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# Role of a *raf* proto-oncogene during *Caenorhabditis elegans* vulval development

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**SUMMARY**

During *Caenorhabditis elegans* vulval induction, multipotent precursors respond to an inductive signal by generating vulval cells as opposed to non-specialized epidermal cells. Both classical and 'reverse' genetic approaches have revealed that a cascade of nematode homologues of mammalian proto-oncogenes is necessary for induction of the vulva. The inductive signal is a growth factor encoded by the *lin-3* gene and its candidate receptor is a tyrosine kinase encoded by the *let-23* gene. *let-23* acts via a Ras protein encoded by the *let-60* gene. A nematode homologue of mammalian *raf* family protein serine/threonine kinases has been cloned and found to be encoded by the *lin-45* gene. Dominant negative *lin-45 raf* mutants prevent vulval induction. A recessive *lin-45 raf* mutation prevents the excessive vulval differentiation caused by activated *ras*, indicating that *raf* might act downstream of *ras* during vulval induction.

## 1. INTRODUCTION

*Caenorhabditis elegans* was originally chosen for an experimental system because of its potential for facile developmental genetic analysis (Brenner 1974). The *C. elegans* genome project (Coulson *et al.* 1988; Sulston *et al.* 1992) and the ability to rapidly make transgenic animals (Fire 1986; Mello *et al.* 1991) has made *C. elegans* an excellent organism for intensive molecular biological study. From combined genetic and molecular biological efforts over the past few years, it has become clear that evolutionarily conserved proto-oncogenes play key roles in *C. elegans* development, and thus the power of *C. elegans* molecular genetics can be applied to the understanding of proto-oncogene function. In particular, genes can be ordered into functional pathways *in vivo* by the construction of doubly mutant strains, and new genes can be identified by extragenic suppressor analysis. Such genetic 'pathway analysis' can complement biochemical studies carried out on mammalian cells. We describe here our recent analysis of the role of a protein serine/threonine kinase gene homologous to the *raf* family. This analysis is primarily done in the context of vulval induction, so we first introduce this aspect of *C. elegans* development.

## 2. VULVAL INDUCTION IN *C. elegans*

Vulval induction in *C. elegans* provides an opportunity to study organogenesis using molecular genetics. The vulva forms from the ventral epidermis after induction by the somatic gonad (reviewed by Greenwald & Rubin 1992; Horvitz & Sternberg 1991). In response to a signal from the gonadal 'anchor cell', three of six vulval precursor cells (VPCs) undergo three rounds of mitosis and generate vulval cells (figure 1). The other three VPCs generate non-specialized epidermis. In the absence of the anchor cell, all VPCs divide once and generate non-specialized epidermis and no vulva is formed. The vulva is required for egg-laying and for copulation with males. However, as *C. elegans* hermaphrodites are self-fertilizing, the vulva is not required for propagation of strains because progeny can hatch internally and escape, and mutants can be easily recognized under a dissecting microscope. In addition to this inductive signal, there are at least two other intercellular signalling pathways. A negative signal prevents VPCs from proliferating and generating vulval progeny in the absence of inductive signal. A lateral signal helps distinguish two types of induced VPCs. Mutants defective in vulval induction are of two major classes. In vulvaless mutants, all VPCs generate non-specialized epidermis. These mutants are either defective in the production of inductive signal or the response to the inductive signal. In multivulva mutants, additional VPCs generate vulval cells. Some of these mutants render the VPCs independent of inductive signal. Others remain signal-dependent. Such a signal-dependent multivulva phenotype might result from excessive signal production or from VPCs that are hypersensitive to inductive signal.

