The *Caenorhabditis elegans* gene *lin-1* encodes an ETS-domain protein and defines a branch of the vulval induction pathway

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The *Caenorhabditis elegans* gene *lin-1* appears to act after the Ras–Raf–MEK–MAPK signaling cascade that mediates vulval induction. We show that *lin-1* is a negative regulator of vulval cell fates and encodes an ETS-domain putative transcription factor containing potential MAPK phosphorylation sites. In *lin-1* null mutants, the vulval precursor cells (VPCs) still respond to signaling from the gonadal anchor cell, indicating that *lin-1* defines a branch of the inductive signaling pathway. We also provide evidence that the inductive and lateral signaling pathways are integrated to control the 1° and 2° vulval cell fates after the point at which *lin-1* acts in the inductive pathway and that VPCs can assess the relative rather than absolute levels of inductive and lateral signaling in determining whether to express the 1° or 2° vulval cell fates.

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Signaling pathways involving receptor tyrosine kinases (RTKs) and Ras proteins have major roles in determining cell fates and in oncogenesis (for review, see Egan and Weinberg 1993; Pawson and Hunter 1994). Such signal transduction pathways have been highly conserved during evolution. For example, *Caenorhabditis elegans* vulval induction is mediated by the *let-23* RTK/*let-60* Ras signaling pathway (for review, see Horvitz and Sternberg 1991; Eisenmann and Kim 1994; Tuck and Greenwald 1994). This pathway acts to specify the fates of a set of six multipotent hypodermal blast cells, P3.p–P8.p, known as the vulval precursor cells (VPCs). The VPCs are considered to be developmentally equivalent, because they can adopt any one of three fates, each of which is characterized by a distinct pattern of cell divisions (lineage) (see Fig. 1; Sulston and White 1980; Sternberg and Horvitz 1986; Thomas et al. 1990). Two of these fates, termed 1° and 2°, are vulval fates, because in wild-type animals the 1° and 2° lineages generate descendants that form the vulva. The third fate, termed 3°, is a non-vulval fate, because the 3° lineage generates descendants that fuse with the hypodermal syncytium and are not part of the vulva.

Vulval fates are induced by signaling from the anchor cell (AC) of the gonad. The inductive signal appears to be

the LIN-3 protein, a member of the epidermal growth factor (EGF) family (Hill and Sternberg 1992), and the apparent receptor for the signal is the LET-23 protein, an RTK of the EGF receptor subfamily (Aroian et al. 1990). LET-23, together with the adapter protein SEM-5 (Clark et al. 1992; Lowenstein et al. 1992), activates LET-60 Ras (Beitel et al. 1990; Han and Sternberg 1990) and the kinase cascade LIN-45 Raf (Han et al. 1993), MEK-2 MAP kinase kinase (MAPKK) (Church et al. 1995; Kornfeld et al. 1995; Wu et al. 1995), and MPK-1 MAP kinase (MAPK) (Lackner et al. 1994; Wu and Han 1994). The ordering of the components of this signaling pathway in *C. elegans* by genetic analysis has been concordant with biochemical studies of similar pathways in other organisms.

In wild-type hermaphrodites, the pattern of vulval fates is invariant: P5.p, P6.p, and P7.p adopt the 2°, 1°, and 2° fates, respectively (Sulston and Horvitz 1977). Two signals are important for this patterning: the inductive signal from the AC described above and a lateral signal between VPCs (Sternberg 1988; Koga and Ohshima 1995; Simske and Kim 1995). The lateral signal appears to be expressed or activated upon reception of the inductive signal, because mutations that reduce vulval induction also reduce lateral signaling (Simske and Kim 1995; Tuck and Greenwald 1995). The identity of lateral signal is as yet unknown, but its receptor appears to be the LIN-12 protein (Greenwald et al. 1983; Sternberg and Horvitz 1989). How the inductive and the lateral signaling pathways are integrated to control vulval

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cell fate and the relative contributions of each pathway to the specification of VPC fate in wild-type hermaphrodites are still unclear.

The identification of genes that act after MPK-1 MAPK during vulval induction is central to understanding how MPK-1 controls vulval induction and how the inductive and lateral signaling pathways are integrated. One candidate is *lin-31*, which encodes a transcription factor of the HNF-3/fork head family (Miller et al. 1993). Another candidate is *lin-25*, which encodes a protein having no significant similarity to other proteins in available data bases (Tuck and Greenwald 1995). Here we describe genetic and molecular studies of a third candidate, *lin-1* (Horvitz and Sulston 1980; Sulston and Horvitz 1981; Ferguson and Horvitz 1985; Ferguson et al. 1987). Our analysis supports the idea that *lin-1*, an ETS-domain putative transcription factor, is a target of the Ras–MAPK cascade. Furthermore, our results indicate that the inductive signaling pathway is branched and that *lin-1* defines one of the branches. Our results suggest that the *lin-1* branch of the inductive pathway activates lateral signaling and that the inductive and lateral signals are integrated after the point at which *lin-1* acts in the inductive pathway. We also present genetic evidence that VPCs can assess the relative rather than absolute levels of inductive and lateral signaling in determining which vulval fate to adopt.

Results

Cloning of *lin-1*

lin-1 had been genetically mapped previously between *daf-1* IV and *lin-22* IV, which had been positioned on both the *C. elegans* genetic and physical maps (Ferguson and Horvitz 1985; Edgley and Riddle 1990; Georgi et al. 1990; L. Wrischnik and C. Kenyon, pers. comm.). *daf-1* and *lin-22* were located on two separate sets of ordered and overlapping YAC and cosmid clones, referred to as contigs. To define the location of *lin-1* with respect to these contigs, we identified and genetically mapped 16 restriction fragment length polymorphisms using cosmids and cosmid subclones from the physical map as probes (see Materials and methods). In collaboration with Alan Coulson (The Sanger Centre, Hinxton, U.K.), we reorganized the physical map in the *lin-1* region and localized *lin-1* to an ~400-kb interval (Fig. 2A). We used cosmids from this interval in germ-line transformation experiments and in a search for allele-specific polymorphisms among the >40 known *lin-1* mutants (Horvitz and Sulston 1980; Ferguson and Horvitz 1985; X. Lu, S.G. Clark and H.R. Horvitz, unpubl.). One cosmid, C37F5, contained rescuing activity and detected polymorphisms in DNA from eight *lin-1* mutants (Fig. 2B; Table 1). Subclones of C37F5 defined a 12-kb *EagI*–*SpeI* fragment that partially rescued the Multivulva (Muv) phenotype of the temperature-sensitive *lin-1* allele *e1275* (Fig. 2B).

