# THE *LIN-3* GENE OF THE NEMATODE C. ELEGANS IS THE VULVAL-INDUCING SIGNAL

Thesis by

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The development of the vulva of the nematode *Caenorhabditis elegans* is induced by a signal from the anchor cell of the somatic gonad. Activity of the gene *lin-3* is required for the Vulval Precursor Cells (VPCs) to assume vulval fates. It is shown here that *lin-3* encodes the vulval-inducing signal.

*lin-3* was molecularly cloned by transposon-tagging and shown to encode a nematode member of the Epidermal Growth Factor (EGF) family. Genetic epistasis experiments indicate that *lin-3* acts upstream of *let-23,* which encodes a homologue of the EGF-Receptor.

*lin-3* transgenes that contain multiple copies of wild-type *lin-3* genomic DNA clones confer a dominant multivulva phenotype in which up to all six of the VPCs assume vulval fates. The properties of these transgenes suggest that *lin-3* can act in the anchor cell to induce vulval fates. Ablation of the gonadal precursors, which prevents the development of the AC, strongly reduces the ability of *lin-3* transgenes to stimulate vulval development. A *lin-3* recorder transgene that retains the ability to stimulate vulval development is expressed specifically in the anchor cell at the time of vulval induction.

Expression of an obligate secreted form of the EGF domain of Lin-3 from a heterologous promoter is sufficient to induce vulval fates in the absence of the normal source of the inductive signal. This result suggests that Lin-3 may act as a secreted factor, and that Lin-3 may be the sole vulval-inducing signal made by the anchor cell.

*lin-3* transgenes can cause adjacent VPCs to assume the 1° vulval fate and thus can override the action of the lateral signal mediated by *lin-12* that normally prevents adjacent 1° fates. This indicates that the production of Lin-3 by the anchor cell must be limited to allow the VPCs to assume the proper pattern of fates of 3° 3° 2° 1° 2° 3°.

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# Cell fate patterning during *C. elegans* vulval development

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# I. Cell fate patterning during C. *elegans* vulval development.


## Summary.

Precursors to the vulva of the *C. elegans* hermaphrodite choose between two vulval fates (1° and 2°) and a non-vulval epidermal fate (3°) in response to three intercellular signals. An inductive signal produced by the anchor cell induces the vulval precursors to assume the 1° and 2° vulval fates. This inductive signal is an EGF-like growth factor encoded by the gene *lin-3*. An inhibitory signal mediated by *lin-15*, and which may originate from the surrounding epidermis, prevents the vulval precursors from assuming vulval fates in the absence of the inductive signal. A short range lateral signal, that acts through the gene *lin-12*, regulates the pattern of 1° and 2° fates assumed by the induced vulval precursors.

The combined action of the three signals precisely directs the six vulval precursors to adopt a 3° 3° 2° 1° 2° 3° pattern of fates. The amount of inductive signal produced by the anchor cell appears to determine the number of vulval precursors that assume vulval fates. The three induced vulval precursors are proposed to adopt the 2° 1° 2° pattern of fates in response to a gradient of the inductive signal, and in response to lateral signalling that inhibits adjacent VPCs from both assuming the 1° fate.


## Introduction.

The nematode *Caenorhabditis elegans* has been used to study the mechanisms that control cell fate determination (reviewed in Greenwald and Rubin 1992, Lambie and Kimble 1991). The development of *C. elegans* follows a largely invariant cell lineage (Kimble and Hirsh 1979, Sulston and Horvitz 1977, Sulston 1976, Sulston et al. 1980, Sulston et al. 1983).

Although some of the cell lineages of *C. elegans* appear to be specified by cell-autonomous mechanisms, other lineages are specified by cell-cell interactions. Some of these interactions were first noted from instances of naturally occurring variation in the lineage in which the development of a cell correlated with its position, thus suggesting that the cell was responding to environmental cues (Sulston et al. 1983). Other examples of cell-cell interactions have been defined by experiments in which groups of cells are ablated by irradiation with a laser microbeam to see if the development of the remaining cells is affected by the change in their environment (Kimble 1981, Schnabel 1991, Sulston et al. 1983, Sulston and White 1980). *C. elegans* is well suited for genetic analysis (Brenner 1974) and many mutations that affect its cell lineage have been identified (Horvitz 1988). The genetic analysis of these mutations and the characterization of the corresponding genes with the techniques of molecular biology has been a driving force in elucidating the mechanisms that control cell fate in *C. elegans*.

The development of the hermaphrodite vulva has been extensively studied by cell ablation experiments and by genetic analysis. The results of these studies support a three signal model of vulval development summarized below (and reviewed in Horvitz and Sternberg 1991) (Fig. 1). The vulva normally develops from three of six ectodermal blast cells called Vulval Precursor Cells (VPCs) that are located within the ventral epidermis. Although in wild-type development each of the VPCs always assumes a particular fate, a variety of experiments indicate that each of the VPCs is equivalent in its ability to assume either of three fates and thus that the VPCs constitute an equivalence group (Sternberg and Horvitz 1986, Sulston and White 1980, Thomas et al. 1990). Each fate consists of a distinct cell lineage that produces a particular set of cell types (Fig. 2). The 1° and 2°

fates both produce vulval tissue although they contribute to different regions of the vulva. The 3° fate produces non-vulval epidermis (Sulston and Horvitz 1977, Sulston 1976). The fate chosen by a VPC depends upon three intercellular signals. The anchor cell of the somatic gonad produces a signal that induces the three more proximal VPCs to assume the 1° and 2° fates (Kimble 1981). An inhibitory signal inhibits the VPCs from assuming vulval fates in the absence of the inductive signal (Herman and Hedgecock 1990). A lateral signal among the induced VPCs regulates the pattern of 1° and 2° fates (Sternberg 1988a). In wild-type development the combined action of the three signals causes the six VPCs to assume a 3° 3° 2° 1° 2° 3° pattern of fates. In the following sections, each of the three signals is discussed separately. This is then followed by a discussion of how the pattern of VPC fates is controlled by the three signals.

**The inductive pathway.**

Cell ablation experiments indicate that the vulva develops as the result of an inductive interaction between the somatic gonad and the vulval precursors (Sulston and White 1980). Kimble showed that the anchor cell of the somatic gonad is necessary and sufficient to induce vulva development (Kimble 1981). If the anchor cell or its precursors are ablated, then the three VPCs that would normally assume the 1° and 2° fates instead assume the 3° fate (Table 1C). This induction occurs one to three hours before the VPCs divide (Kimble 1981). If the anchor cell is ablated after the VPCs have divided, the cell lineage of their daughters is unaffected (Kimble 1981, Sternberg and Horvitz 1986). A large number of mutations that disrupt vulva induction have been characterized (reviewed in (Sternberg and Horvitz 1991)). The cloning of several of these genes indicates that this induction is

mediated by an Epidermal Growth Factor (EGF)-like signalling pathway that includes an EGF-like growth factor encoded by *lin-3* (Hill and Sternberg 1992) and a EGF-Receptor like molecule encoded by *let-23* (Aroian et al. 1990).

*lin-3* encodes the inductive signal.

*lin-3* is proposed to be a nematode member of the Epidermal Growth Factor (EGF) family of growth factors (Hill and Sternberg 1992). This family includes ligands of the EGF-Receptor (EGF-R) such as EGF (Gregory 1975, Savage et al. 1972), Transforming Growth Factor-alpha (TGF-α) (Derynck et al. 1984), Heparin Binding-EGF (HB-EGF) (Higashiyama et al. 1991), amphiregulin (Shoyab et al. 1989); and also ligands of *neu*/HER2, a homologue of the EGF-R, (Holmes et al. 1992, Wen et al. 1992); and the predicted product of the *spitz* locus of *Drosophila* (Rutledge et al. 1992). The EGF growth factors are usually made as membrane spanning proteins that contain at least one extracellular EGF domain (reviewed in (Carpenter and Wahl 1990)). EGF domains are a sequence motif of approximately 50 amino acids that consist of six cysteine residues with semi-conserved spacing (reviewed in (Davis 1990)). In many cases it is known that the EGF domain can be processed away from the rest of the protein to produce a secreted factor that can activate its receptor (reviewed in (Carpenter and Wahl 1990)). In the case of TGF-α, the EGF domain can also activate the receptor without being released from the membrane (reviewed in (Massagué 1990)).

The gene *lin-3* was identified by reduction-of-function mutations that cause a recessive, vulvaless phenotype in which up to all three of the VPCs that usually assume vulval fates instead assume epidermal fates (Ferguson and Horvitz 1985, Horvitz and Sulston 1980, Sulston and Horvitz 1981).

Thus, wild-type *lin-3* activity is required for vulval induction. In contrast, animals bearing *lin-3* transgenes (transgenes made up of wild-type genomic DNA cloned from the *lin-3* locus) can have a dominant multivulva phenotype in which up to all six of the VPCs assume vulval fates (Hill and Sternberg 1992). The multivulva phenotype of the transgenes is proposed to be a gain-of-function phenotype because the transgenes are created by a method that concatenates hundreds of copies of the injected DNA into an extrachromosomal array (Mello et al. 1991). Such an array would likely express levels of Lin-3 protein that are greater than the levels expressed by the two chromosomal copies of the *lin-3* locus. Together, the reduction-of-function and the gain-of-function phenotypes of *lin-3* suggest that the dose of *lin-3* activity controls the number of VPCs that assume vulval fates.

The analysis of *lin-3* transgenes suggests that *lin-3* can act in the anchor cell to cause the VPCs to assume vulval fates (Hill and Sternberg 1992). A transgene in which the *lacZ* gene of *E. coli* is inserted in frame within a *lin-3* genomic DNA clone directs expression of β-galactosidase activity specifically in the anchor cell at the time of vulval induction. This transgene, which should produce a fusion protein that contains the extracellular and transmembrane domains of the Lin-3 protein and a cytoplasmic domain consisting of the β-galactosidase protein, retains the ability to induce vulval fates. This result suggests that expression of *lin-3* in the anchor cell is sufficient to induce vulval fates. This hypothesis is further substantiated by the results of ablation experiments. Ablation of the four gonadal precursor cells at hatching, which prevents the development of the anchor cell, greatly reduces the ability of *lin-3* transgenes to induce vulval development.

It has been proposed that the inductive signal is a secreted factor because induction of vulval fates can occur without apparent direct contact between the anchor cell and the induced VPCs (Sternberg and Horvitz 1986, Sulston and White 1980, Thomas et al. 1990). As mentioned previously, the EGF growth factors can produce secreted factors that consist of an EGF domain. We have made a transgene to test whether a secreted form of the EGF domain of Lin-3, without the rest of the Lin-3 protein, would be sufficient to induce vulval fates (Hill and Sternberg, manuscript in preparation). This transgene uses a heat shock promoter that should express the Lin-3 EGF domain in a tissue general manner (Stringham et al. 1992). This transgene is able to stimulate 1° and 2° vulval fates even when the gonadal precursors have been ablated prior to the development of the anchor cell. This result suggests that *lin-3* can act as a secreted factor in a manner similar to the EGF growth factors. Since overexpression of the EGF domain of Lin-3 is sufficient to induce vulval fates in the absence of the gonad, it is possible that *lin-3* is the only vulval inducing signal made specifically by the anchor cell. It is not yet known if the Lin-3 protein is normally processed *in vivo*.

The response pathway of the VPCs.

A pathway of genes has been characterized that is believed to mediate the response of the VPCs to the inductive and inhibitory signals (Fig. 3). This pathway includes the following genes which are believed to act in the following order on the basis of genetic epistasis experiments: *let-23*, which encodes a homologue of the EGF-Receptor (EGF-R) (Aroian et al. 1990); *sem-5*, which encodes an adapter protein that contains SH2 and SH3 domains (Clark et al. 1992); *let-60,* which encodes a *ras* protein (Han and Sternberg

1990); and *lin-45*, which encodes a *raf* serine-threonine kinase (Han et al. 1993). Wild-type activity of each of these genes is required for vulval induction. A reduction of function mutation in *let-23* is epistatic to the ability of *lin-3* transgenes to stimulate vulval fates indicating that *let-23* acts downstream of *lin-3* (Hill and Sternberg 1992). This pathway is similar to pathways studied in other experimental systems. a *ras* protein, a *raf* kinase, and a Sem-5-like adapter protein also act downstream of the receptors encoded by the *torso* (N. Perrimon, this Volume) and the *sevenless* (E. Hafen, this volume) genes of *Drosophila*, and also downstream of growth factor receptors in mammals (McCormick 1993).