The initial studies of such vulvaless mutants defined



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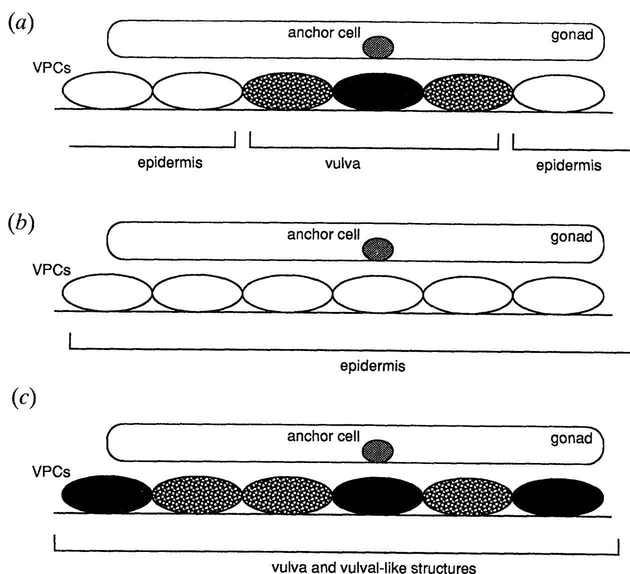


Figure 1. Vulval induction. (*a*) In the wild-type animal, three vulval precursor cells (VPCs) generate vulval progeny. Dark fill represents the central subset of vulval cells (the 1° lineage); stippled fill represents the flanking subsets of vulval progeny (the 2° lineage). More distal VPCs generate non-specialized epidermal progeny (white fill). The anchor cell is located in the center of the somatic gonad and induces vulval formation. (*b*) In vulvaless mutants, the anchor cell and the VPCs are present, but no vulva is formed. (*c*) In multivulva mutants, all six VPCs generate vulval progeny. The pattern is somewhat variable, but adjacent cells are only very rarely both 1° due to a separate signalling pathway.

several genes that are required for vulval induction, in particular, *lin-2*, *lin-3*, *lin-7*, *lin-10*, and *let-23* (table 1) (Aroian & Sternberg 1991; Ferguson & Horvitz 1985; Ferguson *et al.*, 1987; Horvitz & Sulston 1980; Sternberg & Horvitz 1989; Sulston & Horvitz 1981). Initial studies of multivulva mutants defined several genes required to prevent excessive vulval differentiation (Ferguson & Horvitz 1985, 1989; Ferguson *et al.* 1987; Horvitz & Sulston 1980; Sulston & Horvitz 1981). These genes include *lin-1* and *lin-15*. *lin-15* appears to be defective in the negative signalling pathway (figure 2). Mosaic analysis indicates that *lin-15* normally acts in cells other than the VPCs and the anchor cell (Herman & Hedgecock 1990). In *lin-15* mutants, the inductive signal and lateral signalling pathways can still operate, and thus we infer that *lin-15* acts in parallel and antagonistically to the inductive signal (Han *et al.* 1990; Sternberg 1988).

## 3. EXTRAGENIC SUPPRESSORS OF *lin-15* MULTIVULVA PHENOTYPE DEFINE KEY COMPONENTS OF THE SIGNAL TRANSDUCTION PATHWAY

An extensive analysis of existing mutations affecting the extent of vulval differentiation revealed that a *let-23* mutation would suppress the multivulva phenotype of a *lin-15* putative loss-of-function mutation (Ferguson *et al.* 1987). By contrast, mutations of *lin-2*, *lin-3*, *lin-7*, and *lin-10* would not suppress the *lin-15* phenotype (table 2). We therefore screened for mutations that suppress the multivulva phenotype of *lin-15* homozygous hermaphrodites (figure 3). From a screen of approximately 100 000 mutagenized gametes, we recovered ten suppressor mutations. Genetic mapping and complementation analysis revealed that these mutations defined three loci. Two mutations mapped to linkage group II and failed to complement *let-23* (Aroian & Sternberg 1991). Seven mutations caused a semidominant vulvaless phenotype and mapped near *dpy-20* on linkage group IV; six of these caused a recessive lethal phenotype. Subsequent analysis indicated that these were allelic to each other and to recessive lethal mutations of the previously defined *let-60* locus (Han *et al.* 1990). The tenth mutation, *sy96*, defined a new locus on linkage group IV, *lin-45* (Han *et al.* 1993).