We isolated 25 cDNAs that hybridized to the *EagI*–*SpeI*-rescuing fragment. All 25 cDNAs had sequences in

Figure 1. Model for signaling in vulval induction. Outlined is a simplified version of the current model for vulval induction (Horvitz and Sternberg 1991). The six hypodermal blast cells P3.p–P8.p are developmentally equivalent and are referred to as VPCs. As described in the text, VPCs adopt one of three cell fates in response to signaling between the gonadal AC and the VPCs (flared arrowheads), between adjacent induced VPCs (horizontal arrow), and between the syncytial hypodermis and the VPCs (not shown). The characteristic 1°, 2°, and 3° lineages (fates) that the VPCs express in wild-type animals are diagrammed below each cell. The vulval fates are defined after two rounds of division by two criteria: the axis of the third round of nuclear divisions [(T) transverse; (L) lateral; (N) no division] and adherence to the ventral cuticle, indicated by boldface (i.e., L) [nomenclature and criteria according to Sternberg and Horvitz 1986]. The 3° cell fate is nonvulval and the 3° VPC descendants fuse with the hypodermal syncytium without undergoing division in ~50% of wild-type animals, and for P3.p both the S S and S lineages are considered 3°.

common, suggesting that they were transcribed from the same locus. The two longest cDNAs began with the SL1 trans-spliced leader sequence (Krause and Hirsh 1987; for review, see Blumenthal 1995), indicating that they are full-length transcripts. We determined and compared the sequences of the longest cDNA and ~7 kb of the *EagI*–*SpeI*-rescuing fragment (Fig. 3; data not shown). The first 160 bp of the longest cDNA was not contained in the *EagI*–*SpeI* fragment or the C37F5 cosmid, despite the ability of these genomic fragments to at least partially rescue the *lin-1(e1275)* phenotype (Fig. 2C; see Materials and methods). We used polymerase chain reaction (PCR) amplification and Southern blot experiments to determine that the first 160 bp of the predominant *lin-1* message is encoded by two exons located 15–20 kb from the remaining exons (see Materials and methods).

lin-1 encodes an ETS-domain protein containing potential MAPK phosphorylation sites

Conceptual translation of the full-length *lin-1* cDNA revealed a 441-amino-acid open reading frame (ORF) (Fig. 3). To confirm that this ORF encodes the *lin-1* gene product, we determined the genomic sequences of eight *lin-1* alleles that did not show any gross alterations of the *lin-1* genomic region by Southern blot analysis (see Materials

Figure 2. Molecular cloning of *lin-1*. (A) Genetic and physical maps of the *lin-1* region. We tested the cosmids W08E5, C23H5, C51E4, T04C4, M70, and C37F5 for *lin-1*-rescuing activity in germ-line transformation experiments (data not shown). Only C37F5 contained rescuing activity. On the physical map, clone names beginning with "Y" are YAC clones. The smaller clones are cosmids. (npl), Polymorphisms identified and mapped in this study; (eP80), a polymorphism identified previously by D. Pilgrim (pers. comm.); Vertical parallel rules indicate regions of the physical map that contain no cosmids. (B) Abilities of C37F5 subclones to rescue the *lin-1*(e1275) Muv phenotype in germ-line transformation experiments. The orientation shown in B and C is reversed relative to that in A to display the *lin-1* transcript with the 5' end toward the left. (Rescue) The fraction of transgenic lines that were at least partially rescued for the *lin-1*(e1275) Muv phenotype; (*) this *SalI* site is in the vector sequence of C37F5. (C) *lin-1* genomic structure. The exact size of the 15- to 20-kb second intron has not been determined. Exons one and two are not located in the rescuing cosmid C37F5 or in its subclones; exon three contains two in-frame methionine residues amino-terminal of the ETS domain (Figs. 3 and 4). Of 25 cDNAs isolated, 9 of 10 that extended to the exon boundary between exons 2 and 3 had the 6-exon structure drawn with thick lines. One cDNA was SL1 trans-spliced at exon 3, indicated with fine lines and a smaller SL1 label, but we have not determined whether this cDNA represents an alternative splice form or an aberrant transcript. An internal promoter in the second intron may account for the existence of this cDNA as well as for the rescuing activity of genomic fragments lacking the first two exons. (Stippled area) ETS domain; (solid area), 3'-untranslated region.

and methods). Each of the eight mutants contained a single-base change that altered one codon in the putative *lin-1* ORF (Table 1; Fig. 4). Two mutations, *e1777* and *n431*, which have been shown to be suppressed by an amber suppressor tRNA mutation (Ferguson and Horvitz 1985), created the same amber stop codon. These results provide strong evidence that we have identified the *lin-1* gene product.

The amino terminus of the LIN-1 protein has a region of strong similarity to the DNA-binding domain of the ETS family of transcription factors (Fig. 3; for review of ETS proteins, see Seth et al. 1992; Janknecht and Nordheim 1993; Wasylyk et al. 1993). The LIN-1 ETS domain is most similar to the Elk-1/SAP-1/Net subfamily of ETS domains (Dalton and Treisman 1992; Rao and Reddy 1992; Giovane et al. 1994) and appears to be highly diverged from the *Drosophila* Yan ETS domain, despite similarities in the genetic roles of *lin-1* and *yan* in cell fate determination (Lai and Rubin 1992; Tei et al. 1992; Rebay and Rubin 1995; see Discussion). Outside the ETS domain, LIN-1 has no extended similarity to any protein in the available data bases.

Analysis of the LIN-1 sequence with the PESTFIND program (Rechsteiner 1990) revealed three potential PEST sequences (Figs. 3 and 4). PEST sequences are found in many proteins that have short half-lives and have been proposed to mark proteins for rapid turnover (Rechsteiner 1990).

Genetic analyses of *lin-1* indicate that it acts after the *mpk-1* MAPK in the *let-60* Ras signal transduction pathway (Lackner et al. 1994; Wu and Han 1994). LIN-1 has 18 Ser/Thr-Pro consensus MAPK phosphorylation sequences (Fig. 4; for review, see Davis 1993), suggesting that LIN-1 might be a direct substrate for the MPK-1 MAPK. None of the LIN-1 Ser/Thr-Pro sites has the more specific Pro-Xaa-Ser/Thr-Pro consensus MAPK phosphorylation sequence (for review, see Davis 1993).

Molecular analysis of *lin-1* point mutations

As described above, we identified point mutations in eight LIN-1 alleles (Table 1; Figs. 3 and 4). Two mutations, *n303*(R121K) and *n1047*(Y126F), cause conservative substitutions of residues found in all ETS domains (Janknecht and Nordheim 1993; Wasylyk et al. 1993). These changes suggest that the ETS domain is important for *lin-1* function and support the idea that *lin-1* acts as a DNA-binding transcription factor.

The other six mutations all create premature stop codons in the *lin-1* ORF. The *e1275* mutation creates an opal stop codon at position 175, presumably truncating the remaining 266 amino acids. This result was surprising, because *e1275* causes a relatively weak, temperature-sensitive Muv phenotype, which is 65% (*n*=972), 91% (*n*=711), and 99% (*n*=863) penetrant at 15°C, 20°C, and 25°C, respectively. Furthermore, the phenotype caused by *e1275* is much weaker than the non-temperature-sensitive, 100% penetrant Muv phenotype caused by the nonsense alleles *n176*, *n383*, *n431*, and *e1777* (data not shown), despite the fact that the *e1275* protein