A number of other genes have been implicated in the process of vulval induction. The genes *lin-2*, *lin-7*, and *lin-10* are required for wild-type levels of vulval induction (Ferguson and Horvitz 1985). These genes may act at a step near *let-23* in the pathway of vulval induction (Ferguson et al. 1987). The gene *lin-10* encodes a novel protein (Kim and Horvitz 1990). The gene *lin-1* acts downstream of *lin-45*(Han et al. 1993). Wild-type *lin-1* activity acts to repress vulval fates. (Ferguson and Horvitz 1985, Ferguson et al. 1987).

*lin-3* and *let-23* are both required for vulval induction, larval viability (Aroian and Sternberg 1991, Clark, et al. 1988, Ferguson and Horvitz 1985, Herman 1978x, and R. Hill and P. Sternberg, unpublished results), hermaphrodite fertility (Aroian and Sternberg 1991, Ferguson and Horvitz 1985) and proper specification of cell fate in the male B lineage (H. Chamberlin and P. Sternberg, unpublished results). This suggests that the same growth factor and receptor are used together in multiple decisions during the development of *C. elegans*. *let-23* is also required for proper cell fate specification in the P11-P12 equivalence group (Aroian and Sternberg 1991), but it has not been demonstrated whether *lin-3* is also required for

P11-P12 fate specification. *sem-5* (Clark et al. 1992), *let-60* (Beitel et al. 1990, Clark et al. 1988, Han et al. 1990), and *lin-45* (Han et al. 1993) are also required for larval viability and a simple model is that they act downstream of *let-23* in all tissues that use the *lin-3 let-23* pathway. *lin-2*, *lin-7*, and *lin-10*, are unique in that they are only required for vulval induction (Ferguson and Horvitz 1985). In contrast, *sem-5* may be a generalized signal transduction component that acts downstream of multiple receptors since *sem-5* activity is required for the proper positioning of the sex myoblasts (Clark et al. 1992), a process that does not require *lin-3* or *let-23*.

**The inhibitory pathway.**

Genetic experiments suggest that a pathway of genes that includes *lin-15* (Ferguson and Horvitz 1985, Ferguson and Horvitz 1989) produces an intercellular signal that inhibits vulval fates (Herman and Hedgecock 1990). Loss-of-function mutations in the *lin-15* locus cause a multivulva phenotype in which all six VPCs can assume vulval fates (Table 1E1). *lin-15* mutant hermaphrodites display a multivulva phenotype even if the anchor cell has been ablated (Table 1E2). Thus, *lin-15* does not act only in the anchor cell (Ferguson et al. 1987). Genetic mosaic experiments indicate that *lin-15* does not act cell autonomously in the VPCs, and therefore that *lin-15* is part of an intercellular signalling pathway (Herman and Hedgecock 1990). The overall pattern of mosaic phenotypes is complex and has been interpreted to indicate that *lin-15* can act in the epidermal syncytium hyp7, a tissue generated by several different cell lineages (Herman and Hedgecock 1990). hyp7 is the main body epidermis and surrounds all of the VPCs. Thus a simple model is that the inhibitory signal would affect all of the VPCs equivalently and would thus provide no spatial specificity.

The inhibitory signal appears to act in parallel to the inductive signal to regulate the activity of the response pathway of the VPCs. Reduction-of-function mutations in the *let-23* locus that confer a vulvaless phenotype are epistatic to the multivulva phenotype of *lin-15* mutations (Aroian and Sternberg 1991, Ferguson et al. 1987, L. Huang and P. Sternberg, unpublished). Therefore, *lin-15,* like the inductive signal, is an upstream regulator of *let-23*. The inductive and inhibitory signals can regulate vulval fates independently of each other suggesting that they act in parallel. First, in a mutant animal that lacks *lin-15* activity, P6.p will always assume the 1° fate if the anchor cell is present, but can assume the 2° or 1° fate if the anchor cell is absent (Table 1E1,2). This indicates that the inductive signal can influence vulval fate choice independently of *lin-15* (Sternberg 1988a). Second, the multivulva phenotype of putative null mutations of *lin-15* are epistatic to the vulvaless phenotype of strong reduction-of-function *lin-3* genotypes. (Ferguson et al. 1987, R. Hill and P. Sternberg, unpublished results). This suggests that the inhibitory signal can affect vulval fates independently of the inductive signal encoded by *lin-3*. The inhibitory signal does not appear to be a competitive inhibitor of the inductive signal since multicopy *lin-15* transgenes that presumably overexpress the Lin-15 protein do not interfere with vulval induction (L. Huang and P. Sternberg, unpublished results). In wild-type development, a VPC that receives both the inductive signal and the inhibitory signal will assume a vulval fate. Thus, the inhibitory signal prevents the VPCs from assuming vulval fates only in the absence of the inductive signal. One possible interpretation of these observations is that the inhibitory signal negatively regulates the basal activity of *let-23*. The molecular mechanism by which this inhibitory pathway acts is not understood.

**The lateral signal.**

In addition to the inductive and inhibitory signals that originate from tissues other than the VPCs, a lateral signal among the VPCs also regulates VPC fate choice. Evidence for this signal came from observations on the pattern of VPC fates found in *lin-15* mutant animals (Sternberg 1988a). As described above, all six VPCs assume 1° and 2° vulval fates in a *lin-15* mutant animal due to a defect in an inhibitory signal from the surrounding epidermal tissue. In *lin-15* mutant animals, it is commonly observed that adjacent VPCs both assume the 2° fate, but it is rarely observed that adjacent VPCs both assume the 1° fate (Table 1E1). This suggests the action of a signal that prevents adjacent VPCs from both assuming the 1° fate. The action of this signal can also be observed in experiments in which groups of VPCs are isolated in a *lin-15* mutant animal by laser ablation of the other VPCs. A single isolated VPC in a *lin-15* mutant animal assumes the 1° fate (Table 1E3). However, when two adjacent VPCs are left after surgery, one of them will assume the 1° fate and the other will assume the 2° fate (Table 1E4). The lateral signal acts only at a short range and may require direct cell contact since two isolated VPCs in a *lin-15* mutant are only inhibited from both assuming the 1° fate if they are close to each other (Table 1E5).

This lateral signal is believed to act through the gene *lin-12* (Sternberg and Horvitz 1989). *lin-12* encodes a cell surface protein homologous to the protein encoded by the *Notch* locus of *Drosophila* (Greenwald 1985, Yochem et al. 1988). Both *lin-12* and *Notch* function in lateral interactions that control cell fate choice between cells of equivalent developmental potential (Greenwald et al. 1983, Greenwald and Rubin 1992, see P. Simpson, this volume). *lin-12* acts during vulval development to promote the 2° fate. In

animals homozygous for *lin-12* loss-of-function mutations, no VPCs assume the 2° fate, and in animals homozygous for *lin-12* gain-of-function mutations, all six VPCs assume the 2° fate independently of the inductive signal (Table 1F)(Greenwald et al. 1983, Sternberg and Horvitz 1989). *lin-12* also promotes the 2° fate in the AC (1° fate)-Ventral Uterine (VU) precursor (2° fate) equivalence group (Greenwald et al. 1983). Genetic mosaic analysis of *lin-12* activity in the AC-VU decision indicates that *lin-12* acts in a cell autonomous manner to specify the 2° fate (Seydoux and Greenwald 1989). It has thus been predicted that the *lin-12* product acts as the receptor for the lateral signal during vulval development.

Lateral signalling acts through a separate pathway than the inductive pathway. It is not clear precisely where the two pathways converge to control VPC fate although it is likely downstream of *lin-2*, *lin-7*, and *lin-10* (Sternberg and Horvitz 1989). Currently other components of the lateral pathway including the ligand for *lin-12* and genes that act downstream of *lin-12* are not well characterized. One explanation for this is that these other components of the lateral pathway may be required for an essential decision prior to the time of vulval development. The *C. elegans* gene *glp-1* is structurally very similar to *lin-12* and both genes may have originated from a gene duplication of a common ancestor (Yochem and Greenwald 1989). *glp-1* is required zygotically for maintenance of the mitotic germline (Austin and Kimble 1987) and is required maternally for the proper fate specification of early blastomeres (Priess et al. 1987). The *lin-12 glp-1* double mutant has a synthetic zygotic lethal phenotype called Lag (Lambie and Kimble 1991). This indicates that *glp-1* and *lin-12* are functionally redundant for certain essential developmental processes. Other genes that can be mutated to give a Lag phenotype could be shared components of the *lin-12* and *glp-1* pathways

and thus might be required for lateral interactions during vulval development (Lambie and Kimble 1991).

**Patterning of vulval fates.**

Vulval development allows the examination of how different intercellular signals interact to control pattern formation. During vulval development, three intercellular signals direct the six VPCs to adopt the pattern of fates 3° 3° 2° 1° 2° 3°. Mutations that disrupt any of the three signals affects the fates assumed by the VPCs. The wild-type pattern is precise in the number of VPCs induced to assume the 1° and 2° vulval fates, the position of the VPCs induced to assume vulval fates, and in the pattern of 1° and 2° fates assumed by the induced cells. This section summarizes our knowledge of what signals control the extent, location, and pattern of vulval induction.

Extent. As mentioned previously, the genetic dose of *lin-3* is proposed to control the number of VPCs induced to assume vulval fates. This hypothesis is based on the observations that reduction-of-function mutations in *lin-3* reduce the number of the VPCs that assume vulval fates and that multicopy *lin-3* transgenes that presumably overexpress the Lin-3 protein increase the number of VPCs that assume vulval fates (Ferguson and Horvitz 1985, Hill and Sternberg 1992). In *lin-12* mutant animals in which lateral signalling is disrupted, the number of VPCs that assumes vulval fates is near the wild-type level (Table 1F1) (Sternberg and Horvitz 1989). Thus the lateral signal does not appear to regulate the number of VPCs that assume vulval fates. The inhibitory signal acts in a spatially general manner to inhibit vulval fates, but the action of the inhibitory signal is overridden by the inductive

signal. Thus the inhibitory signal ensures that vulval fates are assumed only in response to the inductive signal, but does not itself determine the number of VPCs that assume vulval fates.

Location. The location of the anchor cell determines the location of vulval development. This is illustrated by the pattern of fates assumed by the VPCs in *dig-1* mutant animals. In *dig-1* mutants the position of the gonad and the anchor cell can be shifted anterior and/or dorsal of their wild-type positions (Thomas et al. 1990). In animals in which the anchor cell is shifted anterior of its wild-type position and remains ventral, the pattern of vulval fates is shifted commensurably. For example, when the anchor cell is positioned over P5.p, P5.p assumes the 1° fate and the overall pattern of vulval fates is 3° 2° 1° 2° 3° 3° (Table 1G).

Pattern.

As just discussed, in wild-type development, the induction of 1° and 2° vulval fates is limited to P5.p, P6.p and P7.p because the anchor cell is located above P6.p and because it only makes enough inductive signal to induce the three most proximal VPCs. What signals direct the three induced VPCs to assume a 2° 1° 2° pattern of fates? The experimental analysis of animals that have only one VPC indicates that the inductive signal plays an important role in patterning. The fate of a single, isolated VPC correlates with its distance from the anchor cell: a single VPC that is relatively close to the anchor cell will assume the 1° fate, a single VPC that is further away will assume the 2° fate, and a single VPC that is distant will assume the 3° fate (Table 1H) (Sternberg and Horvitz 1986, P. Sternberg, unpublished results, and M. Herman and H. R. Horvitz, personal communication). Since these

isolated VPCs have no neighboring VPCs, they should not be subject to the effects of the lateral signal. Thus, the fact that the fate of an isolated VPC correlates with its distance from the anchor cell suggests that there is a gradient of inductive signal centered on the anchor cell, and that the VPCs assume different fates in response to different levels of the inductive signal.

