* animals are generated from recombination and that *lin-1* completely suppresses *lin-3* lethality ($P = 0.547$, Fisher's exact test); therefore, we concluded that *lin-1* almost fully suppresses the lethality of *lin-3*. Therefore, if there is an alternative pathway mediating the function of *lin-3* in viability, this pathway should also repress *lin-1*. An alternative possibility is that *lin-3* mediates animal viability using one of the two previously identified pathways, but there are different regulatory mechanisms for the pathway in different developmental processes (N. Hopper, personal communication).

DISCUSSION

lin-3 is required for multiple developmental processes in *C. elegans*. To understand how the structure of *lin-3* contributes to its function, we determined the molecular lesions of *lin-3* mutant alleles and characterized their phenotypes. We also cloned the *lin-3* homolog in *C. briggsae* and inferred the importance of various domains of the protein by comparing the sequence of *lin-3* of *C. elegans* with that of *C. briggsae*.

General model of *lin-3* function: The most notable feature of the EGFs is the EGF motif in the extracellular domain. The scaffold of the tertiary structure of the EGF motif consists of six cysteines, which have conserved spacing and form three disulfide bonds (reviewed by Groenen *et al.* 1994). The EGF motif of LIN-3 is only moderately conserved with that of other members of the EGF family, but the presence of the six cysteines and their spacing are conserved (Hill and Sternberg 1992). This is consistent with the idea that the global structure formed on the basis of three disulfide bonds is the most important in the function of LIN-3. Also consistent is that changing both the third and the fifth cysteines to serines is sufficient to abolish *lin-3* function (Hill and Sternberg 1992). Every EGF has at least one EGF motif. The EGF motif is cleaved from the membrane and then binds its receptor (reviewed by Massagué and Pandiella 1993). LIN-3 has one EGF motif in its extracellular domain, and it has been suggested that this domain is cleaved from its transmembrane precursor to form a soluble factor (Sternberg and Horvitz 1986; Thomas *et al.* 1990; Hill and Sternberg 1992; Katz *et al.* 1995). Four *lin-3* alleles contain molecular lesions in the EGF motif. All four alleles cause severe loss-of-function phenotypes in all developmental processes mediated by LIN-3. Sequence comparison between *C. elegans* and *C. briggsae lin-3* also reveals that the EGF motif is the most conserved region of LIN-3. These results demonstrate that the EGF motif is essential to the function of LIN-3. Since the EGF motif alone can mediate the function of LIN-3 in vulval induction (Katz *et al.* 1995), the EGF motif should be the receptor-binding domain. However, a general caveat in mutant analysis is that a mutation that truncates the mRNA could simply reduce the stability of the message; therefore, all our interpretations of molecular lesions should be taken with caution.

The cytoplasmic domain of LIN-3 may function in the maturation, membrane localization of LIN-3 precursor, and regulation of the cleavage of the EGF motif. For example, TGF-α, a representative member of the EGF family, is synthesized as a transmembrane precursor, pro-TGF-α, which later undergoes protein cleavage to form a soluble protein. The C-terminal valine is required for maturation and intracellular routing of pro-TGF-α (Briley *et al.* 1997). The cytoplasmic domain of pro-TGF-α is also associated with two proteins, and this protein complex has kinase activities (Shum *et al.* 1994). The cytoplasmic domain of LIN-3 has no homology to that of TGF-α or of any other known protein, but showed a high degree of similarity between the *C. elegans* LIN-3 and its *C. briggsae* homolog. Three alleles of *lin-3* contain molecular lesions in this domain. *sy52* and *s751* have the same mutation, which could delete the N-terminal half of the cytoplasmic domain. Both cause defects in all developmental processes that require *lin-3*. The N terminus of the cytoplasmic domain could function in the localization of the LIN-3 precursor on the cell membrane. *n1058* is a mutation that could delete the C-terminal half of the cytoplasmic domain. It causes tissue-specific phenotypes and will be discussed later.

The sequence similarity between *C. elegans* and *C. briggsae lin-3* indicates that there are other domains important for the function of *lin-3*, *e.g.*, the transmembrane domain, although no mutant allele has been yet isolated with mutations in this domain.