Table 1. Molecular lesions of *lin-1* mutants

Allele	Mutagen ^a	Class ^b	Molecular lesion
A. Gross DNA alterations present in eight <i>lin-1</i> alleles^c			
<i>sy254</i>	psln	strong Muv, null	exons 3 and 6 deleted, 4 and 5 rearranged
<i>n304</i>	EMS	strong Muv, null	exon 3 and 4 deleted, 5 and 6 rearranged
<i>m546</i>	EMS	strong Muv	exon 3 and 4 rearranged
<i>ar147</i>	X-ray	strong Muv	exon 4, 5, and 6 rearranged/duplicated
<i>n1140</i>	EMS	strong Muv	exon 4, ~100-bp deletion
<i>n381</i>	EMS	strong Muv	exon 5 rearranged/duplicated
<i>n2692</i>	EMS	strong Muv	exon 5 rearranged/duplicated
<i>n2701</i>	EMS	strong Muv	exon 5 and 6 rearranged/duplicated
B. Base and codon changes in eight <i>lin-1</i> point mutants^d			
<i>n757</i>	EMS	strong Muv ^e	W 105 (TGG) → Amber (TAG)
<i>n303</i>	EMS	strong Muv	R 121 (AGA) → K (AAA)
<i>n1047</i>	EMS	strong Muv	Y 126 (TAT) → F (TTT)
<i>e1275</i>	EMS	ts, weak Muv	R 175 (CGA) → Opal (TGA)
<i>n176</i>	EMS	strong Muv	R 255 (CGA) → Opal (TGA)
<i>n383</i>	EMS	strong Muv	Q 298 (CAA) → Ochre (TAA)
<i>n431</i>	EMS	am, ^f strong Muv	Q 309 (CAG) → Amber (TAG)
<i>e1777</i>	EMS	am, ^f strong Muv	Q 309 (CAG) → Amber (TAG)

^aMutagen used in the isolation of the allele; (psln) trimethylpsoralen; (EMS) ethyl methanesulfonate.

^b(Strong Muv) Animals have a 100% penetrance of large ventral bumps at 20°C (data not shown); (ts) temperature-sensitive; (weak Muv) the penetrance of the Muv phenotype is <100% (see text), and the ventral protrusions of these animals are smaller and fewer than those of animals homozygous for strong alleles (data not shown).

^cAlterations were detected using Southern blot and PCR analyses (see Materials and methods). The detailed natures of the lesions in these mutants have not been determined.

^dThe amino acid positions affected by each mutation are as in Fig. 3

^e*n757* has not been tested for amber suppressibility.

^f*n431* and *e1777* have been shown to be suppressed by the amber suppressor *sup-7* (Ferguson and Horvitz 1985).

product is predicted to be considerably shorter than the protein products predicted for these other alleles. One possible explanation for these results is that the presumed deletion of the two carboxy-terminal PEST sequences from the *e1275* protein product renders it more stable than the *n176*, *n383*, *n431*, and *e1777* products.

n304 and *sy254* are *lin-1* null alleles

To define more clearly the role of *lin-1* in vulval development, we identified apparent *lin-1* molecular null alleles, designated as *lin-1(0)*. We examined the *lin-1* genomic regions of 35 *lin-1* mutants using exon-specific probes on Southern blots and by PCR amplification of individual exons. One mutation, *n304*, was deleted for exons three and four, which contain the ETS domain, and rearranged in exons 5 and 6 such that they cannot be amplified by PCR using exon-specific primers (Table 1; Materials and methods; data not shown). Another mutation, *sy254*, was deleted for exons 3 and 6 and rearranged in exons 4 and 5. The first two exons, which are separated from the rest of the gene by the ~15- to 20-kb second intron and encode 38 amino acids, were not deleted or disrupted in any of the 35 *lin-1* mutants that we examined.

Both *n304* and *sy254* appear to be null mutations for the following reasons: First, the coding sequence for all but the first 38 amino acids of LIN-1 was deleted or disrupted severely in both *n304* and *sy254* DNA. Second, in

all genetic tests done to date, *n304* and *sy254* behave identically to each other and to six other *lin-1* mutations that cause gross rearrangements in the *lin-1* locus (Tables 1 and 2; data not shown). There are currently no chromosomal deletions that eliminate the *lin-1* region completely, so we could not examine the phenotype of animals hemizygous for *n304* or *sy254*.

lin-1 negatively regulates vulval cell fates

lin-1(0) hermaphrodites have a 100% penetrant Muv phenotype characterized by a squarish protruding vulva and one to three additional large ventral protrusions (Tuck and Greenwald 1995; data not shown). The protrusions are pseudovulvae resulting from ectopic VPC induction. In wild-type animals, only three of six VPCs adopt vulval fates after inductive signaling from the gonadal AC (Sulston and White 1980; Kimble 1981). In contrast, in *lin-1(0)* mutants almost all VPCs adopted vulval fates even in the absence of the AC and the rest of the somatic gonad (Table 2). Specifically, 320/324 VPCs adopted 1° or 2° vulval fates or underwent multiple rounds of division characteristic of vulval rather than nonvulval fates. Thus, *lin-1* is an important negative regulator of the 1° and 2° vulval cell fates. However, in these *lin-1(0)* animals, many of the VPC lineages were abnormal (102/324), and a few VPCs still adopted the 3° fate (4/324) (Table 2). Abnormal lineages and the presence of

lin-1 ETS acts in a branched signaling pathway

Figure 3. *lin-1* cDNA sequence and alignment of ETS domains. (Top) The sequence of the longest *lin-1* cDNA. Conceptual translation begins with the first in-frame ATG codon. The nucleotide (above) and amino acid (below) positions are indicated at the right. SL1 sequences are double underlined; PEST sequences are indicated by a bracketed underline; the exon 2–3 splice site is shown by an inverted arrowhead; the ETS domain is indicated by a bracketed bold underline. The PESTFIND scores (Rechsteiner 1990) of the indicated PEST sequences, starting from the amino-terminal sequence, are 31, 6, and 17. Scores >0 indicate potential PEST sequences. The GenBank accession nos. for the *lin-1* cDNA and genomic sequences are U38935, U38936, and U38937. (Bottom) Alignment of the *lin-1* and other ETS domains. (% Identity) Percent identity between the indicated ETS domain and the *lin-1* ETS domain; (C.e.) *C. elegans*; (Dros.) *Drosophila melanogaster*; (ELK1) from Rao and Reddy (1992); (SAP1) from Dalton and Treisman (1992); (Ptd2a) from Klaes et al. (1994); (ETS2) from Watson et al. (1988); (FLI1A) from Delattre et al. (1992), Watson et al. (1992); (Yan A) Lai and Rubin (1992); (Tei et al. (1992); (PU1) from Ray et al. (1990); (ETS consensus) invariant residues in ETS domains (Wasylyk et al. 1993).

occasional 3° fates are not characteristic of all mutations that cause a Muv phenotype. For example, in the *lin-15(n309)* mutants examined by Sternberg (1988; Table 1D,E) and Sternberg and Horvitz (1989; Table 2C,D), there were only 3/137 abnormal VPC lineages and all 137 VPCs adopted vulval fates. The high frequency of abnormal lineages and the occurrence of 3° fates in *lin-1(0)* mutants combined with the results of laser-ablation experiments presented below demonstrate that while

lin-1 plays a crucial role in VPC fate determination, other genes appear to act in parallel to *lin-1* in controlling the vulval/nonvulval cell fate decision.

VPCs are responsive to inductive signaling in *lin-1* null mutants

If AC–VPC signaling induces VPCs to adopt vulval fates through a linear signal transduction pathway that inac-

Figure 4. Schematic diagram of the LIN-1 protein. The positions of the ETS domain, potential MAPK phosphorylation sites (Ser/Thr–Pro), PEST sequences, point mutations, and location of the splice site that joins exons 2 and 3 are shown. (aa) Amino acids.