The inhibitory signal mediated through the *lin-15* pathway does not have an important role in vulval fate patterning. Although all six VPCs assume vulval fates in *lin-15* mutant animals, the patterning of fates is still normal. In a *lin-15* mutant animal, the inductive signal still promotes P6.p to assume the 1° fate and the lateral signal still functions to prevent P5.p and P7.p from assuming the 1° fate. Thus if the anchor cell is present in a *lin-15* mutant animal, then P5.p, P6.p, and P7.p, will assume the same 2° 1° 2° pattern of fates that they assume in wild-type animals (Table 1E1). However, if the anchor cell is ablated, then P5.p, P6.p, and P7.p can each assume either the 1° or 2° fate (Table 1E2) (Sternberg 1988a).

The lateral signal appears to play an important role in the patterning of vulval fates assumed by the induced VPCs. In *lin-12* loss-of-function mutant animals, the pattern of fates is usually 3° 3° 1° 1° 1° 3° (Table 1F1). Thus, lateral signalling may be necessary to specify the 2° fate. Moreover, the lateral signal is believed to measure differences in the amount of inductive signal received between two VPCs and to act in response to this difference to direct the VPCs to assume different fates. This model is supported by indirect evidence that certain levels of the inductive signal can specify a VPC to have different fates depending upon the states of the VPC's neighbors. For example, a VPC in the position of P5.p always assumes the 2° fate in wild-type development. But an isolated VPC in this position has been observed to assume the 1° fate in animals in which the other VPCs have been ablated (P.

Sternberg, unpublished observations). This isolated VPC may assume the 1° fate for either of two reasons; it might be receiving more inductive signal than it does in wildtype because it lacks neighbors that absorb the inductive signal, or it may receive the same amount of inductive signal as it does in wildtype, but lacks lateral signalling from its neighbors that specify it to be 2°. Another example is the pattern of vulval fates in *dig-1* mutant animals in which the anchor cell is displaced to the dorsal side of the animal (Thomas et al. 1990). In these animals the VPC closest to the anchor cell is at a distance equal to or greater than the distance of P5.p is to the anchor cell in a wild-type animal. Thus, no VPC in these *dig-1* mutant animals should receive more inductive signal than P5.p in a wild-type animal. Yet, although P5.p always assumes the 2° fate in wild-type development, VPCs at an equivalent distance from the anchor cell in *dig-1* mutants often assume the 1° fate. Thus it is possible that there are intermediate doses of the inductive signal that will specify a VPC to have the 1° fate in the context of neighboring VPCs that are receiving less inductive signal, but that will specify a VPC to have the 2° fate in the context of neighboring VPCs that are receiving more inductive signal.

The action of the lateral signal in vulva development is also modeled on the role of *lin-12* in the AC-VU equivalence group (Greenwald et al. 1983). In this equivalence group, either the cell Z1.ppp or Z4.aaa can assume the fate of the anchor cell with approximately equal likelihood. Lateral signalling among these two cells mediates this decision without apparent influence from outside cells (Kimble 1981, Seydoux and Greenwald 1989). Although either cell can assume either fate, the lateral signal ensures that only one cell always assumes the fate of the anchor cell and that the other cell always assumes the fate of VU (Kimble and Hirsh 1979). Based upon this

observation, it has been proposed that the lateral signal acts in a feedback loop that establishes mutually exclusive states in adjacent cells. In this model both cells start with the ability to produce both the lateral signal and the receptor for the lateral signal. Cells that receive the lateral signal down-regulate the activity of the lateral signal and up-regulate the activity of the receptor. Conversely, cells that produce the lateral signal up-regulate the activity of the lateral signal and down-regulate the activity of the receptor. This feedback mechanism should result in only one cell producing active lateral signal and with the other cell producing only active receptor (Seydoux and Greenwald 1989, Sternberg 1988b). In vulval development, the outcome of lateral signalling among the VPCs is invariant and is believed to be biased by the inductive signal produced by the anchor cell. It is proposed the lateral signalling initially acts in proportion to the amount of inductive signal received by each VPC. The feedback mechanisms of the lateral signal then act to accentuate the differences in the amount of lateral signalling between the VPCs. This ensures that adjacent cells assume different fates in response to different levels of the inductive signal (Sternberg and Horvitz 1989).

Another way of comparing the relative contribution of the inductive signal and the lateral signal in controlling pattern formation is by examining the genetic interactions of the two signals. In animals with *lin-12* gain-of function mutations, all six VPCs usually assume the 2° fate. There is also usually no anchor cell in these animals and thus no inductive signal (Table 1F2). However, in those *lin-12* gain-of-function mutant animals that have an anchor cell, P6.p always assumes the 1° fate (Table 1F3) (Sternberg and Horvitz 1989). Thus, for a single cell, the action of the inductive signal in specifying the 1° fate overrides the action of the lateral signal in specifying the 2° fate. A function of the lateral signal in patterning though, is to prevent

two adjacent VPCs from both assuming the 1° fate. Thus an important experiment is to determine if the lateral signal can still promote adjacent cells to assume different fates when they both receive high levels of the inductive signal. In *lin-3* transgenic animals, which overexpress the inductive signal in the anchor cell, adjacent VPCs often both have the 1° fate (Table 1I) (Hill and Sternberg, manuscript in preparation). In this situation, two adjacent VPCs both assume the 1° fate, even though each of them should be laterally signalling their neighbors to be 2°. Thus, either a high level of the inductive signal specifies the 1° fate and overrides the action of the lateral signal; or, alternatively, Lin-3 protein might bind to, and inhibit the action of, either the ligand or the receptor of the lateral signal. In either case, the dose of the inductive signal must be limited to achieve wild-type patterning in which P5.p and P7.p assume the 2° fate.

Summary of 1° and 2° fate patterning.

The pattern of 1° and 2° fates assumed by the VPCs is determined both by the action of an inductive signal encoded by *lin-3*, and a lateral signal that acts though *lin-12*. The inductive signal appears to provide the specific information that sets up the pattern of fates assumed by the VPCs. First, the inductive signal originates only from the anchor cell and is the only signal that clearly provides spatially specific information. Second, the fact that the fate of an isolated VPC correlates with its distance from the anchor cell, suggests that different doses of the inductive signal promote the 1°, 2°, and 3° fates, and that a spatial gradient of the inductive signal promotes the VPCs to assume a graded pattern of fates. Third, a high level of the inductive signal specifies the 1° fate and overrides the action of the lateral signal in specifying the 2° fate. This indicates that the production of the inductive

signal must be limited to get VPCs with the 2° fate. The properties of the lateral signal and *lin-12* are intriguing. On the one hand the lateral signal seems to reinforce a pattern that has already been determined by the inductive signal; i. e., the lateral signal ensures that P5.p and P7.p assume the 2° fate in response to intermediate amounts of the inductive signal, and that P6.p assumes the 1° fate in response to high amounts of the inductive signal. On the other hand, wild-type activity of the gene *lin-12*, which is believed to encode the receptor for the lateral signal, is required to specify the 2° fate. Thus a VPC that receives a dose of the inductive signal that promotes the 2° fate still apparently requires input from the lateral signal to assume the 2° fate. The mechanism by which an isolated VPC assumes the 2° fate is unclear, since it lacks neighbors with which it can laterally signal. Such a VPC may engage in autocrine signalling through *lin-12* to assume the 2° fate. The inductive signal and the lateral signal appear to cooperate to promote the graded 2° 1° 2° pattern of fates assumed by the induced VPCs. The use of two signals to promote this pattern may ensure that the process of pattern formation has a reproducible outcome. Thus for example, the lateral signal could ensure that the VPCs assume a 2° 1° 2° pattern of fates over a broad dose range of the inductive signal, as long as the inductive signal is distributed in a gradient.

Our current model of VPC fate determination is summarized in Fig. 1. An inhibitory signal that arises in a spatially general manner inhibits the VPCs from assuming vulval fates in the absence of the inductive signal. Localized production of the inductive signal encoded by *lin-3* controls the number and location of VPCs that assume the vulval fates. Lateral signalling among the VPCs acts in response to a spatial gradient of inductive signal to specify P5.p and P7.p to assume the 2° fate.

**Prospects.** A question for the future is how the different signals interact to control the fate of the VPCs. The molecular cloning of several key genes in these intercellular pathways means that the interactions of these signals can be analyzed both genetically and by the techniques of molecular biology. The molecular mechanism by which the inhibitory signal represses the response pathway of the VPCs has not been established. Vulva development provides an example where the outcome of lateral interactions among cells of equivalent developmental potential appears to be controlled by the action of an outside signal. Elucidating the mechanisms by which the lateral signal and inductive signal interact to control the choice between 1° and 2° will be a goal of future research. The use of genetics to identify additional components of the lateral signalling pathway will be important in understanding the mechanism of action of the lateral signal. Genetic screens for suppressers of existing mutations that disrupt vulval development should be useful in identifying new genes involved in the pathways of vulval fate determination.

**Section II. The EGF family of growth factors.**

The Epidermal Growth Factor (EGF)-like growth factors are inter-cellular signalling molecules. The structural hall mark of the EGF-like growth factors is the EGF repeat, a self-folding structural domain of approximately 50 amino acids that has six cysteine residues with semi-conserved spacing. The EGF-like growth factors are made as membrane-bound precursors and can be proteolytically processed to release the EGF repeat as a secreted factor, although processing is not necessary for activation of the EGF-like growth factors. The EGF-like growth factors act through the EGF-Receptor (EGF-R) subfamily of tyrosine kinase receptors. Activation of the EGF-R results in a diverse set of physiological responses in different cell types including growth, inhibition of growth, induction and repression of gene expression, and differentiation. The EGF-like growth factors are generally implicated as important proto-oncogenes and are over-expressed or mis-expressed by a wide variety of tumor cell lines. During development the EGF-like growth factors are expressed in a wide variety of tissues, but in general the function of these inter-cellular signalling molecules in development is not established.

**EGF/urogastrone**

The best characterized members of the EGF family of growth factors are Epidermal Growth Factor and Transforming Growth Factor-alpha (TGF-$\alpha$). Murine epidermal growth factor was discovered during the purification of Nerve Growth Factor (NGF) as a novel set of activities distinct from NGF (Cohen 1960, Levi-Montalcini and Cohen 1960). This activity was noted to have a direct mitogenic effect on epidermal cell types and was thus termed

epidermal growth factor (Cohen 1964). EGF was purified to homogeneity (Savage and Cohen 1972) and the primary sequence of the 53 amino acid EGF polypeptide was reported in 1972 (Savage et al. 1972). The sequence contains 6 cysteine residues which form disulfide bonds in the pattern 1 to 3, 2 to 4, and 5 to 6 (Savage et al. 1973). Human EGF was characterized as urogastrone, which is an activity found in urine that inhibits gastric acid secretion ((Sandweiss 1943) cited in (Gregory 1975)). The amino acid sequence of urogastrone was discovered to be identical to that of murine EGF at 38 of 53 positions. This result and the fact that murine EGF mediated the effects of urogastrone established urogastrone as the human EGF equivalent (Gregory 1975).

The cloning of a cDNA for the EGF precursor revealed that it could encode a protein of over 1200 amino acids, relatively large compared to the size of EGF (Gray et al. 1983, Scott et al. 1983). The precursor has two hydrophobic stretches similar to a signal peptide sequence and a trans-membrane domain, which suggests that the EGF precursor is a membrane-spanning protein. The sequence of mature EGF is located near, and to the amino terminus, of the membrane-spanning domain of the precursor. The EGF precursor was noted to contain 8 regions that have a primary amino acid structure and a spacing of six cysteine residues similar to that of mature EGF. The structural motif of these 6 cysteine residues was named an EGF repeat and are also called EGF-like domains (Doolittle et al. 1984, Gray et al. 1983, Scott et al. 1983). To date there is no evidence that the other EGF repeats present in the EGF precursor are processed to form soluble factors. EGF repeats have since been noted to be present in the extracellular domains of a diverse set of proteins of different functions including many proteins that are not growth factors (reviewed in (Carpenter and Wahl 1990, Davis 1990)).