The *let-23* mutations are non-null alleles of *let-23* (Aroian & Sternberg 1991). The *let-60* mutations are dominant negative alleles of *let-60 ras* (Han *et al.* 1990; Han & Sternberg 1991). S. Clark & R. Horvitz (personal communication) have obtained a number of extragenic suppressors of a temperature-sensitive *lin-15* allele; some of these overlap with the set we identified; others define new genes such as *sem-5* (Clark *et al.* 1992).

Table 1. *Summary of genes in the vulval induction pathway*

| | | vulval induction phenotype[b] | | |
|---|---|---|---|---|
| gene | product[a] | lf | gf | role |
| *lin-3* | ligand for EGF-R | vul | muv | inductive signal |
| *lin-23* | EGF-receptor | vul | ? | receptor |
| *sem-5* | SH3-SH2-SH3 | vul | ? | transducer |
| *let-60* | *ras* | vul | muv | transducer |
| *lin-45* | *raf* | vul | ? | transducer |
| *lin-1* | uncloned | muv | ? | neg. reg. of vulva differentiation |
| *lin-15* | novel | muv | ? | neg. reg. of *let-23* |

[a] Product, homology or similarity. EGF, epidermal growth factor; EGF-R, EGF-receptor. Ligands for human EGF-R include transforming growth factor α, heparin-binding epidermal growth factor, amphiregulin and EGF.
[b] lf, reduction- or loss-of-function phenotype; gf, gain-of-function phenotype; neg. reg., negative regulator; role, proposed role based on genetic studies; vul, vulvaless; muv, multivulva; ?, gain-of-function mutants not available.

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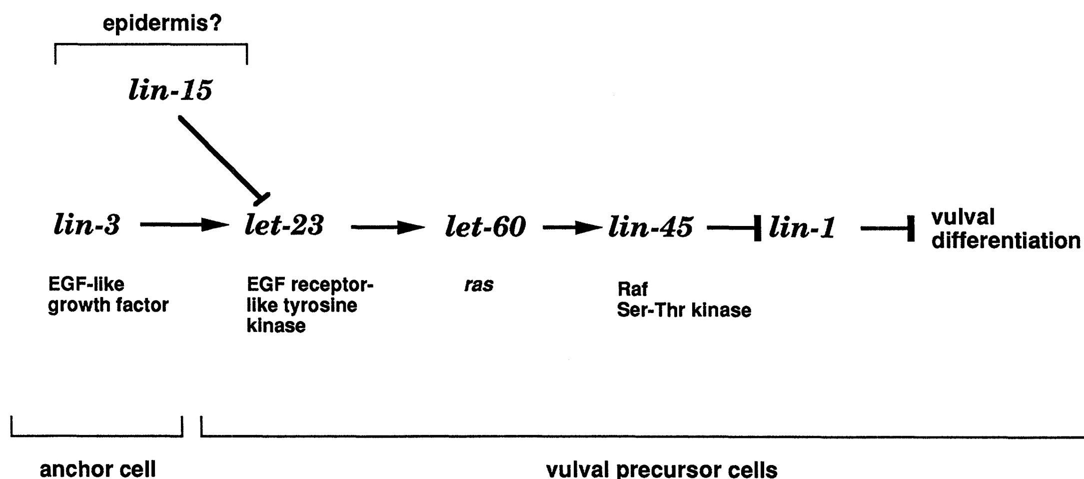


Figure 2. Vulval induction pathway. The key known players in the vulval induction are shown. An arrow indicates a positive effect; a bar represents negative regulation. *lin-15* has been proposed to act in cells other than the VPCs and the anchor cell, presumably in the epidermis adjoining the VPCs.

## 4. A MOLECULAR PATHWAY OF VULVAL INDUCTION

Molecular cloning of four of the genes defined by recessive vulvaless mutants has revealed that these genes encode proteins homologous to mammalian intercellular signalling and signal transduction components. The vulval induction pathway thus provides an excellent opportunity to study a cascade of genes in the context of the normal development of an organism. The identification of these four components is described below.