Table 2. VPC lineages in *lin-1* mutants

Genotype	AC	Gonad	P3.p	P4.p	P5.p	P6.p	P7.p	P8.p
WT [‡]	+	+	<div>3° S S S</div>	<div>3° S S</div>	<div>2° LLTN</div>	<div>1° TTTT</div>	<div>2° NTLL</div>	<div>3° S S</div>
A. <i>sy254</i>	+	+	LLTO	OLTT	LLLO	TTTT	TTLL	OTTL
	+	+	SLLL	LLON	LLLT	TTTT	NLOL	LLOL
	+	+	SSOT	LOLL	LLOT	TOTT	OOLL	TLLL
	+	+	SSLL	LLTO	OLOT	TTOT	NTLL	TDOL
	+	+	LOTO	LOOO	LTIN	TOTT	OOTL	OLLL
	+	+	LOOL	LLOL	OLLO	TTTT	NTLL	OLOL
n304	+	+	S OO	OOLL	LOTN	TTTT	TOLT	LL S
	+	+	LLLT	LTOT	LLOO	OTTT	TTOL	LLOL
	+	+	LTOO	LLTT	LLTT	TTTO	NOLL	TTLL
	+	+	LTLL	LOTL	LLLN	TTTT	NTOL	TTTL
	+	+	S LT	LLLT	LTTT	TTTT	TTLL	LLOT
	+	+	S	LOTL	LLTN	TTTL	TTTL	LLTL
B. <i>sy254</i>	-a	-a	LLTO	LLTO	LLLO	TLLL	OOTT	LTOT
	-	-	LLLO	LLTO	TTOT	LLLO	TTTL	OLOO
	-	-	S LO	LOTL	LLOT	LOTT	LOOT	LLTD
	-	-	S TO	LTTT	LLON	TOOT	TOLL	TOOL
	-	-	LLTT	LOTL	OOTL	LLON	DTTL	LLLL
	-	-	TTOL	LILL	TTOO	TOLO	TOLL	LOLL
n304	-	-	LLLT	LLTO	OTTO	LLLO	DLLL	LTTL
	-	-	S LT	LLOD	OTTO	LLLO	LLOL	LLLL
	-	-	LTOO	TLTL	LLLL	LLLT	TTTO	LLLD
	-	-	LLOO	LLTT	LLLL	TTTT	LLLL	LTOO
	-	-	SSOO	LLLL	LLOO	OTOT	LLOL	LLOO
	-	-	LLOL	LLTL	OLOL	LLTT	LLLL	LLLL
C. <i>sy254</i>	+b	-b	S OL	LTTT	LLTO	TTTO	LLLL	LOTO
	+	-	LLTL	LLOO	TTTT	LTOO	NLLO	LLTO
	+	-	S LL	LLOO	LTTD	TTTT	OOLL	DLOL
	+	-	S	LDTT	LLOD	TTTT	OTLL	LLTO
	+	-	S LO	LOOD	LOOD	TTLO	TTLL	LLOO
	+	-	LOOL	LDTO	LLOT	TTTT	TTLL	LLOO
n304	+	-	LLTO	LLOO	LLOT	TTTO	LLLL	LLOO
	+	-	S OO	S S	LLOO	TOTT	NPLL	S OL
	+	-	LOLO	LOTO	LLOT	TTTL	LLLL	LOTO
	+	-	S OO	LDTT	LLLO	LTTT	TTTL	DLTT
	+	-	SSLL	LTTT	LLTN	TTTT	NTLL	S TO
	c	-	S OD	LLLO	LLLO	LOTT	OOLL	LLOT
d	+	-	LTOO	LLOT	LOTT	TTTL	TOLL	OTTL

tivates *lin-1*, then in the absence of *lin-1* VPCs should be insensitive to the presence or absence of AC signaling. However, in examining the pattern of the vulval cell fates in *lin-1(0)* animals we found that P6.p, the VPC closest to the AC, adopted the 1° fate in 11 of 12 animals (Table 2A). In contrast, the other VPCs in these animals often adopted the 2° fate. These observations suggested that in the absence of *lin-1* function, either P6.p was intrinsically biased to adopt the 1° fate or intercellular signaling independent of *lin-1* function caused P6.p to adopt the 1° fate. To test whether the gonadal AC might be the source of such a signal, we ablated the somatic gonad precursor cells Z1 and Z4 using a laser microbeam. In operated animals, P6.p adopted the 1° fate in 4/12 cases and the 2° fate in 5/12 cases (Table 2B). Thus, one or more of the Z1/Z4 descendants signals P6.p to adopt the 1° fate. To demonstrate that the AC in the absence of other Z1/Z4 descendants is capable of signaling P6.p to adopt the 1° fate, we individually ablated in *lin-1(0)* animals all Z1/Z4 descendants except the AC. In these animals P6.p adopted the 1° fate in 11/13 cases and never adopted the 2° fate (Table 2C). Furthermore, in the 2/13 cases in which P6.p adopted hybrid 1°/2° fates, the AC was positioned abnormally between P6.p and an adjacent VPC. These results demonstrate that P6.p, and presumably the other VPCs, is responsive to AC signaling even in the absence of *lin-1* function. Thus, the AC-to-VPC signal transduction pathway cannot be a simple linear pathway in which all of the signaling passes

through *lin-1*. The AC–VPC signal transduction pathway must branch at some point, and *lin-1* must act in and therefore define one of the branches.

Relative levels of *lin-1* and the *lin-12* pathway specify VPC fates

The two major signaling pathways known to be involved in specifying VPC fates are the Ras-mediated inductive and the *lin-12*-mediated lateral signaling pathways (for review, see Sternberg and Horvitz 1991; Kenyon 1995). Lateral signaling can specify the 2° vulval cell fate in the absence of Ras pathway signaling in the presumptive 2° VPCs during vulval induction (Koga and Ohshima 1995; Simske and Kim 1995) and prevents two adjacent induced VPCs from both adopting the 1° fate (Sternberg 1988). However, there is evidence that the inductive AC signal is spatially graded and that the decision between the 1° and 2° fate can be determined by the strength of Ras pathway signaling (Sternberg and Horvitz 1986; Thomas et al. 1990; Katz et al. 1995). It is not clear to what extent each of these pathways contributes to the 2°/1°/2° pattern of fates seen in wild-type vulval development. It is also not understood how the VPCs integrate the inductive and lateral signals to specify the 1° or 2° fates. For example, do VPCs have set responses to specific levels of either the inductive or lateral signals, or can VPCs compare the relative levels of the inductive and lateral signals and select the appropriate fate?