Still, the EGF repeat is the defining structural feature of the EGF-like growth factors: it is the ligand domain of these factors, and the analysis of the structure of the precursors of different EGF-like growth factors (below) indicates that the EGF repeat is the only feature that all have in common.

**Transforming growth factor-alpha**

Transforming growth factor-alpha (TGF-α) was first characterized as sarcoma growth factor, an activity present in the conditioned media of 3T3 cells transformed with Moloney murine sarcoma virus (De Larco and Todaro 1978). Sarcoma growth factor was shown to be mitogenic for fibroblasts, and to inhibit the binding of $^{125}$I-EGF to its receptor, but to be immunologically distinct from EGF. Sarcoma growth factor also had the striking ability to allow normal rat fibroblasts to form colonies in soft agar, (De Larco and Todaro 1978), which EGF was not able to do, and was thus renamed transforming growth factor (De Larco et al. 1980). Similar activities were also found in the supernatants of several human tumor cell lines which indicated that transforming growth factor was likely of mammalian and not of viral origin (De Larco et al. 1980). During the purification of transforming growth factor it was demonstrated that its different activities were mediated by different molecules. TGF-α was shown to be a mitogen for fibroblasts, and to inhibit the binding of $^{125}$I-EGF to the EGF-R, but highly purified TGF-α preparations could not support soft-agar colony formation. TGF-β could not inhibit binding of $^{125}$I-EGF, but could act either with TGF-α or with EGF to promote soft agar colony formation (Anzano et al. 1983). Further analysis confirmed that TGF-α acted through the same receptor as EGF (Carpenter et al. 1983, Massagué 1983). The amino acid sequence of the 50 amino acid TGF-α polypeptide showed that it had 32-36% sequence identity with EGF

including the conservation of the spacing of the 6 cysteine residues (Marquardt et al. 1984, Marquardt et al. 1983, Massagué 1983). The molecular cloning of a TGF-α cDNA showed that TGF-α was made as a 159 (human) or 160 (rat) amino acid membrane spanning protein (Derynck et al. 1984, Lee et al. 1985). The TGF-a precursor is palmitoylated and is found on the plasma membrane (Bringman et al. 1987). The TGF-α precursor contains only a single EGF repeat, and does not share sequence similarity to the EGF precursor outside of the EGF repeat.

**Viral EGF-like growth factors.**

A search through the protein databases for additional proteins that contained EGF repeats indicated that the previously determined nucleotide sequence of the LTR of the vaccinia virus (Venkatesan et al. 1982) could encode a 140 amino acid membrane spanning protein with an EGF repeat. The EGF repeat of this protein has 35% amino acid identity with EGF and TGF-α. On the basis of this observation, the supernatant of vaccinia-virus-infected cells was analyzed for the presence of EGF-like growth factor activities. An activity called Vaccinia Virus Growth Factor (VVGF) was discovered which was mitogenic for 3T3 cells and which would competitively inhibit the binding of $^{125}$I-EGF to 3T3 cells. The direct amino acid sequence of VVGF showed that it could be derived from proteolytic processing of the protein encoded within the LTR of vaccinia virus (Stroobant et al. 1985). Two other pox viruses have been noted to contain genes that could encode EGF-like growth factors. Shope fibroma virus could encode a protein of 84 amino acids called Shope Fibroma Virus Growth Factor (SFGF) (Chang et al. 1987) and myxoma virus could encode a protein of 85 amino acids called Myxoma Virus Growth Factor (MVGF) (Upton et al. 1987). The EGF repeats of SFGF

and MVGF show about 80% amino acid identity to each other and about 40% identity to the EGF repeat of murine EGF. MVGF and SFGF are unique among the EGF-like growth factors in that they both lack putative membrane spanning domains. Synthetic MVGF and SFGF can both competitively inhibit binding of $^{125}$I-EGF to human A-431 cells and are mitogenic for normal rat fibroblasts, although SFGF is 200-fold less active, and MVGF is 10-fold less active than EGF in these assays (Lin et al. 1988, Lin et al. 1991). The lower activity of MVGF and SFGF could be due to the fact that both proteins are from viruses that infect rabbits and are being tested on human and rat cells.

**Amphiregulin and EGF-like growth factors with basic regions.**

A number of EGF-like growth factors that have basic regions have been characterized. The first member of this class is amphiregulin, which was purified from TPA-stimulated human breast carcinoma cells. Amphiregulin is a secreted factor of 84 or 78 amino acids. The carboxy terminus of the secreted factor contains an EGF repeat with 30% identity to human EGF. The amino terminus of amphiregulin extends farther than EGF and contains a 21 amino acid region with 14 basic amino acids (Shoyab et al. 1989). Amphiregulin is derived from a 252 amino acid membrane-spanning precursor (Plowman et al. 1990). Schwannoma Derived Growth Factor (SDGF) is 76% identical to amphiregulin and the SDGF precursor is 70% identical to the amphiregulin precursor. Mature SDGF has a stretch of basic amino acids to the amino terminus of the EGF repeat that is identical to amphiregulin at 18 of 21 amino acid residues (Kimura et al. 1990). This high level of sequence similarity between the two molecules suggests that SDGF may be the rat orthologue of amphiregulin. Amphiregulin can competitively

inhibit, but not completely, the binding of $^{125}$I-EGF to A-431 cells (Shoyab et al. 1989). Recent reports confirm that at least some of the activities of amphiregulin are mediated through the EGF-R (Johnson et al. 1993). Amphiregulin can mediate some of the activities of EGF but not others: amphiregulin is a mitogen for the murine keratinocyte cell line Balb/MK, but can not act with TGF-β to promote colony formation in soft agar. Amphiregulin inhibits the growth of some human carcinoma cell lines, although the mechanism of growth inhibition is not clear (Shoyab et al. 1988).

SDGF was purified by its ability to bind heparin columns (Kimura et al. 1990). Amphiregulin can also bind heparin based upon its identity with Keratinocyte Autocrine Factor (KAF). KAF is produced by and is a mitogen for normal human keratinocytes. KAF was purified on the basis of its ability to bind heparin and was shown to be identical in sequence to amphiregulin (Cook et al. 1991). Heparin-sulfate blocks the ability of purified KAF to competitively inhibit the binding of $^{125}$I-EGF and also the ability of KAF to act as a mitogen for AKR-2B cells. Heparin-sulfate, however, does not block the mitogenic effect of EGF on AKR-2B cells (Cook et al. 1991). The different activities of EGF and amphiregulin/KAF in bioassays could be due to factors that regulate the action of amphiregulin.

Heparin-Binding EGF was purified from a human macrophage-like cell line on its ability to bind heparin. HB-EGF is a secreted factor generated from a 208 amino acid precursor. HB-EGF is distinct in sequence from amphiregulin but is similar to amphiregulin in that it has a stretch of basic amino acids to the amino terminus of the EGF repeat. HB-EGF is also a competitive inhibitor of $^{125}$I-EGF, suggesting that it also acts through the EGF-R (Higashiyama et al. 1991).

**The EGF-R subfamily**

Three additional receptors have been identified in mammals that are members of the EGF-R subfamily called *neu*/HER2/p185$^{erb}$B2 (Bargmann et al. 1986, Yamamoto et al. 1986), HER3/p160$^{erb}$B3 (Kraus et al. 1989, Plowman et al. 1990) and HER4/p180$^{erb}$B4 (Plowman et al. 1993). HER2 is not activated by EGF-R ligands (Yamamoto et al. 1986). The extracellular domains of HER3 and HER4 are more related to each other than they are to HER2 and to the EGF-R (Plowman et al. 1993). The amino acid sequence of ligands of HER3 and HER4 have not been reported. HER4 is not activated by EGF or amphiregulin. A partially purified heparin-binding factor has been isolated that activates HER4, but which does not activate HER3, HER2 or EGF-R (Plowman et al. 1993).

**Ligands of *neu*/HER2**

Ligands of HER2 have recently been described as Neu Differentiation Factor (NDF) (Wen et al. 1992), heregulin (Holmes et al. 1992), Glial Growth Factor (GGF) (Marcchionni et al. 1993), and betacellulin (Sasada et al. 1993). NDF was purified from *ras* transformed rat fibroblasts (Peles et al. 1992, Yarden and Peles 1991). NDF induces phosphorylation of HER2 and cross-linking studies show that it binds HER2. Heregulin was purified from human breast carcinoma cells (Holmes et al. 1992) and GGF was purified from bovine pituitary glands (Marcchionni et al. 1993). HER2-activating factors that may be the same protein as NDF have been biochemically characterized (Davis et al. 1991, Dobashi et al. 1991, Lupu et al. 1990). The ligands of HER2 do not activate the EGF-R, for example Heregulin-$\alpha$ does not compete with $^{125}$I-EGF for binding to A-431 cells (Holmes et al. 1992). There may be some cross-talk between the receptors, however, for example,

activation of the EGF-R by amphiregulin causes phosphorylation of HER2 (Johnson et al. 1993).

The structure of the HER2 ligands indicates that they belong to the EGF family of growth factors but that they are structurally unique. NDF is an integral membrane protein that contains a single EGF repeat proximal and to the amino terminus of the trans-membrane domain. The EGF repeat of rat NDF meets the consensus for EGF repeats used as growth factors. It has 26% amino acid identity with the EGF repeat of rat TGF-α over 51 amino acids, whereas rat TGF-α shows 33% amino acid identity to rat EGF over the same interval. NDF, like SFGF and MVGF, contains 13 amino acid residues between the third and fourth cysteine residues, whereas EGF and most other EGF-R ligands have only 10 amino acids in this region. NDF also contains a single Immunoglobulin-like (Ig) domain that is present in the mature secreted forms of the molecules (Wen et al. 1992). None of the EGF-R ligands have an Ig domain. The analysis of heregulin and GGF indicates that they are produced by the same gene as NDF and that this gene undergoes a complex pattern of alternative splicing. Surprisingly, different heregulin cDNAs would produce proteins that would differ in the carboxy-half of the EGF repeat. This half of the EGF repeat is usually the more conserved half of the EGF repeat, and for the EGF-R ligands is important in receptor recognition. The predicted heregulin proteins also differ in the 20 amino acid domain between the EGF repeat and the membrane-spanning domain, where proteolytic processing would need to occur to release the mature growth factor from the cell. Some heregulin cDNAs also contain a stop codon after the EGF repeat and before the membrane spanning domain (Holmes et al. 1992). It is thus conceivable that different heregulin proteins would have different biological activities. None of the NDF or heregulin cDNAs would

encode a hydropathic amino terminal signal peptide(Holmes et al. 1992, Wen et al. 1992). The analysis of glial growth factor includes the sequence determination of bovine genomic DNA and the analysis of cDNAs from a variety of tissues and species (Marcchionni et al. 1993). These results indicate that there are additional 5' exons present that were not detected in the analysis of heregulin and NDF. The most 5' exon could encode a signal peptide and a kringle domain. Novel transcripts were discovered that encode the kringle domain and the Ig domain but not the EGF repeat. The three cytoplasmic exons of Glial Growth Factor also undergo alternative splicing. The functional significance of the diverse splice variants is not established. The heregulin-β3 and GGFH#B1 cDNAs that do not encode signal peptides and that contain stop codons between the EGF repeat and the transmembrane domains do not produce secreted protein in transfection studies (Holmes et al. 1992, Marcchionni et al. 1993).

**Processing of the EGF growth factors.**

The EGF-like growth factors can act as secreted factors that are derived by proteolytic processing from integral membrane proteins. Different EGF-like growth factors are processed at different types of amino acid sequences. For example, TGF-α is processed at small hydropathic sequences (Derynck et al. 1984, Lee et al. 1985) and EGF is processed at dibasic residues (Gray et al. 1983, Scott et al. 1983). The processing of TGF-α has been studied most extensively. Immunolocalization experiments confirm that the TGF-α precursor (pro-TGF-α ) is found on the plasma membrane of cells (Bringman et al. 1987). In transfected CHO cells that express pro-TGF-α, processing of pro-TGF-α to release the sequence amino-terminal to the EGF repeat occurs quickly with a half-life of 30 minutes. The processing that

releases mature TGF-α from the membrane occurs slowly, with a half-life of 2 hours (Teixidó et al. 1990). Stimulation of the transfected CHO cells with serum, or the calcium ionophore A23187, or with the phorbol ester PMA causes a rapid release of TGF-α from the cell surface (Pandiella and Massagué 1991, Pandiella and Massagué 1991). These results suggest that the processing mechanisms that release TGF-α from a cell are regulated and can be activated by signal transduction pathways. The processing appears to occur at the cell surface. Treatment of transfected CHO cells with brefeldin A which blocks golgi transport does not prevent the release of previously synthesized pro-TGF-α from the cell in response to PMA stimulation. But TGF-α made after the treatment with brefeldin A is not processed and released in response to PMA stimulation (Bosenberg et al. 1992). The agents chloroquine and leupeptin that disrupt lysosome function also do not prevent PMA from stimulating the release of TGF-α from the cell (Bosenberg et al. 1992).