The *lin-3* gene encodes an inductive signal for vulval development. *lin-3* is required for induction of the vulva (Ferguson & Horvitz, 1985, Ferguson *et al.* 1987; Horvitz & Sulston 1980; Sulston & Horvitz 1981), and its overexpression in transgenic nematodes results in hyperinduction of the vulva (Hill & Sternberg 1992). Thus, the level of *lin-3* activity specifies the extent of vulval differentiation. The product of *lin-3* inferred from translation of cDNA sequence is a putative growth factor precursor with the architecture of precursors to ligands of the human epidermal growth factor (EGF) receptor. An amino-terminal signal sequence is followed by a single EGF repeat and a transmembrane domain. We do not yet know whether Lin-3 acts in a membrane-bound or diffusible form. *lin-3* is expressed in the anchor cell at the time of vulval induction as indicated by histochemical staining of animals carrying a *lin-3-lacZ* reporter construct (Hill & Sternberg 1992).

The *let-23* gene encodes a candidate receptor for the *lin-3* inductive signal. *let-23* is required for vulval induction (Aroian & Sternberg 1991; Ferguson & Horvitz 1985). Recessive mutations in the *let-23* gene result in a failure of vulval induction and hence an egg-laying defect. *let-23* is required for the excessive vulval differentiation caused by overexpression of *lin-3* (Hill & Sternberg 1992). *let-23* encodes a receptor tyrosine kinase of the EGF-receptor subfamily (Aroian *et al.* 1990). These proteins have a large extracellular domain that has two cysteine-rich regions with characteristic spacing of cysteine residues (reviewed by Ullrich & Schlessinger 1990). A domain between the cysteine-rich domains is thought to bind ligand. A single helix transmembrane domain connects the

Table 2. *Epistasis table*

(muv, multivulva; vul, vulvaless; n.d., not determined. Data from Aroian & Sternberg (1991), Ferguson *et al.* (1987), Han *et al.* (1990, 1993), Han & Sternberg (1990), Hill & Sternberg (1992).)

| | multivulva | | | |
|---|---|---|---|---|
| vulvaless | *lin-3* transgene[a] | *lin-15* | *let-60* (gf) | *lin-1* |
| *lin-3* (lf) | muv | muv | muv | muv |
| *let-23* (lf) | vul | vul | muv | muv |
| *let-60* (lf) | n.d. | vul | muv | muv |
| *lin-45* (lf) | n.d. | vul | vul | muv |

[a] *lin-3* multivulva mutant generated by high copy transgene.

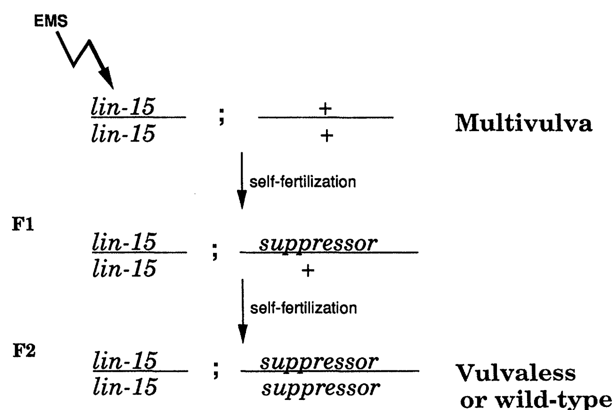


Figure 3. *lin-15* reversion experiment. The grandprogeny of mutagenized *lin-15* homozygous hermaphrodites were screened for reversion of the multivulva phenotype. The $F_2$ typically arise one week after mutagenesis. Some suppressors are recessive; others are dominant and were recovered as heterozygotes. EMS, ethylmethane sulphonate.

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extracellular domain to a cytoplasmic tyrosine kinase domain. The carboxyltermini of this family contains the regulatory autophosphorylation sites as well as domains necessary for substrate interaction and negative regulation (Schlessinger & Ullrich, 1992).