Table 2. (Continued)

Genotype	AC	Gonad	P3.p	P4.p	P5.p	P6.p	P7.p	P8.p
D. <i>sy254</i>	–e	–e	LLLO	S TT	TTTO	LLOL	LLTT	OOLL
	–	–	S SS	LLTO	OLLT	OOTO	TLOT	DLTO
	–	–	OLTT	S TO	TLTT	TTOT	TLLL	DTSL
	–	–	SSOT	LLTT	LLTT	OODO	LLLL	LOTT
	–	–	SSLT	LLLT	LDOO	LTTT	LMTL	LTTO
	–	–	S LL	LLLN	LLOT	TTTL	DLTD	LLTO
	–	–	SOTO	LLLT	LLOT	LLOO	LLOT	LLOO
	–	–	LLOL	LLOO	TOTO	LLOO	LTTT	LLOD
	–	–	LLOO	LLLO	LLTO	TTOT	NLLL	DTTL
	–	–	S S	OTOL	LLTO	TTTT	TTLL	OTTL
<i>n304</i>	–	–	S OD	LLTO	LLOO	TLOL	TTOT	LLLL
	–	–	SSOO	LLLN	OOOT	LLLD	TTTT	SSLO

The symbols and conventions used are essentially those defined by Sternberg and Horvitz (1989) and are explained in Fig. 1 and below. (O) An oblique division axis; (D) the division axis was not observed. (Heavy-lined box) 1° lineages; (fine-lined box) 2°; (shaded box) 3°. Some lineages were not interpretable as 1°, 2°, or 3° and are not boxed. (AC) Presence or absence of anchor cell; (gonad) presence or absence of the descendants of the somatic gonad precursor cells Z1 and Z4, excluding the AC. (†) Canonical wild-type (WT) lineage observed by Sulston and Horvitz (1977), with 1°, 2°, and 3° fate designations (Sternberg and Horvitz 1986) indicated above each VPC lineage. (a) The somatic gonad precursor cells Z1 and Z4, which produce the AC, were ablated during the L1 stage; (b) all descendants of Z1 and Z4 except the AC were ablated (see Materials and methods); (c) the AC in this animal was located between P6.p and P7.p; (d) the AC in this animal was located between P5.p and P6.p; (e) all descendants of Z1 and Z4 were ablated as in part C, except that the AC was also ablated. Because P6.p could adopt either the 2° fate (2/12 cases) or the 1° fate (5/12 cases) in part D, we conclude that the AC, and not any residual debris from the other somatic gonad cells, induced P6.p to adopt the 1° rather than 2° fate in part C.

When a wild-type VPC is induced to adopt a vulval fate by activation of the inductive RTK/Ras pathway, the VPC activates the lateral signaling pathway. In *lin-1(0)* mutants, almost all VPCs adopt vulval fates, although it appears that not all aspects of vulval cell fates are induced correctly in *lin-1(0)* mutants (see above). To determine whether lateral signaling is activated correctly in *lin-1(0)* mutants, we examined VPC lineages in *lin-1(0)*

animals. We observed only 3 cases of adjacent 1° VPCs among 59 pairs of VPCs that adopted vulval fates (Table 2A). This result suggests that lateral signal function was largely normal in *lin-1(0)* mutants. We then determined that lateral signaling in *lin-1(0)* mutants is dependent on the presence of *lin-12*, which encodes the putative receptor for the lateral signal (Sternberg 1988; Yochem et al. 1988; Seydoux and Greenwald 1989). *lin-12* activity is

Table 3. VPC lineages in *lin-12*; *lin-1* mutants

Genotype	AC	P3.p	P4.p	P5.p	P6.p	P7.p	P8.p
WT [±]	+	3°	3°	2°	1°	2°	3°
	+	S S or S	S S	LLTN	TTTT	NTLL	S S
<i>lin-12(0)</i> ¹	++	3°	~3°/3°	1°	1°	1°/~3°	3°
A. <i>lin-12(0); lin-1(0)</i>	++	1°/~3°	1°/~3°	1°	1°	1°	1°/3°/~3°
<i>lin-12(0); lin-1(n304)</i>	++	S OO	LTTL	OTLT	DOOT	TTTS	OL S
	++	SSOT	DTTO	LLTT	TTTT	LTDL	OL S
	++	LTLO	OOLL	LLTT	TLTT	TOOL	S
	++	S LT	SSOT	LLTL	LTOT	TTTT	TLLL
	++	SSSS	S LT	OOOO	TTTT	TTLL	S S
	++	LLLL	OTTO	LOOT	TTTL	OOLL	LLLL
<i>lin-12(d)/+</i> ²	-	~2°	2°	2°	2°	~2°	2°/3°
B. <i>lin-12(d)/+; lin-1(0)</i>	-	1°/3°	2°/~2°	2°	1°	2°	1°
<i>lin-12(d)/+; lin-1(n304)</i>	-	LOTT	LLOO	TTLO	TTTT	OTOO	TOLL
	-	LLOT	TLTT	LLLL	OTTT	NLLL	DOOL
	-	S	LOTN	LLTT	LLOT	TTTL	TTTL
	-	S S	LDOS	LLLN	TTOT	DLLL	LLOL
	-	LLOL	LLON	LLTT	TTTT	NLLL	TTOO
	-	LLLT	LLLO	LLTN	TTOT	OLLL	LTTO
<i>lin-12(d)/lin-12(d)</i> ¹	-	2°	2°	2°	2°	2°	2°
C. <i>lin-12(d)/lin-12(d); lin-1(0)</i>	-	~2°	2°	~2°/2°	1°/2°	2°	~2°
<i>lin-12(d)/lin-12(d); lin-1(sy254)</i>	-	LLOO	LLOO	LTTT	OOLL	NLLL	OOLL
	-	LLTN	LLTN	LLON	LOLL	NOLL	LLNL
	-	LLNO	LLTN	LLON	OOTT	LOLL	NOLL
	-	LLNO	LLON	LLON	OOTT	NOLL	NOOL
	-	LOTN	OTLL	LLTT	OOTT	NOOL	OTLL
	-	LLOO	LLTT	LOLT	LTOT	NTLL	TOLL

VPC lineages and our interpretations of these lineages are shown. Single-mutant data are from ¹Sternberg and Horvitz (1989) and ²Mango et al. (1991). Symbols are as in Fig. 1 and Table 2, with the following exceptions: (AC) Animals have zero (-), one (+), or two (+ +) anchor cells resulting from cell fate transformations caused by *lin-12* mutations (Greenwald et al. 1983). ~[2°] VPCs expressed hybrid lineages with the 2° characteristics of undergoing multiple rounds of division and having at least one L descendant; ~[3°] VPCs expressed hybrid lineages with the 3° characteristic of having one or more S descendants. We determined VPC lineages in *lin-12*; *lin-1* double mutants derived from heterozygous parents (see Materials and methods). The complete genotypes of lineaged animals were *lin-12(0)*; *lin-1(sy254)*, [*unc-36(e251)lin-12(n941)*]; *lin-1(sy254)*; *lin-12(d)* +; *lin-1(n304)*, [*unc-32(e189) lin-12(n137)/qC1*]; *lin-1(n304)*; *lin-12(d)*; *lin-1(sy254)*, [*unc-32(e189)lin-12(n137)*; *lin-1(sy254)*].

required for VPCs to adopt the 2° fate in a wild-type background, because no VPCs in *lin-12(0)* animals adopted the 2° fate (Greenwald et al. 1983). Similarly, in *lin-12(0); lin-1(0)* animals, 0/36 VPCs adopt the 2° fate (Table 3A). This observation suggests that VPCs adopt the 2° fate in *lin-1(0)* mutants as a result of lateral signaling.