Site directed mutagenesis experiments indicate that the carboxy terminal Val residue of the cytoplasmic domain of the TGF-α precursor is the major recognition signal of this processing pathway (Bosenberg et al. 1992). The cytoplasmic domain of TGF-α is 34 amino acids long and is highly conserved (Derynck et al. 1984, Lee et al. 1985). Replacement of the carboxy terminal Val with Gly, Ser, or Glu blocks processing of pro-TGF-α, replacement with Ala, Met, Phe, or Trp reduces processing, and replacement with Leu and Ile does not block processing (Bosenberg et al. 1992). In contrast most internal deletions of the cytoplasmic domain do not block processing. The processing pathway appears to be associated with the cell since supernatants from cells that are undergoing processing can not process the pro-TGF-α found on other cells (Bosenberg et al. 1992). CHO cells do not

normally express TGF-α and it has not been confirmed that TGF-α processing is regulated in a similar manner in cells that normally express TGF-α. The enzymes responsible for the processing of the precursors have not been identified for any of the EGF-like growth factors.

Processing of the EGF-like growth factors does not appear to be required to allow the EGF repeat to bind the receptor. Two groups made site directed mutants of TGF-α in which the processing sites were disrupted and demonstrated that cells which express these mutants do not release processed TGF-α into the culture media. Unprocessed mutant TGF-α purified from these cells can induce phosphorylation of the EGF-R, and can induce colony formation in soft agar in the presence of TGF-β. Moreover, unprocessed mutant TGF-α on the surface of live cells can induce phosphorylation of the EGF-R on other cells but the supernatant of the cells expressing the mutant TGF-α could not induce phosphorylation of the EGF-R on other cells (Brachmann et al. 1989, Wong et al. 1989). It has also been shown that purified full length EGF precursor is also biologically active (Mroczkowski et al. 1989). These studies raise the possibility that the EGF-like growth factors act as membrane bound factors *in vivo*.

**TGF-α knockouts.**

Little is known about the function of the EGF-like growth factors *in vivo* and during ontogeny. The EGF-like growth factors are expressed in complex patterns which have been reviewed elsewhere (Carpenter and Wahl 1990, Derynck 1992). Briefly, EGF is expressed at high levels in the mouse in the submaxillary gland and in the kidneys (Rall et al. 1985) and is believed not to be expressed in the embryo (Carpenter and Wahl 1990). TGF-α RNA is detected in unfertilized mouse embryos but goes away after fertilization.

TGF-α RNA and protein is seen in the mouse blastocysts (Rappolee et al. 1988). TGF-α RNA is detected by in situ hybridization at day 9-10, but not at days 12, 14, and 16 of mouse embryogenesis. It is detected in the otic vesicle, cells lining the oral cavity and pharyngeal pouch, the syncytiotrophoblast layer of the placenta, the first and second brachial arches, and the developing mesonephric tubules of the developing kidney (Wilcox and Derynck 1988). TGF-a TGF-α is produced by normal keratinocytes and is present in skin biopsies (Coffey et al. 1987) and is detected *in vivo* in wound macrophages (Rappolee et al. 1988). Glial Growth Factor is detected by *in situ* hybridization in the developing nervous system of the mouse, but not in non-neural tissues. Expression appears to be high in developing motor neurons and primary sensory neurons (Marcchionni et al. 1993).

Two groups have reported on the phenotypes of mice in which function of the TGF-α gene has been disrupted by homologous recombination (Luetteke et al. 1993, Mann et al. 1993). Despite the extensive expression of TGF-α in the embryo, mice homozygous for the TGF-α knockout are viable, fertile, healthy, and have normal wound healing. These mice show susceptibility to corneal inflammation, have a wavy coat, curly whiskers, and disorganization of the hair follicles, but otherwise appear to be normal. The TGF-α mutation is allelic to the mouse mutant waved-1. It is possible either that TGF-α has no important role in development other than those indicated by the phenotype of waved-1, or that TGF-α acts redundantly with other EGF-like growth factors during development. The analysis of the null phenotype of the other growth factors and their receptors will be necessary to determine the function of these proteins in development.

**EGF-like proteins in *Drosophila*.**

The *spitz* locus of *Drosophila* encodes a 26-kD membrane spanning protein with a single EGF-like repeat that is structurally similar to TGF-α and Lin-3 (Rutledge et al. 1992). The EGF repeat of *spitz* has 23% amino acid identity with the EGF repeat of human TGF-α and 32% amino acid identity with the EGF repeat of Lin-3. mRNA for *spitz* is expressed ubiquitously in the embryo and is present in high levels in the procephalic region, ventral midline, and mesodermal layers. A polyclonal antisera to a *spitz*-T7 fusion protein detects *spitz* protein ubiquitously in the embryo (Rutledge et al. 1992). The *spitz* gene belongs to the *spitz* group of mutations that also includes *Star (S)*, *single-minded (sim), pointed (pnt), rhomboid (rho),* and the maternally-acting gene *sichel (sic)* (Mayer and Nüsslein-Volhard 1988). Amorphic mutations of *spitz* cause a zygotic embryonic lethal phenotype in which there are defects in the specification of structures in the ventral-lateral blastoderm. This includes a loss of the Keilin's organs in the thoracic segments and a loss of ventral structures in the head. Pole cell transplantation experiments indicate that *spitz* is required for the development of the female germline (Mayer and Nüsslein-Volhard 1988). *spitz* is also required in the development of the nervous system. In *spitz* mutants the MGM glial cells fail to migrate which results in a fusion of the anterior and posterior commissures (Klämbt et al. 1991). The set of tissues that express *spitz* is wider than the set of tissues affected by loss of *spitz* function, and thus other genes may control where *spitz* is active (Rutledge et al. 1992).

The molecular identity of the *spitz* gene product suggests that it encodes a ligand of the *Drosophila* EGF-Receptor encoded by the *DER* locus (Livneh et al. 1985). It has not been yet been demonstrated that the *spitz* protein directly binds the *DER* protein or that *spitz* acts genetically upstream

of *DER*. *DER* performs a large number of developmental functions and has been genetically characterized as *faint little ball (flb)*, alleles of which cause a zygotic embryonic lethal phenotype (Nüsslein-Volhard et al. 1984, Schejter and Shilo 1989) as *torpedo (top)*, alleles of which cause a female sterile phenotype (Price et al. 1989, Schüpbach 1987) and as *Ellipse*, alleles of which cause dominant defects in the development of the eye (Baker and Rubin 1989). In *flb* mutant embryos, there is a defect in germ-band extension, a loss of anterior and ventral cuticle, degeneration of the amnioserosa, and a loss of ventral cuticle that is similar to, but more severe than, the defect caused by the *spitz* group of mutants (Clifford and Schüpbach 1992, Nüsslein-Volhard et al. 1984, Raz and Shilo 1992). An analysis using temperature-sensitive *flb* alleles indicates that *DER* function is required for sequential steps in the development of the CNS and that loss of *DER* function after the early steps confers a phenotype in which the commissures are fused in a manner similar to the *spitz* phenotype (Raz and Shilo 1992). The severe phenotype of amorphic *DER* mutations suggest that the *Drosophila* EGF-R is involved in more developmental processes than *spitz*, although maternal contributions of *spitz* could mask the full role of *spitz* in zygotic development. This could suggest that *DER*, like the EGF-R of mammals uses multiple ligands. Amorphic mutations in the *gurken* locus confer a female sterile phenotype equivalent to the phenotype of the *top* alleles of *DER* (Schüpbach 1987). It has been reported that *gurken* encodes a TGF-α like protein and thus the *gurken* protein is a second candidate ligand for the *DER* protein (S. Neuman-Silberberg, T. Schüpbach, personal communication). *gurken* acts cell autonomously to the germline and *DER* acts cell autonomously to the soma which is consistent with the model that *gurken* and *DER* are involved in inter-cellular signalling during germ-line development (Schüpbach 1987).

**Figures.**

Figure 1. Three-signal model of VPC fate determination. The six VPCs choose between three possible fates, 1°, 2° and 3° in response to three intercellular signals. A spatially graded inductive signal made by the anchor cell induces the three more proximal VPCs to assume 1° and 2° vulval fates. An inhibitory signal from the syncytial epidermis prevents the VPCs from assuming vulval fates in the absence of the inductive signal. A lateral signal acts among the VPCs to promote the 2° fate. Each VPC is believed to laterally signal its neighbors in proportion to the amount of inductive signal that it receives.

**Figure 1. Three-signal model of VPC fate determination.**

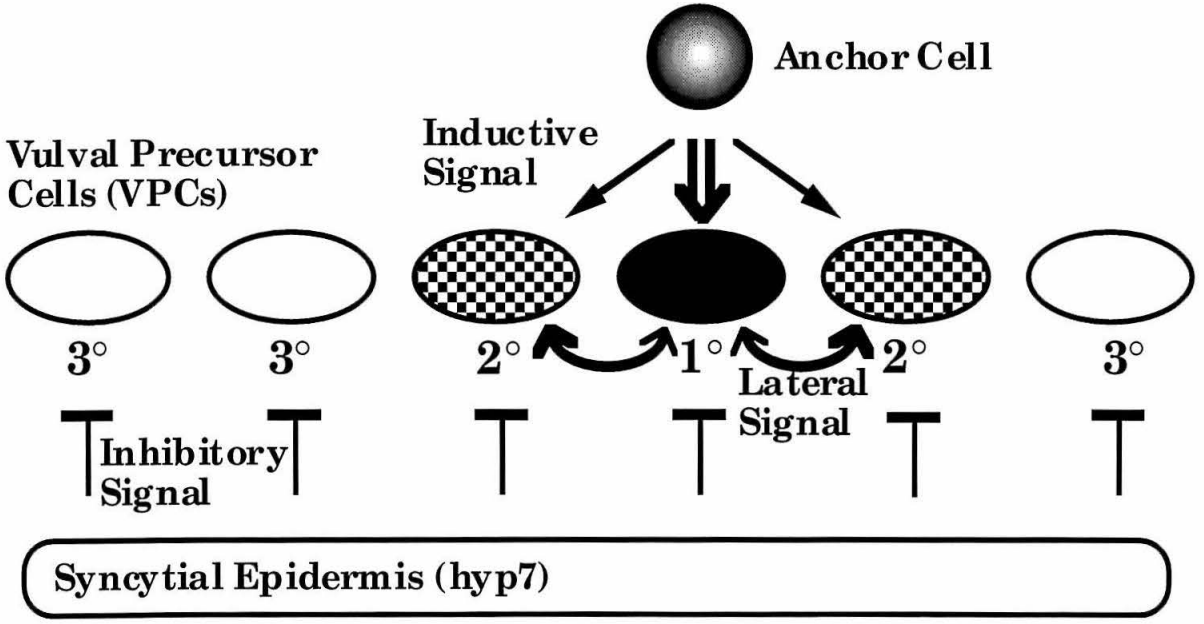

Figure 2. Schematic representation of the fates of the VPCs in wild-type vulval development. The name, relative position, and fate of each cell is shown. The lineage associated with each fate is drawn below the VPCs. Vertical lines represent cells and horizontal lines represent cell divisions. P5.p, P6.p, and P7.p contribute to the vulva and the other VPCs form epidermis. P3.p can assume the 3° fate or fuse directly to the syncytial epidermis without dividing (Sulston and Horvitz 1977, Sulston and White 1980). A unique characteristic associated with the final division of each cell, or with the terminal progeny, is indicated by a single letter code beneath the lineage, (convention of Sternberg and Horvitz, 1986): S, cell fuses with the syncytial epidermis hyp7; L, nucleus divides longitudinally in the anterior-posterior axis of the body, underline indicates that the daughter nuclei adhere to the ventral cuticle; T, nucleus divides transversely in the left-right body axis; N, nucleus does not divide. AC: Anchor Cell.