The *sem-5* gene is also necessary for vulval induction. The *sem-5* gene encodes a protein consisting of two SH3 domains flanking one SH2 domain (Clark *et al.* 1992). *sem-5* is a homologue of the human GRB-2 protein, which binds the carboxyl tail of the human EGF-receptor via its SH2 domain (Lowenstein *et al.* 1992). This observation, coupled with the finding that over-expression of GRB-2 and mammalian Ras in cultured fibroblasts results in cellular transformation, suggests that *sem-5*/GRB-2 is also involved in tyrosine kinase-mediated signalling pathways. While ordering of *sem-5* with respect to *let-23* cannot yet be done, it is likely to act similarly during vulval induction, by analogy with GRB-2.

The *let-60* gene acts like a switch controlling vulval differentiation (Beitel *et al.* 1990; Han *et al.* 1990). High activity of *let-60* leads to excessive vulval differentiation irrespective of the presence of inductive signal (multivulva phenotype). Low *let-60* activity leads to epidermal differentiation as opposed to vulval differentiation. *let-60* encodes a Ras protein (Han & Sternberg 1990). Mutations that by genetic criterion appear to increase *let-60* activity are activating mutations (Gly 13 to Glu) analogous to weak activating mutations in mammalian Ras proteins (Beitel *et al.* 1990). Activated *let-60 ras* mutants bypass the need for *lin-3*, *let-23* and *sem-5* (Clark *et al.* 1992; Han *et al.* 1990). Moreover, overexpression of the wild-type *let-60* gene bypasses requirement for at least *lin-3* and *let-23* (Han & Sternberg 1990). Thus, *let-60* likely acts downstream of these other three components during vulval induction. Dominant negative mutations of *let-60 ras* have also been selected *in vivo* (Beitel *et al.* 1990; Han *et al.* 1990). These mutations map to residues analogous to those that when mutated interfere with guanine nucleotide binding in the mammalian and yeast Ras proteins (Han & Sternberg 1991).

From the molecular identities of these four genes and their mutant phenotypes, it is clear that signalling pathways similar to mammalian tyrosine kinase-mediated mitogenic signalling exist in *C. elegans*. The study of these components, as well as the identification of other interacting gene products, should enhance our understanding of the function of these genes in both mammalian cell signalling and nematode development.

## 5. MOLECULAR CLONING OF A *C. elegans raf* HOMOLOGUE

In addition to a classical genetic approach (from mutation to clone), we undertook a 'reverse genetic' (from clone to function) approach to identify other anticipated components of this signalling pathway. Given the striking similarity of the *lin-3–let-23–sem-5–let-60* pathway with that of the EGF-receptor-mediated pathway in mammalian cells, we reasoned that a Raf serine/threonine kinase might act in the vulval induction pathway. The Raf serine/threonine kinase has been implicated in EGF-receptor mediated signal transduction by the observations that it complexes with the human EGF-receptor and is phosphorylated upon EGF stimulation of cells (App *et al.* 1991; Li *et al.* 1991; Morrison 1990). Experiments with dominant negative Raf mutants and anti-Raf antibodies have also suggested that Raf acts downstream of both receptor tyrosine kinase and the Ras proto-oncogene. For these reasons, we cloned a *C. elegans* Raf homologue, *Ce-raf*, by polymerase chain reaction using degenerate oligonucleotide primers designed against regions in the kinase domain highly conserved among the Raf proteins from various species (Han *et al.* 1993). Sequence of a full-length cDNA revealed a protein with similarity in overall architecture to the Raf family of kinases. The three conserved regions present in all members of the Raf family are present in