Because *lin-1(0)* mutations cause many of the effects of activating the Ras pathway, including activation of the lateral signaling pathway, we explored the relationship between the inductive and lateral signaling pathways by examining double mutants between *lin-1(0)* alleles and dominant activated *lin-12* mutations (Greenwald et al. 1983; Greenwald and Seydoux 1990). Our results suggest that the effective increase in Ras pathway activity caused by a *lin-1(0)* mutation can override limited, but not large, increases in *lin-12* activity. Specifically, 16/36 VPCs adopted the 1° fate in *lin-12(d)/+; lin-1(0)* hermaphrodites (Table 3B), whereas no VPCs adopted the 1° fate in *lin-12(d)/+* animals (Mango et al. 1991). However, in *lin-12(d); lin-1(0)* hermaphrodites only 3/36 VPCs adopted the 1° fate, whereas 33/36 VPCs adopted 2° or 2°/1° hybrid fates (Table 3C). These observations suggest that if a VPC receives both inductive signaling from the AC and lateral signaling from adjacent VPCs, then the relative strengths of these signaling inputs can be assessed by a VPC while it decides its fate.

Discussion

LIN-1 is an ETS-domain protein that negatively regulates vulval cell fates

Genetic epistasis experiments suggest that *lin-1* acts after *let-60* Ras, *mek-2* MEK, and *mpk-1* MAPK in the receptor tyrosine kinase/Ras pathway that mediates vulval induction (Han et al. 1990; Lackner et al. 1994; Wu and Han 1994; Kornfeld et al. 1995; Wu et al. 1995). We have identified mutations that delete almost all of the *lin-1* gene and are therefore likely to be molecular null alleles. In these *lin-1(0)* mutants, almost all VPCs adopt vulval fates, even in the absence of the AC. Thus, *lin-1* is a negative regulator of the 1° and 2° vulval cell fates. *lin-1* may function by actively promoting the 3° fate in the absence of Ras pathway activity, by actively preventing the 1° and 2° vulval cell fates until Ras pathway activity releases this inhibition, or by a combination of these mechanisms. *lin-1* is likely to act as a transcription factor, because *lin-1* encodes a protein containing an ETS DNA-binding domain and because two strong loss-of-function *lin-1* alleles appear to result from point mutations that cause conservative substitutions of amino acids found in all ETS domains.

LIN-1 is probably regulated by the Ras pathway through MAPK phosphorylation because LIN-1 appears to act after a MAPK, LIN-1 has 18 potential MAPK phosphorylation sites and ETS-domain proteins in other systems have been shown to be controlled by MAPK phosphorylation (Brunner et al. 1994; O'Neill et al. 1994; Tre-

isman 1994). However, none of the eight *lin-1* point mutations that we have identified affect potential MAPK phosphorylation sites. All five point mutations outside the ETS domain result in termination codons. Whereas the lack of missense mutations outside the ETS domain may be coincidental, it is possible that many of the 18 potential MAPK phosphorylation sites are subject to phosphorylation and that elimination of any given site does not reduce LIN-1 activity significantly.

Similarities between lin-1 and other ETS-domain proteins

The signal transduction pathways mediating *C. elegans* vulval induction and *Drosophila* R7 photoreceptor induction are remarkably similar. In both, a receptor tyrosine kinase apparently interacts with an SH3-SH2-SH3 domain protein that activates a Ras, Raf, MEK, and MAPK kinase cascade (for review, see Dickson and Hafen 1994). Both pathways are negatively regulated by ETS-domain proteins: *C. elegans* by LIN-1 and *Drosophila* by Yan (Lai and Rubin 1992; Tei et al. 1992; Rebay and Rubin 1995). However, the ETS domains of LIN-1 and Yan are not very similar, and LIN-1 and Yan have no obvious similarity outside their ETS domains. Furthermore, the position of the ETS domain, which appears to be characteristic of some ETS subfamilies (Janknecht and Nordheim 1993; Wasylyk et al. 1993), is very different in LIN-1 and Yan: The Yan ETS domain is centrally located, whereas the LIN-1 ETS domain is near the amino terminus. Despite their apparently similar genetic roles, it is not clear how similarly *lin-1* and Yan act at the molecular level. The LIN-1 ETS domain is more similar to the ETS domain of *Drosophila* Pointed, which functions as a positive regulator of R7 cell fate (Brunner et al. 1994; Klaes et al. 1994). However, LIN-1 and Pointed share no significant similarity outside of the ETS domain, and the ETS domain of Pointed is located carboxy-terminally rather than amino-terminally as in LIN-1. It is possible that neither Yan nor Pointed is a direct homolog of LIN-1 and that Yan, Pointed, and LIN-1 lie at the point of divergence between the vulval and R7 conserved signal transduction cascades as these cascades act on cell-type-specific transcription factors.

The LIN-1 ETS domain is most similar to the Elk-1, Net, and SAP-1 ETS domains, and the ETS domains of all of these proteins are similarly located at their amino termini (Dalton and Treisman 1992; Rao and Reddy 1992; Giovane et al. 1994). However, LIN-1 does not have the non-ETS regions of similarity shared among Elk-1, SAP-1, and Net and required for the formation of the serum response factor ternary complex (for review, see Treisman 1994). Apart from the ETS domain, LIN-1 has no significant similarity to proteins in the current data bases. As LIN-1 does not obviously fit into any current family of ETS-domain proteins and does not appear to have direct homologs in other species at this time, it may define a new subfamily of ETS-domain proteins.

The inductive signaling pathway is branched

If inactivation of *lin-1* were the sole function of the inductive signaling pathway, VPCs lacking *lin-1* activity should be insensitive to the presence or absence of an AC. However, we found that the AC induced P6.p to adopt a 1° rather than 2° fate in *lin-1(0)* mutants, indicating that VPCs do not require *lin-1* to respond to AC signaling. Therefore, the inductive pathway cannot be a simple linear pathway, and *lin-1* defines one branch of the inductive pathway. We consider two alternative models for this branching.

If the LIN-1 protein is a direct target of the MPK-1 MAPK, the inductive pathway might branch at any of several points prior to *lin-1*. In mammalian Ras pathways, branching can occur at RTKs, Ras, and MAPK (Pawson 1992; Blenis 1993; Rodriguez-Viciana et al. 1994). In the *C. elegans* vulval induction pathway, Kornfeld et al. (1995) have argued that the pathway does not have an essential branch before *mek-2*, because *mek-2* null alleles cause the same phenotype as killing the AC. This observation suggests that the inductive pathway branches at *mek-2* or *mpk-1*. If LIN-1 is a target of the Ras pathway, then additional targets of the Ras pathway would act in parallel to LIN-1 to specify VPC fate and *lin-1* would define a divergent branch of the inductive pathway (Fig. 5A).