## Figure 2. Lineages of the VPCs

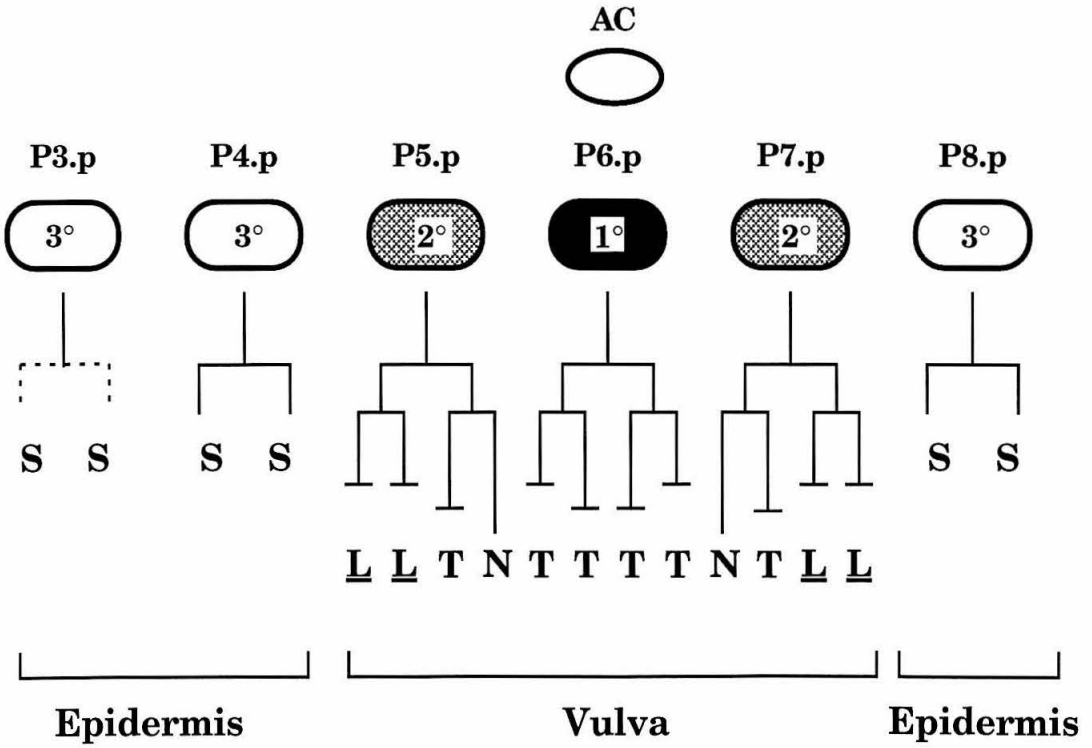

Figure 3. Simplified pathway of vulval induction. The gene *lin-3* encodes a EGF-like growth factor made by the anchor cell that is proposed to activate *let-23*. *lin-15* negatively regulates the basal activity of *let-23*. *let-23* encodes an EGF-Receptor-like tyrosine kinase. An adapter protein encoded by *sem-5* is proposed to couple the action of *let-23* to *let-60*. *let-60* encodes a *ras* protein that acts upstream of the *raf* ser-thr kinase encoded by *lin-45*. Activity of this pathway eventually results in inactivation of *lin-1*. *lin-1* promotes the epidermal fate and inhibits the vulval fates.

Figure 3. Simplified pathway of vulval induction.

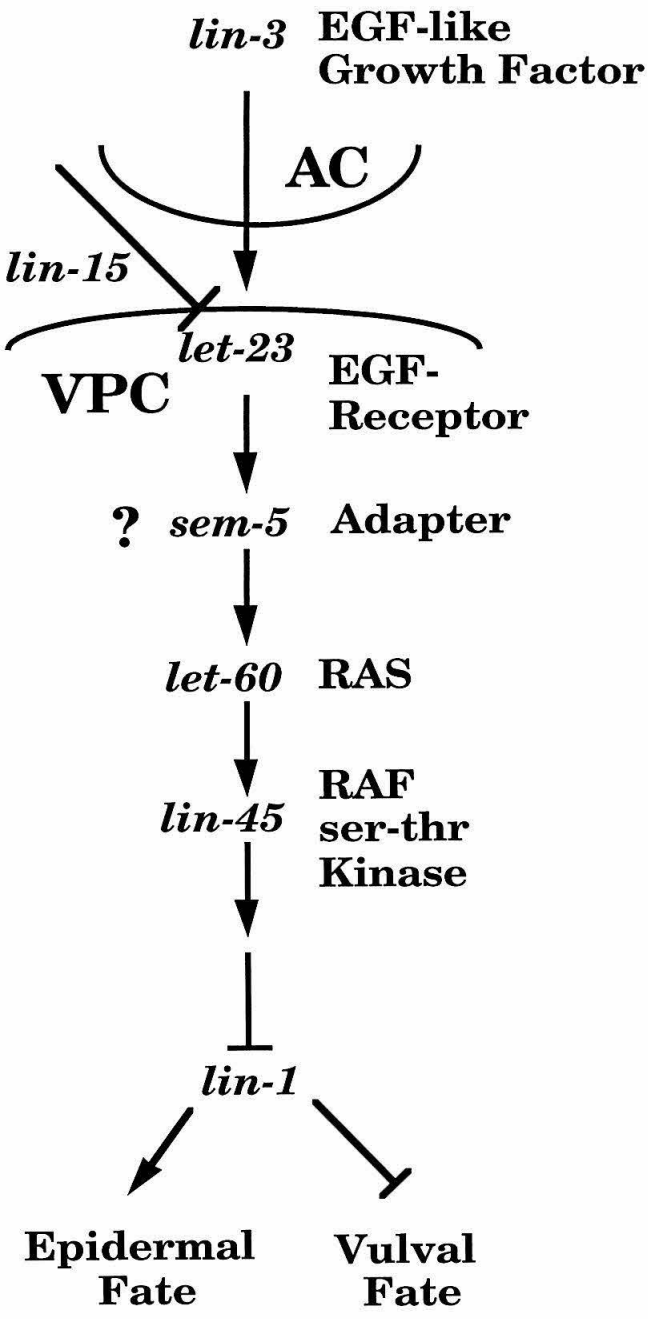

Table 1. Representative vulval fate patterns seen in different genotypes or after cell ablation. A. In wild-type development the six VPCs assume the pattern of fates 3° 3° 2° 1° 2° 3° (Sulston and Horvitz 1977, Sulston 1976, Sulston and White 1980). B. The VPCs can display regulation of fates upon ablation of VPCs prior to the time of vulval induction. A cell that normally adopts the 3° or 2° fate can assume the 1° fate if the cell that normally assumes the 1° fate is destroyed. A cell that normally assumes the 3° fate can replace a 2° cell. A 1° cell will not replace a 2° or 3° cell, and a 2° cell will not replace a 3° cell. The regulating cell will usually assume the position, as well as the fate, of the cell that it is replacing. The abilility of the VPCs to undergo replacement regulation supports the model that the VPCs are equivalent in their potential to assume either of the three fates (Sternberg and Horvitz 1986, Sulston and White 1980). C. Ablation of the AC or its precursors before or during the first several hours of the L3 stage prevents P5.p, P6.p and P7.p from assuming vulval fates. Therefore, the vulval fates are specified by an inductive signal from the AC of the somatic gonad (Kimble, 1981, Sulston and White, 1980) D1. Recessive mutations in the genes *lin-2, lin-3, lin-7, lin-10, lin-45, let-23, let-60,* and *sem-5*, and dominant negative mutations in *let-60* can cause a vulvaless phenotype in which fewer than the normal number of VPCs assume vulval fates, even though the AC and the VPCs are present (Aroian and Sternberg, 1991, Beitel, et al., Clark, et al., 1992, Ferguson and Horvitz, 1985, Han, et al., 1990, Han, et al., 1993, Horvitz and Sulston, 1980, Sulston and Horvitz, 1981). These genes are involved either in the generation of the inductive signal by the AC, or the response to the inductive signal by the VPCs. D2. Recessive mutations in *lin-1*, and *lin-15*, and gain-of-function mutations of *let-60*, confer a multivulva phenotype in which greater than the normal number of VPCs assume vulval

fates (Beitel, et al. 1990, Ferguson and Horvitz 1985, Han, et al. 1990). E. In *lin-15* mutant animals all six VPCs assume vulval fates (E1) and can do so independently of the inductive signal (E2) (Ferguson and Horvitz ,1985, Ferguson, et al., 1987). In *lin-15* mutant animals it is commonly observed that two adjacent VPCs both assume the 2° fate, but it is rarely observed that two adjacent VPCs both assume the 1° fate. This suggests the action of a lateral signal among the VPCs that prevents two adjacent VPCs from both assuming the 1° fate. 1. In *lin-15* mutant animals, the patterning of P5.p, P6.p, and P7.p is normal if the AC is present. P6.p always assumes the 1° fate and P5.p and P7.p assume the 2° fate. 2. If the AC is absent, then the patterning of P5.p, P6.p and P7.p is disrupted so that any of these cells can assume either the 1° or 2° fate. Thus, the VPCs can still respond to the inductive signal in *lin-15* mutant animals. 3. An isolated VPC in a *lin-15* mutant assumes the 1° fate. 4,5. Two isolated VPCs in a *lin-15* mutant animal will usually both assume the 1° fate if they are not in close proximity to each other, but only one of the cells will assume the 1° fate if they remain in close contact with each other. Thus, the lateral signal between the VPCs appears to act only at a close range and may require direct cell contact (Sternberg, 1988a). F. The *lin-12* activity promotes the 2° fate in the VPC equivalence group and in the AC (1°) VU (2°) equivalence group (Greenwald, et al. 1983). 1. In animals homozygous for null mutations of *lin-12* approximately three VPCs are induced to assume 1° lineages and there are often two or more ACs. 2. In animals with dominant gain-of-function mutations in *lin-12,* all six VPCs assume 2° fates and there is often no AC. 3. In some animals of the genotype *lin-12(d)/lin-12(0)* ACs are present. When an AC is present, P6.p assumes the 1° fate and when the AC is absent P6.p assumes the 2° fate. Therefore the inductive signal can override the action of

*lin-12(d)* mutations to specify the 1° fate (Sternberg and Horvitz 1989). G. The AC is the organizing center of vulval fate patterning. When the AC is shifted to a more anterior position in *dig-1* mutant animals, the pattern of vulval development is usually commensurably shifted (Thomas, et al. 1990). H. The fate of an isolated VPC correlates with its distance from the AC. A VPC that is close to the AC will assume the 1° fate, a VPC that is relatively further away will assume the 2° fate, and a VPC that is far from the AC will assume the 3° fate. This suggests that the inductive signal is distributed in a spatial gradient centered on the AC and that the VPCs can assume different fates in response to different levels of the inductive signal (Sternberg and Horvitz, 1986). I. In animals bearing multicopy *lin-3* transgenes, which presumably overexpress Lin-3 protein from the anchor cell, adjacent VPCs often assume the 1° fate. Therefore, high levels of the inductive signal can override the effect of the lateral signal and specify two adjacent cells to both have the 1° fate (Hill and Sternberg, manuscript in preparation). AC: Anchor Cell.