Tissue-specific effects of *lin-3*: LIN-3 mediates at least four developmental processes during *C. elegans* development. Generally, the different responses elicited by intercellular signaling often result from differences in the properties of the target tissues. For example, tissues may differ in which receptors they express. In *C. elegans*, however, there is only one known receptor of LIN-3, which is encoded by *let-23*. But one receptor can nonetheless activate different signal transduction pathways in different developmental processes. In the development of both the vulva and male spicules, LIN-3 activates a Ras/Raf/MAPK pathway downstream of LET-23 (Chamberlin and Sternberg 1994; reviewed by Sundaram and Han 1996). LIN-3 regulates hermaphrodite fertility by activating a different pathway downstream of LET-23 that involves inositol (1,4,5) trisphosphate-3-kinase (IP3K) and IP3 receptor (Clandinin *et al.* 1998). Tissues may also differ in which transcription factors are preprogrammed to be activated in response to a growth factor, and we believe this is how vulval induction differs from spicule development. We do not yet know which pathway is activated by LIN-3 to keep the animals viable. However, the observation that *let-23(gf)* and *let-60(gf)* mutations failed to suppress the lethal phenotype

of *lin-3* indicates that either *lin-3* may have to go through a third pathway to maintain animal viability, although this alternative pathway also leads to the repression of *lin-1*, or that the downstream pathway is under regulation by different mechanisms in different developmental processes.

However, it is also possible that the tissue-specific effects of a growth factor are generated by differential regulation of expression or different functional mechanisms in different tissues. Some *lin-3* alleles do not have equal effects on the four developmental processes that require *lin-3* (Table 1), indicating that the alleles have tissue-specific effects. One explanation of the tissue specificity is that different tissues may require different LIN-3 thresholds. If so, a mutation in *lin-3* that lowers the activity of LIN-3 could result in the activity of LIN-3 being lower than the threshold in one tissue but still being higher than that of another. If this is the only reason for LIN-3 tissue specificity, the thresholds for the four developmental processes should be able to form one rank from low to high, and the *lin-3* mutations should also form one single allelic series on the basis of how much they lower the LIN-3 activity. However, different allelic series are formed for different developmental processes (Figure 4). We regard this as evidence that different thresholds for different developmental processes may explain some of the tissue-specific effects of *lin-3* alleles, but it cannot explain all. The allelic series in different processes suggests that LIN-3 acts in different manners for different developmental processes. We discuss several possible mechanisms by which mutant alleles of *lin-3* could generate tissue-specific effects.

Different thresholds for different developmental processes: *n378* has a mutation that changes the amino acid immediately after the signal sequence and might affect the amount of LIN-3 localized on cell membrane. *n378* causes a severe Vul phenotype, but very weak spicule defects, and both phenotypes are enhanced when *n378* is *in trans* to the null allele *n1059*. Animal fertility and viability are affected by neither *n378* nor *n378/n1059* (Ferguson and Horvitz 1985; Table 1). Therefore, the vulva and the male spicules are the only two tissues affected by *n378*. The thresholds of LIN-3 required for viability and fertility may be very low and are exceeded by the amount of LIN-3 protein made by *n378* and *n378/n1059*. The threshold for spicule development may be higher, and that for vulval induction is even higher. Different thresholds may generate different magnitudes of downstream responses and activate different sets of genes. The apparent differential thresholds may also reflect the different stringencies of the requirement of *lin-3* function in different developmental processes. For example, one developmental process that requires LIN-3 and other intercellular signals may require less LIN-3 than a process that depends solely on LIN-3. *lin-3* is absolutely required for vulval induction. Worms carrying strong *lin-3* alleles, *e.g.*, *n378/n1059*, have essentially no vulval induction (Ferguson and Horvitz 1985; Table 1). On the other hand, the role of *lin-3* in male spicule development may not be essential (Chamberlin and Sternberg 1993, 1994). It is possible that multiple signals regulate the fate of anterior cells in the B lineage, and the defects caused by *lin-3* mutations can be partially compensated by other factors.