Alternatively, *lin-1* may not be directly regulated by the Ras pathway and may act by antagonizing the activity of Ras pathway targets (Fig. 5B). In this case, the branch of the inductive pathway defined by *lin-1* is a convergent branch. One way to distinguish between the diverging and converging branch models is to determine

Figure 5. Alternative models for the role of *lin-1* in vulval induction. (A) *lin-1* defines one of two branches of the inductive signal transduction pathway. In this model, *lin-1* is a direct target of *mpk-1* MAPK, and the inductive pathway branches divergently at *mpk-1*. As discussed in the text, the inductive pathway may branch at points other than, or in addition to, *mpk-1*. *lin-1* and *mpk-1* each act through an unknown number of genes to control vulval cell fate. Arrows indicate positive regulatory interaction; T-headed arrows indicate negative regulatory interaction. (B) *lin-1* defines a convergent branch of the inductive pathway. In this model, a *lin-1* pathway and the inductive signaling pathway known previously converge to regulate vulval cell fate. (?) The activity of *lin-1* could be actively regulated by signaling processes other than the inductive signaling pathway from the anchor cell.

biochemically if *lin-1* activity is regulated by the Ras pathway. If *lin-1* does define a branch that converges with the RTK/Ras signaling pathway, then *lin-1* would be superficially similar in function to the synthetic Muv (synMuv) genes, which also negatively regulate vulval cell fates (Ferguson and Horvitz 1989; Herman and Hedgecock 1990; Clark et al. 1994; Huang et al. 1994). However, the synMuv genes appear to attenuate signaling through the RTK/Ras signaling pathway, because mutations in these genes require the activities of *let-23* RTK and *let-60* Ras to cause VPCs to adopt vulval cell fates (Ferguson et al. 1987; Beitel et al. 1990; Han et al. 1990). In contrast, *lin-1* appears to control vulval cell fates by acting after the known genes that function in Ras pathway signal transduction, because *lin-1* mutations cause VPCs to adopt vulval fates even in animals that are defective in the activities of the known Ras pathway signaling genes, including *let-23* RTK, *let-60* Ras, and *mpk-1* MAP kinase (Ferguson et al. 1987; Han et al. 1990, 1993; Lackner et al. 1994; Wu and Han 1994; Kornfeld et al. 1995; Wu et al. 1995).

Integration of the inductive and lateral signaling pathways

Cell-fate decisions can involve the reception and integration of multiple extracellular signals (Herman and Hedgecock 1990; Rusch and Levine 1994; Firtel 1995). During *C. elegans* vulval induction, two signals have been proposed to act in specifying the 2°/1°/2° pattern of vulval fates: a graded signal from the AC and a *lin-12*-mediated lateral signal between VPCs (Sternberg and Horvitz 1989). Sternberg and Horvitz (1986), Thomas et al. (1990), and Katz et al. (1995) have provided evidence that a graded AC signal can specify the 2° cell fate, whereas Simske and Kim (1995) and Koga and Ohshima (1995) have argued that the lateral signal is sufficient to specify the 2° cell fate during vulval induction. The ability of VPCs to assess and integrate these two signals and whether a graded AC signal, a lateral signal, or both specify the invariant 2° cells fates in wild-type animals have not been established.

We have shown that in *lin-1(0)* mutants, lateral signaling appears to be activated relatively normally: In *lin-1(0)* animals, adjacent VPCs rarely both adopt the 1° fate, whereas in double mutants lacking *lin-1* and *lin-12*, adjacent VPCs frequently both adopt the 1° fate. These results suggest that inactivation of *lin-1* leads to at least a transient activation of the lateral signal, because VPCs lacking *lin-1* still signal neighboring VPCs to adopt the 2° fate, even in the absence of inductive signaling from the AC. Thus, the *lin-1* branch of the inductive pathway may play an important role in regulating lateral signal activity. One possibility is that the LIN-1 protein is a transcriptional repressor of the lateral signal.

The relatively normal functioning of lateral signaling in *lin-1(0)* animals also suggests that the LIN-12 protein, the presumptive receptor for the lateral signal (Greenwald et al. 1983; Seydoux and Greenwald 1989; Sternberg and Horvitz 1989), specifies the 2° fate after or in

parallel to the action of LIN-1 in the inductive pathway. This inference is supported by our observation that even in the absence of *lin-1* activity, a high level of *lin-12* activity can cause most VPCs to adopt the 2° fate instead of the 1° fate.

In responding to inductive and lateral signaling, do VPCs assess the relative or absolute activity levels of these signaling pathways? In otherwise wild-type animals, even one copy of a dominant-activated *lin-12* mutation is sufficient to cause almost all VPCs to adopt 2° or 2°/1° fates (Greenwald et al. 1983; Sternberg and Horvitz 1989; Mango et al. 1991). In contrast, we found that in animals carrying one, but not two, copies of the activated *lin-12* mutation, activation of the inductive signaling pathway by *lin-1(0)* mutations caused many VPCs to adopt the 1° fate. Similar dose-dependent effects of activated *lin-12* mutations have also been seen in animals in which the RTK/Ras pathway has been activated by mutations in the *lin-15* locus (Sternberg and Horvitz 1989; see Tables 1E,F; 2C,D; 3F,G), which negatively regulates the RTK/Ras pathway by as yet undetermined mechanisms (Clark et al. 1994; Huang et al. 1994). Together these results suggest that the relative rather than the absolute levels of the inductive and lateral signaling can be assessed by the VPCs while they decide their fates. These results therefore support models of vulval development in which both graded AC signals and lateral signals contribute to specifying the remarkably invariant pattern of cell fates in wild-type hermaphrodites.

Materials and methods

Strains and cultures

C. elegans variety Bristol strain N2 was the wild-type parent of all strains used in this work except where indicated. In particular, polymorphism mapping was done using *C. elegans* strain variety DP13, which was derived from RW7000 by D. Pilgrim (pers. comm.). Strains were handled and maintained as described by Brenner (1974). Animals were grown at 20°C, except where indicated. The genetic markers used were described by Wood (1988), except where noted, and are as follows: LG III *unc-36(e251)*; *dpy-19(e1259)*; *unc-32(e189)*; *lin-12(n137, n941)*; *qC1* (balancer chromosome, Austin and Kimble 1989; Edgley et al. 1995); LG IV *lin-1(e1026, e1275, e1777, n176, n303, n304, n383, n431, n753, n1047, n1054)* (Ferguson and Horvitz 1985); *lin-1(n1140)* (J. Park and E. Ferguson, pers. comm.); *lin-1(n1814, n1815, n1816, n1817, n1848)* (S.G. Clark and H.R. Horvitz, pers. comm.); *lin-1(n2694, n2695, n2696, n2698, n2699, n2700, n2701, n2702, n2703, n2704, n2705)* (X. Lu and H.R. Horvitz, pers. comm.); *lin-1(ar147)* (this study), *lin-1(sy254)* (G. Jongeward and P.W. Sternberg, pers. comm.); *lin-1(m546)* (M. Edgley, pers. comm.); *lin-22(n372)*; *unc-33(e204)*.

The cosmids and YACs used in this work were obtained from Alan Coulson (The Sanger Centre, Hinxton, UK).

General DNA manipulation and analyses

General DNA manipulation and analyses were done using the methods described by Sambrook et al. (1989). Subclones were made using the pBluescript SK(+) and SK(−) vectors (Stratagene Cloning Systems, La Jolla, CA). Probes for Southern blots were made either by random priming from a cloned DNA tem-

plate using [α -³²P]dATP or by PCR amplification of a template substituting [α -³²P]dATP for dATP in the reaction mix.

Positional cloning of *lin-1*

To obtain genetic and physical markers to locate *lin-1* on the physical map, we identified restriction fragment length polymorphisms using random primed probes from cosmids and other clones to probe genomic Southern blots of *Eco*RI-digested N2 and DP13 genomic DNA. Polymorphisms were mapped in 22 Unc non-Muv and Muv non-Unc recombinant progeny from N2 *lin-1(e1275)* *lin-22(n372)* *unc-33(e204)*/DP13 *lin-1(+)* *lin-22(+)* *unc-33(+)* parents. The detailed methods and results of these experiments have been described by Beitel (1994).