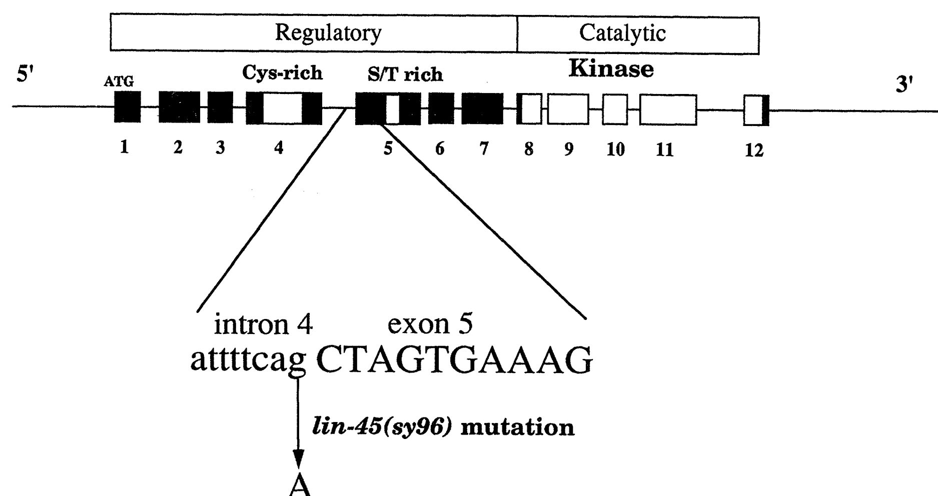


Figure 4. Domain structure of Raf and the *lin-45(sy96)* mutation. The 12 exons of *Ce-raf* are shown with key domains marked. CR1 is a cysteine-rich domain. CR2 is a serine- and threonine-rich domain. The kinase domain is CR3. From Han *et al.* (1993).

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the *C. elegans* homologue. The carboxyl-terminal kinase domain (conserved region 3) had 59% amino acid identity with the kinase domains of the human and *Drosophila* proteins. Conserved regions 1 and 2 (CR1 and CR2) are in the amino-terminal half of the Raf proteins and serve a regulatory function (figure 4). Deletion of CR1 and/or CR2 in mammalian Raf proteins results in a constitutive, oncogenic form of the kinase (Heidecker *et al.* 1990; Stanton *et al.* 1989). Expression of a kinase-inactive form, or of just CR1, interferes with activity of Raf, i.e. these forms are dominant negative. The CR1 domain includes a cysteine-rich putative metal binding finger and presumably interacts with regulatory factors that normally activate wild-type Raf (Morrison, 1990). Protein kinase C (PKC) has a similar metal-binding finger that has been shown to bind zinc, and also is the site of interaction with phorbol ester, activators of PKC (Ono *et al.* 1989). By analogy with PKC, CR1 might be a site for interaction with phospholipid or a protein activator.

## 6. DOMINANT NEGATIVE MUTANTS OF *RAF* INTERFERE WITH VULVAL INDUCTION

To test whether Ce-Raf has a role in vulval induction or other aspects of *C. elegans* development, we engineered a dominant negative version of *raf* (Han *et al.* 1993). Specifically, we mutated *in vitro* the critical lysine of the ATP-binding site to a tryptophan codon. By analogy with dominant negative mutants in a number of other kinases, such a mutation would inactivate the kinase, but leave sites of interaction with regulatory factors intact. Thus, if the kinase-defective protein titrates a positively acting factor, then the endogenous pathway is disrupted. We constructed transgenic animals carrying the dominant negative version of *raf* driven by its own regulatory sequences (the mutation was made in a 9.7 kb genomic fragment, and is thus likely to be under normal regulation). About 40% of such transgenic animals are egg-laying defective. Examination of such transgenic animals under Nomarski optics indicates that the egg-laying defect is due to a failure in vulval induction: approximately one-third lack any vulval differentiation. Thus, the dominant negative version of *raf* causes a similar phenotype to loss-of-function mutations in the *lin-3* growth factor, *let-23* tyrosine kinase and *let-60 ras*, as well as dominant negative *let-60 ras* mutants.