Regulation of expression in different tissues: The expression of *lin-3* is temporally and spatially regulated (Hill and Sternberg 1992; Chang *et al.* 1999; R. Hill, J. Liu and P. W. Sternberg, unpublished data). For example, in vulval induction, *lin-3* is only expressed in the somatic AC during a short time window. *lin-3* may contain elements that interact with tissue-specific regulatory factors of transcription. One mutant allele, *lin-3(e1417)*, has no mutation(s) in the coding region and, thus, is inferred to have lesions in a noncoding region. It has vulval-specific defects, but retains activity in viability, fertility, and male spicule development. Two models may explain the effects of *e1417*. First, the expression of *lin-3* could be regulated in a tissue-specific manner, and in *e1417*, the noncoding region required specifically for AC expression is impaired. Alternatively, the expression of *lin-3* may be moderately reduced in all tissues in *e1417* animals, but vulval induction requires a higher level of LIN-3. In the former case, we would expect no phenotype other than Vul in *e1417/n1059* animals. If the latter is true, we would expect to observe other phenotypes in *e1417/n1059*. We observed that there are indeed no other defects in *e1417/n1059* besides a Vul phenotype (Ferguson and Horvitz 1985; Table 1). This observation is consistent with the hypothesis that *e1417* has mutations in a region that specifically affects *lin-3* expression in the AC.

The range of signaling: The alternative splicing region between the EGF motif and the transmembrane domain of LIN-3 is unique among EGFs (reviewed by Massagué and Pandiella 1993), but reminiscent of that in another growth factor, the kit ligand (KL). Two KL proteins are encoded by two mRNAs that are generated by alternative splicing at a position similar to that of LIN-3. The longer protein is cleaved, but the shorter one is relatively resistant to protein cleavage and stays predominantly on the cell membrane (Huang *et al.* 1992). It has been suggested that the alternative splicing in *lin-3* determines whether LIN-3 has long- or short-range of signaling (J. Liu and P. W. Sternberg, unpublished results). The protein with the amino acids encoded by the alternatively spliced region is a short-range signal and can induce fewer VPCs than the protein without these amino acids. The two LIN-3 proteins could have different susceptibilities to cleavage, similar to those of KL. Other possibilities do exist; *e.g.*, the two proteins may differ in their stability or abilities to diffuse. Whether or how the two LIN-3 proteins generate different functions

is unknown. However, it has been shown that in PC12 cells, different durations of one signaling pathway activation can lead to different outcomes of the signal transduction (reviewed in Marshall 1995). In the case of KL, the secreted KL and the transmembrane KL cannot substitute for each other for their respective functions (Wehrle-Haller and Weston 1995). The transmembrane form elicits a sustained phosphorylation of its receptor, while the soluble form elicits a transient one (Miyazawa *et al.* 1995). It is conceivable that the two LIN-3 proteins may use a similar mechanism to generate different cellular responses: while a diffusable signal generates fast effects over a long range, the locally concentrated signal generates sustained responses.

n1058 may truncate the C-terminal half of the cytoplasmic domain of LIN-3. It causes completely penetrant hermaphrodite sterility and a strong male spicule defect, but only mild vulval defects and weak lethality (Ferguson and Horvitz 1985; Table 1). Two simple possibilities exist: First, the truncated protein in *n1058* is not functional, and the functions in viability and vulval induction are mediated by a small amount of full-length protein produced due to incomplete elimination of splicing. However, *sy51*, having a mutated splice donor, does cause completely penetrant lethality, indicating that the residual wild-type protein is unlikely to be sufficient to sustain viability. A more plausible possibility is that the viability and partial vulval induction observed in *n1058* animals are mediated by the truncated protein. We propose that the C-terminal half of the cytoplasmic domain, or part of it, is required for the functions of *lin-3* in only a subset of developmental processes: it is required for fertility and male spicule development, but not for vulval induction and viability. We do not yet know whether the C-terminal portion of the cytoplasmic domain of LIN-3 interacts with tissue-specific regulatory factors to affect secretion, cellular localization, or stability of LIN-3 protein. Interestingly, the C-terminal half of the cytoplasmic domain of LIN-3 is also required for the long-range signaling of LIN-3 (J. Liu and P. W. Sternberg, unpublished results). A possibility is that different developmental processes prefer different signaling ranges or signaling modes.

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