Germ-line transformation experiments

Germ-line transformation experiments were done using CB1275 *lin-1(e1275)* raised at 15°C and the method of Mello et al. (1991). We placed injected animals at 20°C and scored the F₁ “roller” progeny for the Muv phenotype. Transmitting lines were established, and F₂ rescue of the *e1275* Muv phenotype was scored at 20°C and 25°C. In no case was complete rescue of the *e1275* mutant phenotype observed. “Rescued” animals no longer had the *e1275* Muv phenotype but usually had protruding vulvae and were egg-laying defective.

cDNA library screening and DNA sequence determination and analysis

We screened cDNA libraries obtained from Barstead and Maruyama (1989; Maruyama and Brenner 1992) using the 12.0-kb *Eag*I–*Spe*I-rescuing fragment labeled by random priming. We isolated a total of 25 independent cDNAs.

We determined cDNA and genomic DNA sequences using single- and double-stranded templates processed by the Applied Biosystems Prism Cycle Sequencing protocols and a model 373A DNA sequencer (Applied Biosystems, Inc., Foster City, CA). We assembled DNA sequence using the XBAP program (Dear and Staden 1991). We analyzed sequences using the GeneWorks program (IntelliGenetics, Inc., Mountain View, CA) and the network BLAST sequence comparison service provided by the National Center for Biotechnology Information accessed with the network BLAST client written by W. Gilbert (University of New Hampshire, Durham, NH).

We determined the 3′ and 5′ end sequences of all 25 cDNAs using the forward and reverse primers for the pBluescript plasmid. We determined the sequence of the longest of those cDNAs using nested deletions generated by exonuclease III digestion (Sambrook et al. 1989) and sequence for the 12-kb *Eag*I–*Spe*I C37F5 genomic fragment using the method of shotgun cloning into M13 described by Wilson et al. (1994).

We determined the genomic sequences of eight *lin-1* alleles that were not polymorphic by Southern blot analysis. We selected *n431* and *e1777* because they are suppressible by an amber-suppressor tRNA mutation, and *e1275* because it was the only known temperature-sensitive *lin-1* mutation (Ferguson and Horvitz 1985). We chose the remaining alleles because they had been characterized previously by Sulston and Horvitz (1981) and Ferguson and Horvitz (1985). We determined the complete sequences of the exons and at least 15 bp on either side of exon splice sites using the cycle sequencing protocol of Craxton (1991) and [γ -³³P]ATP as a label. All differences from the N2 sequence are reported in the text.

Determination of the genomic organization of lin-1

The determination of the genomic organization of *lin-1* has been described in detail by Beitel (1994). Briefly, the first 160 bp of the longest cDNA is derived from two exons separated from the remaining exons by the 15- to 20-kb second intron. The size of the second intron was estimated from the minimal size of genomic DNA fragments that hybridize to both the second and third exons and from the observation that we were unable to recover genomic DNA containing both the second and third exons from a λ gt11 library. To confirm that the structure of the longest cDNA accurately represented the predominant *lin-1* message, we analyzed the 5' and 3' sequences of the 25 cDNAs we had isolated. With one exception, the structures of these cDNAs were consistent with that of the longest cDNA, including the structures of nine cDNAs long enough to span the second/third exon boundaries. The one exceptional cDNA had 7 bp of SL1 *trans*-splice sequence joined to the third *lin-1* exon (Fig. 2C).

Southern blot analysis of lin-1 alleles

We examined *lin-1* region genomic DNA from strains containing the following alleles: *ar147*, *e1026*, *e1275*, *e1777*, *m546*, *n176*, *n303*, *n304*, *n381*, *n383*, *n431*, *n753*, *n757*, *n1047*, *n1054*, *n1140*, *n1814*, *n1815*, *n1816*, *n1817*, *n1848*, *n2692*, *n2693*, *n2694*, *n2695*, *n2696*, *n2698*, *n2699*, *n2700*, *n2701*, *n2702*, *n2703*, *n2704*, *n2705*, and *sy254*. We used PCR-labeled exon-specific fragments to probe Southern blots of genomic DNA from the above strains digested with *Eco*RI and with *Xba*I and *Spe*I.

Phenotypic analysis of strains

The phenotypes of homozygous strains were determined by placing one to six L4 hermaphrodites per plate and placing groups of plates at 15°C, 20°C, or 25°C. Hermaphrodites were transferred to fresh plates once or twice per day until the hermaphrodites no longer produced progeny. Progeny were scored for larval phenotypes ~2 days after hatching. We examined larval viability in *lin-1(0)* mutants because mutations that reduce the activities of some genes in the vulval induction signaling pathway result in a rod-like larval lethality that is suppressed by reduction-of-function *lin-1* mutations (Ferguson and Horvitz 1985; Beitel et al. 1990; Han et al. 1990; Aroian and Sternberg 1991; Clark et al. 1992; Kornfeld et al. 1995). Neither *n304* nor *sy254* caused any larval lethality (data not shown), demonstrating that *lin-1* is not required in the Ras-mediated process controlling larval viability.

We scored animals for vulval phenotypes within 24 hr of the L4 molt to adulthood. Animals were scored as Muv if they had ventral protrusions (pseudovulvae) in addition to a vulva or if they had ruptured and could not be directly scored for the presence of pseudovulvae. Animals were scored as egg-laying defective if progeny hatched within the parent. Unlike animals with a Muv phenotype caused by mutations in other genes such as *let-60* Ras, almost all *lin-1(0)* animals have protruding vulvae and those that do not rupture are egg-laying defective (data not shown). These observations suggest that *lin-1(0)* mutations either do not induce VPCs to execute normal vulval fates or that *lin-1* activity is required for correct vulval morphogenesis.

Lineage analysis and laser ablation experiments

The methods for observing VPC lineages and the criteria and nomenclature for assigning 1°, 2°, and 3° fates to lineages are described by Sternberg and Horvitz (1989). Laser ablations of cells was done by the method of Sulston and White (1980) using

the laser microbeam system described by Avery and Horvitz (1987). We ablated the gonadal precursor cells Z1 and Z4 using the method of Sternberg (1988) and ablated the AC and all Z1 and Z4 descendants except the AC using the method of Kimble (1981). To control for debris left by the Z1/Z4 descendant ablations, we ablated all the Z1/Z4 descendants including the AC in some animals.

Construction and lineage analysis of double mutants

lin-12;lin-1 double mutant strains were constructed using standard genetic techniques (Wood 1988). Important details are as follows: *unc-36(e251) lin-12(n941)*; *lin-1(sy254)* animals segregating from the strain *unc-36(e251) lin-12(n941)/unc-32(e189)*; *lin-1(sy254)* were identified for lineage determination as *Unc-36* animals with two ACs at the early L3 stage. *unc-32(e189) lin-12(n137) qC1*; *lin-1(sy254)* heterozygotes had the large, irregularly spaced pseudovulva phenotype characteristic of *lin-1(0)* mutants.

unc-32(e189) lin-12(n137); *lin-1(sy254)* animals segregating from the strain *unc-32(e189) lin-12(n137)/qC1*; *lin-1(sy254)* were identified for lineage determination as *Unc-32* animals with no ACs at the early L3 stage. *unc-32(e189) lin-12(n137)*; *lin-1(sy254)* homozygotes usually had the small, evenly spaced pseudovulva phenotype of *lin-12(n137)* mutants.

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