Table 1. Vulval fate patterns.

| | P3.p | P4.p | P5.p | P6.p | P7.p | P8.p |
|---|---|---|---|---|---|---|
| A. wild-type | 3° | 3° | 2° | AC 1° | 2° | 3° |
| B. Replacement Regulation: | | | | | | |
| 1. P6.p ablation | 3° | 2° | 1° | AC x | 2° | 3° |
| 2. P5.p ablation | 3° | 2° | x | AC 1° | 2° | 3° |
| C. AC ablation. | 3° | 3° | 3° | x 3° | 3° | 3° |
| D. 1. vulvaless | 3° | 3° | 3° | AC 3° | 3° | 3° |
| 2. multivulva | 1° | 2° | 2° | AC 1° | 2° | 1° |
| E. 1. *lin-15* AC+ | 1° | 2° | 2° | AC 1° | 2° | 1° |
| 2. *lin-15* AC- | 1° | 2° | 1° | x 2° | 1° | 2° |
| 3. *lin-15* Isolated VPC | x | x | x | x x | 1° | x |
| 4. *lin-15* VPCs close | x | x | x | x x | 1° | 2° |
| 5. *lin-15* VPCs distant | x | 1° | x | x x | 1° | x |
| F. 1. *lin-12(0)* | 3° | 3° | 1° | AC AC 1° | 1° | 3° |
| 2. *lin-12(d)* | 2° | 2° | 2° | 2° | 2° | 2° |
| 3. *lin-12(0)/lin-12(d)* AC+ | 2° | 2° | 2° | AC 1° | 2° | 2° |

| | | | | | | |
|---|---|---|---|---|---|---|
| G. *dig-1* anterior AC. | 3° | 2° | AC 1° | 2° | 3° | 3° |
| H. Isolated VPCs. | | | | | | |
| 1. Proximal VPC | x | x | x | AC 1° | x | x |
| 2. Intermediate VPC | x | x | 2° | AC x | x | x |
| 3. Distal VPC | 3° | x | x | AC x | x | x |
| I. *lin-3* transgene. | 2° | 2° | 1° | AC 1° | 2° | 2° |

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# Chapter 2

## Molecular cloning of the gene *lin-3*.

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# The gene *lin-3* encodes an inductive signal for vulval development in *C. elegans*.

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**The *lin-3* gene is necessary for induction of the *C. elegans* vulva by the anchor cell. It encodes a molecule similar to epidermal growth factor and to transforming growth factor-α and acts through the epidermal growth factor receptor homologue *let-23*. Expression of *lin-3* in the anchor cell stimulates vulval induction; *lin-3* may encode the vulval inducing signal.**

Induction is a general developmental mechanism in which one set of cells specifies the fate of another set of cells. The vulval development of *C. elegans* hermaphrodites is an intensively-studied example of induction (reviewed in (Horvitz and Sternberg 1991)). The vulva normally forms from a subset of six tripotential cells called the vulval precursor cells (VPCs) that are located within the ventral epidermis. Each VPC can have an epidermal fate in which it produces two progeny that fuse with the syncytial epidermis, hyp7. Alternatively, each VPC can have either of two vulval fates in which it produces seven or eight vulval progeny. In a wild-type animal, the three VPCs that are closer to the anchor cell of the somatic gonad have vulval fates and the three VPCs farther from the AC have epidermal fates (Fig. 1a). If the anchor cell is destroyed early in development, then all six VPCs have the epidermal fate, indicating that the vulval fates are induced by a signal from the anchor cell (Kimble 1981). This vulval inductive signal can act directly on each VPC and may be spatially graded (Sternberg and Horvitz 1986). The vulval inductive signal may be a secreted factor because vulval induction can occur in *dig-1* mutant animals when there is no apparent contact between the anchor cell and the VPCs (Thomas et al. 1990).

Many genes involved in the process of vulval induction have been defined by mutations that fall into two broad classes (Horvitz and Sternberg 1991). Vulvaless (Vul) mutations decrease the number of VPCs that assume vulval fates, whereas multivulva (Muv) mutations increase the number of VPCs that assume vulval fates. Mutations that partially lower *lin-3* activity confer a Vul phenotype in which the VPCs that would normally form the vulva instead usually form epidermis, even though the anchor cell is present (Ferguson and Horvitz 1985, Ferguson et al. 1987, Horvitz and Sulston 1980, Sulston and Horvitz 1981). Although *lin-3* affects other developmental processes, for example a putative null mutation in *lin-3* results in lethality early in postembryonic development (Ferguson and Horvitz 1985), only the role of *lin-3* in vulval development is characterized here. A genetic pathway of vulval induction has been derived from the epistasis displayed by the Vul and Muv mutations (Aroian and Sternberg 1991, Ferguson, et al. 1987, Han et al. 1990). These experiments suggest that *lin-3* acts before *let-23* and *let-60*, which are other genes necessary for vulval induction. *let-23* encodes a homologue of the epidermal growth factor receptor (EGF-R) subfamily and is the proposed receptor for the inductive signal (Aroian et al. 1990, Aroian and Sternberg 1991), and *let-60* encodes a *ras* protein that acts downstream of *let-23* as a switch to control VPC fate (Beitel et al. 1990, Han, et al. 1990, Han and Sternberg 1990). Several other genes that are believed to act downstream of *lin-3* during vulval induction have been molecularly characterized: *lin-10* encodes a novel protein (Kim and Horvitz 1990) that is necessary for wild-type levels of vulval induction; *sem-5* encodes a novel protein with SH2 and SH3 domains (Clark et al. 1992). To establish further the role of *lin-3* in vulval development, we have cloned the *lin-3* locus and

examined the genetic properties of *lin-3* transgenes. Our analysis indicates that *lin-3* has a structure similar to certain mammalian growth factors, and that *lin-3* can act as a signal *in vivo*.

**Cloning of the *lin-3* locus**

We cloned the *lin-3* locus by transposon tagging and DNA-mediated transformation (Fig. 2). We obtained the transposon-induced allele *lin-3(sy91::Tc1)* by mating *lin-3* males with hermaphrodites of the strain RW7096, which has high rates of transposition of the transposon Tc1 (Mori et al. 1988). *sy91* confers a recessive Vul phenotype (Fig. 3b) and was recovered because it fails to complement the paternal *lin-3* mutation. Genetic mapping showed that the Tc1 insertion called *syP1* cosegregates with the mutation *sy91* (Fig. 2 legend). DNA sequences that flank *syP1* were used to identify the genomic clones λPS#1 and λPS#2. Two observations confirm that these genomic clones cover at least part of the *lin-3* locus. First, λPS#1 detects the polymorphism *syP2* associated with the mutation *lin-3(n1058)* and which is located 2.5 kilobases (kb) from the *syP1* Tc1 insertion. Second, both genomic clones can rescue the Vul phenotype of *lin-3* mutations when introduced as extrachromosomal multicopy transgenes. A 5-kb subclone called pRH9 and a 3.3-kb subclone called pRH19, both of which cover the location of *syP1* and *syP2*, can also rescue *lin-3*. pRH19 was used to isolate the cDNA clones pRH39 and pRH40. We determined the nucleotide sequences of the cDNA clone pRH39 (Fig. 4) and of the genomic region equivalent to pRH19 (data not shown). The two cDNAs appear to encode full length Lin-3 proteins. The results of site-directed mutagenesis experiments confirm that pRH39 and pRH40 are *lin-3* cDNAs. We introduced mutations into the genomic clone pRH9 that would change the sense of two codons of the transcription unit

defined by the cDNAs (Fig 4 legend). These mutations abolish the ability of this clone to rescue the Vul phenotype of *lin-3(n378)* when introduced as a transgene (Fig 4 legend). Partial sequencing of *lin-3* genomic DNA indicates that the region of pRH39 and pRH40 that encodes the signal peptide is not located within pRH9 or pRH19. One explanation for the ability of these genomic clones to rescue *lin-3* is that they contain a genomic region that has characteristics of a 5' exon that could encode an alternative signal-peptide-like sequence (Fig. 4 legend). This region could be an alternative 5' end of the *lin-3* locus or a cryptic 5' end used only by the transgenes.

**Lin-3 structure is similar to EGF-R ligands**

The Lin-3 protein predicted from nucleotide sequence is a membrane-spanning protein of 438 or 423 amino acids with a single extracellular EGF repeat. Two forms of the protein are encoded by alternatively spliced transcripts and differ in the presence or absence of 15 amino acids between the EGF repeat and the transmembrane domain. The cytoplasmic domain of Lin-3 comprises 181 amino acids of novel sequence (Fig. 4). The EGF repeat of Lin-3 has statistically significant sequence similarity to many of the EGF repeats of other genes (Fig. 5). An EGF repeat is a motif consisting of six cysteine residues with semi-conserved spacing (reviewed in (Carpenter and Wahl 1990)). EGF repeats are present in a variety of proteins of many different functional classes (Carpenter and Wahl 1990, Davis 1990). Lin-3 does not have overall sequence similarity to any characterized gene, and is most likely a new member of the EGF group of growth factors. The EGF repeat of Lin-3 has Tyr13 and Arg41, which are conserved preferentially among the EGF repeats used as growth factors (Fig. 5). Also, the overall structure of Lin-3 is most similar to the structure of the growth factor

precursors, which beyond the presence of at least one EGF repeat and a transmembrane domain have little sequence similarity common to the entire class.

The EGF group of growth factors includes EGF itself (Gregory 1975, Savage et al. 1972), transforming growth factor-α (TGF-α) (Derynck et al. 1984), heparin-binding EGF (HB-EGF) (Higashiyama et al. 1991), amphiregulin (Shoyab et al. 1989), and vaccinia virus growth factor (VVGF) (Stroobant et al. 1985), which are known ligands of the EGF-R, as well as rat *neu* differentiation factor (NDF) (Wen et al. 1992) and the human heregulins (Holmes et al. 1992), which are ligands of *neu*/HER2, one of two other members of the EGF-R subfamily. The membrane-proximal EGF repeat is known in several cases to be the receptor-binding domain of these growth factors. Most of these molecules can undergo proteolytic processing to release this EGF repeat as a secreted molecule. In the case of TGF-α, the unprocessed membrane bound form can also activate the receptor (reviewed in (Massagué 1990)). Processing of Lin-3 has not been demonstrated. The alternative versions of the Lin-3 protein differ in the region between the EGF repeat and the membrane-spanning domain where processing would have to occur. The different Lin-3 proteins could differ in their ability to be processed. The use of alternative splicing to produce membrane bound ligands that differ in their ability to be processed has been demonstrated for the Kit-ligand (Flanagan et al. 1991), but has not been seen with the EGF group of growth factors.

The phylogenic relationship of *lin-3* to the EGF group of growth factors remains unclear. *lin-3* is not a clear homolog of any one member of the group and a simple hypothesis is that *lin-3* is diverged from a common ancestor of the diverse mammalian growth factors. By RDF2 analysis (Pearson and

Lipman 1988), the EGF repeat of Lin-3 is most similar to the EGF repeats of the growth factors from myxoma virus (MVGF) (Upton et al. 1987) and Shope fibroma virus (SFGF) (Chang et al. 1987). These two genes differ from the other EGF-like growth factors in that they lack possible membrane-spanning domains. MVGF has been shown to bind the EGF-R at a 200-fold lower affinity than EGF itself (Lin et al. 1991), but the function of SFGF is unknown.

### *lin-3* transgenes cause excess vulval induction

Transgenes containing wild-type *lin-3* DNA confer a dominant multivulva (Muv) phenotype (Fig. 3c) in which up to all six of the VPCs have vulval fates. The transgenes are created by a method that concatenates about a hundred copies of the injected DNA into an extrachromosomal array (Mello et al. 1991) and thus are likely to express levels of a Lin-3 protein that are higher than the levels of Lin-3 expressed in a wild-type animal. We believe the transgenes cause excess vulval induction because the process of vulval induction is sensitive to overexpression of Lin-3. In contrast, mutations that lower *lin-3* activity decrease the number of VPCs that have vulval fates (Ferguson and Horvitz 1985, Ferguson, et al. 1987, Horvitz and Sulston 1980, Sulston and Horvitz 1981). Thus, the amount of Lin-3 protein produced may control the number of VPCs that have vulval fates.