## 7. *lin-45* ENCODES *raf*

Physical mapping of the *raf* homolog placed it in a 500 kb interval on the physical map of *C. elegans* chromosome IV. This interval of the physical map corresponds to a 0.5 map unit interval on the genetic map that contains *lin-45* (figure 5). We therefore tested whether a raf genomic clone could complement the vulvaless and lethal phenotypes of the *lin-45(sy96)* mutation. By injecting a genomic clone of *raf* as well as marker DNA into animals carrying a *lin-45* mutation, we determined that a *raf* clone at high level could rescue the mutant phenotypes of the *lin-45* mutation (Han *et al.* 1993).

To confirm that *lin-45* encodes *raf* and is not merely rescued by *raf* overexpression, we sequenced genomic DNA from a *sy96* strain. The *sy96* mutation is a G to A transition in the absolutely conserved AG dinucleotide at the 3′ splice acceptor of intron 4 of *Ce-raf*. Because analysis of other AG to AA mutations in *C. elegans* has revealed that normal splicing can occur in the absence of the G nucleotide (Aroian *et al.* 1993), we analysed RNA products made in a *lin-45(sy96)* strain. Approximately 95% of the transcripts splice to an AG four nucleotides downstream, and thus result in a shift of reading frame leading to premature termination. The other 5% included transcripts that presumably encode functional or wild-type protein. The predominant transcript might encode a truncated protein that contains just CR1, which might be expected to be dominant negative (Bruder *et al.* 1992). However, the *sy96* mutation is recessive. Even two mutant copies of *lin-45(sy96)* are complemented by one copy of *lin-45*(+) in partial triploids (Han *et al.* 1993). Although we cannot exclude the possibility that the *sy96* mutation is a recessive interfering mutation, we con-

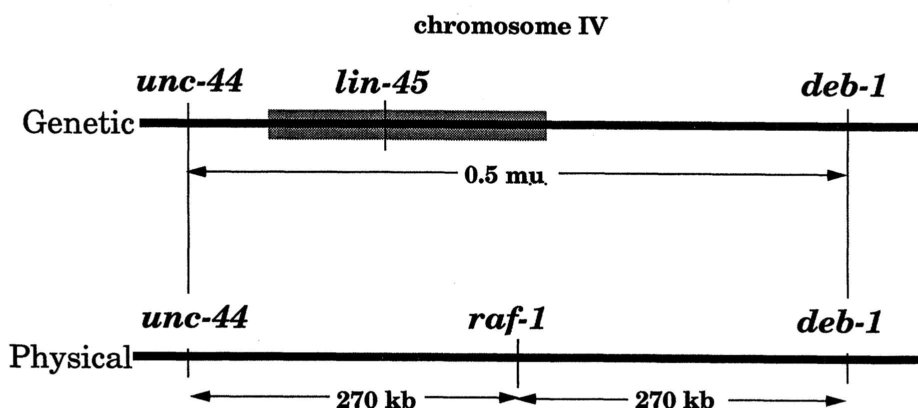


Figure 5. Genomic map of *lin-45/raf* region. The Genetic and Physical maps in the immediate vicinity of *lin-45 raf* are shown. *unc-44* and *deb-1* are markers that correlate the genetic and physical maps as they are defined by both molecular clones and genetic mutations. m.u., map units; kb, kilobase pairs of DNA.

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clude that *sy96* severely reduces activity of *lin-45 raf* and thus *lin-45* is necessary for vulval induction.

## 8. PATHWAY ANALYSIS

To test where *lin-45 raf* acts with respect to *let-60 ras*, we constructed double mutants carrying an activated *let-60 ras* mutation and the recessive *lin-45(sy96)* mutation (table 2) (Han *et al.* 1993). Such strains display less vulval differentiation than an activated *let-60* mutant. Because the *lin-45* mutation is not an RNA null, we cannot distinguish between the possibilities that *lin-45* is completely or only partially required for *let-60 ras* action. In either case, we conclude that *lin-45 raf* acts downstream of *let-60 ras* (figure 2), or it acts in parallel to *let-60 ras* with a convergent target.

## 9. DOWNSTREAM GENES

The *lin-1* gene is defined by recessive reduction-of-function mutations that result in a multivulva phenotype (Ferguson & Horvitz 1985; Horvitz & Sulston 1980). The absence of *lin-1* activity renders vulval differentiation independent of *lin-3*, *let-23*, *let-60* and *lin-45* (Ferguson *et al.* 1987; Han *et al.* 1990, 1993). Thus, formally, *lin-1* is a negative regulator of vulval differentiation whose action is antagonized by *let-60* and *lin-45*. Whether *lin-1* is a direct target of *lin-45* and *let-60* is an open question.

## 10. GENERAL ROLES OF THE PATHWAY

Loss-of-function mutations in *lin-3*, *let-23* and *let-60* are lethal (Aroian & Sternberg 1991; Beitel *et al.* 1990; Clark *et al.* 1988; Ferguson & Horvitz 1985; Han *et al.* 1990; Herman 1978). The severe reduction-of-function allele of *lin-45 raf* causes 90% of homozygotes to die, suggesting that the null phenotype is lethal. Additional genetic analysis will be required to demonstrate this point conclusively.

We have been able to study the effects of these essential genes on vulval induction by taking advantage of special alleles. For *lin-45*, we isolated an allele that makes some product because we selected for viability. For *let-60*, gain of function (activated) alleles and dominant negative alleles have defects in vulval induction. For *let-23*, three alleles were obtained as viable vulvaless mutations; these mutations either partially decrease activity of the protein or only affect the activity of the protein in certain tissues. For *lin-3*, either partial reduction-of-function or tissue-specific mutations have allowed us to see its role in vulval induction. For *lin-3* and *let-60*, overexpression of the wild-type gene results in a multivulva phenotype; vulval induction is very sensitive to changes in the activity of these proteins.

These genes are also required for induction during male spicule development (H. Chamberlin & P. W. Sternberg, unpublished observations) and specification of the P12 neuroectoblast (Aroian & Sternberg 1991; Fixsen *et al.* 1985; Han *et al.* 1993). The larval lethality prevents examination of potential other phenotypes during embryonic development, as an embryo could survive due to its maternal contribution of gene product.

## 11. Raf IN SIGNAL TRANSDUCTION

Our results support the emerging view that Raf acts downstream of Ras in response to tyrosine kinases. For example, in mammals, a dominant negative mutant of Ras blocks NGF-stimulated phosphorylation of Raf-1 (Troppmair *et al.* 1992; Wood *et al.* 1992). Also, Raf-1 is required for growth of serum-stimulated or Ras-transformed NIH-3T3 cells, as demonstrated by a dominant negative Raf mutant (Kolch 1991). Thus, Raf acts in tyrosine-kinase and Ras-mediated signalling pathways. Activated Raf renders PC12 cell differentiation independent of Ras (Troppmair *et al.* 1992). Also, dominant negative Raf mutants block serum-, TPA- and Ras-induced expression from oncogene-induced promoters (Bruder *et al.* 1992). Thus, Raf is likely to be required for Ras action. Our results strongly support the conclusion that for many cell types, the effect of Ras is primarily exerted through Raf. In *Drosophila*, Raf is required for pattern formation in embryos. *Drosophila* Raf acts downstream of the tyrosine kinases encoded by the *torso* (Siegfried *et al.* 1990) and *sevenless* loci (Hafen *et al.*, this symposium). Thus, a role for Raf downstream of tyrosine kinases is likely to be general to metazoans.

## 12. CONCLUSIONS

We have demonstrated that a nematode homologue of the *raf* proto-oncogene plays a crucial role in vulval induction using both classical and reverse genetics. By cloning a *raf* homologue and engineering a dominant negative mutation, we showed that Raf is involved in vulval induction. By analysing extragenic suppressor mutations that decrease signal transduction we identified a mutation in the *raf* gene, *lin-45*. By a genetic epistasis test we have shown that *lin-45 raf* is needed for the excessive vulval differentiation caused by activated *ras*. We have thus placed *raf* firmly in a tyrosine kinase-*ras*-mediated signal transduction pathway *in vivo*.

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