### The *lin-3* transgenes act via *let-23*

We ordered the action of *lin-3* before the action of *let-23* based on a test of genetic epistasis using the dominant Muv phenotype of the *lin-3* transgenes. The *let-23* mutation *sy97* causes a highly penetrant Vul phenotype such that more than 99% of *sy97* homozygotes fail to lay eggs (the Egl phenotype)

(Aroian and Sternberg 1991). 90% of animals bearing the transgene *syEx13,* which contains wild-type *lin-3* DNA, are Muv. *let-23(sy97)* homozygotes carrying the transgene *syEx13* are not Muv and 99.7% are Egl (see Fig. 3 legend). Therefore the *lin-3* transgenes can only stimulate vulval fates if *let-23* is functional and thus *lin-3* is likely to be an upstream activator of *let-23*. Previous experiments in which Vul mutations of *lin-3* and *let-23* were indirectly ordered also suggest that *lin-3* acts before *let-23* (Ferguson, et al. 1987). In contrast to the behavior of the *lin-3* transgenes, multicopy *let-60* transgenes cause a Muv phenotype even in a *let-23(sy97)* mutant (Han and Sternberg 1990).

**Anchor cell expression of *lin-3::lacZ***

To determine where the *lin-3* transgenes are expressed, the *lacZ* gene of *Escherichia coli* was inserted in-frame into the first cytoplasmic exon of *lin-3* (Fig. 6). The *lin-3::lacZ* fusion should produce a protein consisting of the extracellular and transmembrane domains of *lin-3* followed by a β-galactosidase domain encoded by *lacZ*. In transgenic animals this fusion produces detectable β-galactosidase activity while maintaining the ability to stimulate excess vulval development. pRH36 contains the *lin-3::lacZ* fusion within a 5 kb *lin-3* genomic region equivalent to pRH9, whereas pRH56 contains an additional 4.6 kb of 5' genomic sequence (shown in Fig. 2). A transgene derived from pRH36 shows specific expression of β-galactosidase in the anchor cell. This expression is detectable during the lethargus between the L2 and L3 larval stages (Fig 6a, b) and remains detectable in the L4 stage when vulval morphogenesis is nearly complete (Fig 6d). Ablation experiments indicate that vulval induction occurs late in the L2 lethargus or early in the L3 (Kimble 1981) and thus the transgene is expressed before or

during the time of vulval induction. pRH56 directs other localized expressions (data not shown) as would be expected, given that *lin-3* has other developmental phenotypes (Ferguson and Horvitz 1985). Nonetheless, the only expression near the VPCs is in the anchor cell. The expression patterns of pRH36 and pRH56 suggest that the 5 kb *lin-3* region represented by pRH9 could be a minimal region sufficient for vulval rescue which might not contain all control sequences required for other expressions. As the pRH36 transgene can stimulate excess vulval development (Fig. 6d) while displaying specific expression in the anchor cell, we infer that the anchor cell expression is relevant for vulval development.

### *lin-3* transgenes may act in the anchor cell

To test whether the ability of the transgenes to cause a Muv phenotype was dependent upon the inductive signal, we ablated the gonad precursors, and hence the anchor cell, in early L1 animals that carried *lin-3* transgenes. Gonad-ablated *syEx13* animals had an average of 23% induction compared with 143% induction in mock-ablated *syEx13* animals (see legend of Fig. 3 for explanation of scoring). Specifically, 12 of 15 ablated animals had no vulval differentiation and three animals had three to five VPCs generating vulval progeny. 7 of 15 mock-ablated *syEx13* animals had wild-type vulval induction and eight had vulval induction greater than wild-type (Fig. 3d). Therefore, the *lin-3* transgene either acts in the gonad to stimulate vulval development, or acts elsewhere and requires the gonad to become active. Because *syEx13* contains the same *lin-3* genomic region as a *lin-3::lacZ* fusion that is expressed specifically in the anchor cell, we believe that *lin-3* acts in the anchor cell. These ablation studies do not indicate the site of action for the residual activity of the *lin-3* transgenes in the three ablated animals with

vulval development. It is possible that the transgenes have erratic ectopic expression, or that the VPCs are responding to an overexpression of Lin-3 in other tissues that do not normally induce the vulva. We conclude that *lin-3* can act in a non-cell autonomous manner to stimulate vulval development.

### *lin-3* as inductive signal

We propose that *lin-3* encodes the vulval-inducing signal from the anchor cell defined by the ablation experiments of Kimble (Kimble 1981). This hypothesis is supported by several observations. First, the vulvaless phenotype of mutations that lower *lin-3* activity indicates that *lin-3* is normally necessary for the specification of vulval fates (Ferguson and Horvitz 1985, Ferguson, et al. 1987, Horvitz and Sulston 1980, Sulston and Horvitz 1981). Second, presumptive overexpression of *lin-3* by multicopy transgenes causes increased levels of vulval induction. Together these two facts suggest that the dose of *lin-3* determines the extent of vulval development. Third, genetic epistasis experiments indicate that *lin-3* acts genetically upstream of *let-23* in a common pathway. Fourth, *lin-3* appears to be a new member of the EGF group of growth factors and *let-23* is homologous to the EGF-receptor (Aroian, et al. 1990). Thus the molecular identities of *lin-3* and *let-23* and their genetic order of action suggest that *lin-3* could encode a ligand for the receptor encoded by *let-23*. Fifth, *lin-3::lacZ* fusions are expressed in the AC at the time of vulval induction. Sixth, ablation experiments indicate that the *lin-3* transgenes can act in the gonad to stimulate vulval development. *lin-3* has the correct genetic properties, molecular structure, and pattern of expression to be this inductive signal.

In addition to the inductive signal encoded by *lin-3*, two other intercellular signals have been proposed to regulate the fates of the VPCs. First, a lateral

signal mediated through *lin-12* regulates the pattern of fates assumed by induced VPCs and appears to act after *lin-3* (Greenwald et al. 1983, Sternberg 1988, Sternberg and Horvitz 1989). Second, an inhibitory signalling pathway defined by mutations in *lin-15* promotes epidermal fates (Ferguson and Horvitz 1985, Ferguson and Horvitz 1989, Sternberg 1988, Sternberg and Horvitz 1989). The inhibitory signal may originate from the surrounding epidermis and thus would inhibit all the VPCs equally (Herman and Hedgecock 1990). Genetic epistasis experiments indicate that *lin-15*, like *lin-3*, acts to control *let-23* activity (Aroian and Sternberg 1991, Ferguson, et al. 1987, and L. Huang & P. Sternberg, unpublished results). Given that *lin-3* and *lin-15* are both regulators of *let-23*, it is possible that they either act in parallel and antagonistically on *let-23*, or that they act in series such that *lin-3* is a negative regulator of *lin-15*. We propose that *lin-3* and *lin-15* act in parallel on *let-23*. First, the nature of the the *lin-3* and *let-23* proteins suggest Lin-3 could be a direct ligand of Let-23. Second, the inductive signal can influence the fates of VPCs in a *lin-15* mutant suggesting that the inductive signal does not require functional *lin-15* to influence the fates of the VPCs (Han, et al. 1990, Sternberg 1988).

We propose that the VPCs possess a response pathway that originates with the receptor tyrosine kinase encoded by *let-23* and that acts through *let-60 ras* to specify vulval fates (Fig. 1b). In our view, the activity of this response pathway is controlled by two antagonistic upstream pathways. An inhibitory pathway mediated by *lin-15* lowers the basal activity of the response pathway in all VPCs in the absence of the inductive signal. We propose that *lin-3* is the inductive signal produced by the anchor cell and that binding of Lin-3 to Let-23 activates Let-23 and the response pathway. Because the inductive signal can apparently act at a distance (Thomas, et al.

1990) Lin-3 may be proteolytically processed to release the EGF repeat as a secreted factor. It is possible though, that some wild-type induction uses a membrane bound form of Lin-3. For example, the VPC directly beneath the anchor cell, P6.p, could be induced by membrane-bound Lin-3 while the VPCs one cell distal to the anchor cell, P5.p and P7.p, could be induced by secreted Lin-3.

The *lin-3* transgenes can stimulate all the VPCs in a given animal to have vulval fates. This observation confirms that each VPC is competent to respond to the inductive signal. Since this induction can occur when all six VPCs are present, we argue against the possibility that intercellular signalling among the VPCs has an absolute ability to limit the number of VPCs that differentiate as vulval tissue. Thus, during normal development the extent of vulval differentiation might be limited by the level of inductive signal.

## Figure Legends

**Fig. 1. Model of vulval induction.**

**a.** A schematic diagram of the anatomy of an early L3 *C. elegans* hermaphrodite at the time of vulval induction. Each of six Vulval Precursor Cells (VPCs) located within the ventral epidermis have the potential to assume an epidermal fate or either of two vulval fates. The anchor cell (AC) is located ventrally in the somatic gonad and is the source of an inductive signal necessary for the VPCs to assume vulval fates. In wild-type vulval animals the three VPCs closest to the AC (P[5-7].p) assume vulval fates and the three other VPCs have epidermal fates. The AC and the VPCs are separated by the body cavity. The model of vulval induction is based on many studies reviewed in reference (Horvitz and Sternberg 1991). The drawing is based on anatomical studies described in reference (Wood 1988) and work by Lynn Carta (personal communication).

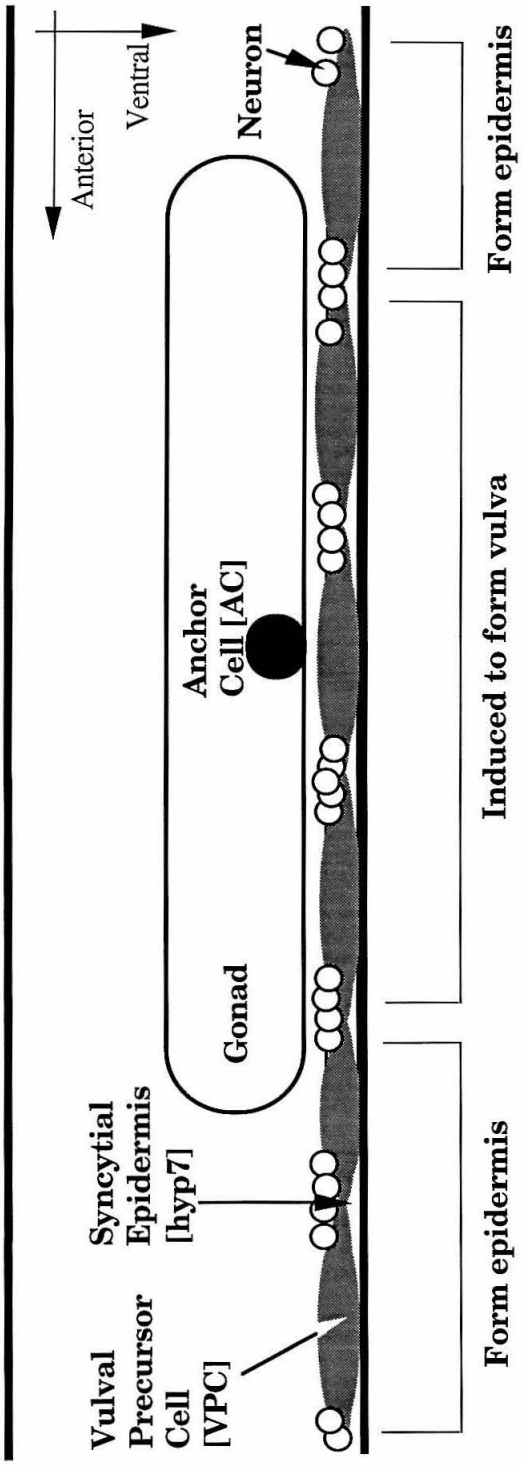



Figure 1a. Schematic diagram of the anatomy of vulval induction.

**Fig. 1. b.** Schematic model of vulval induction. As described in the text, we propose that *lin-3* is the inductive signal from the anchor cell that is necessary for the specification of vulval fates. *lin-3* has structural similarities to the EGF group of growth factors and is expressed in the AC. The EGF repeat of *lin-3* may be released from the surface of the AC by proteolytic processing to generate a secreted factor. *lin-3* is genetically upstream of *let-23* and *let-60,* which are proposed to act in the VPCs. *let-23* encodes a nematode homolog of the EGF-R and is proposed to be the receptor for the inductive signal. The binding of *lin-3* to *let-23* is proposed to activate *let-23* which then stimulates vulval fates by activation of *let-60 ras*. Genetic experiments indicate that *lin-15* inactivates *let-23* in the absence of *lin-3* activity and suggest that *lin-15* may act in the surrounding epidermis, hyp7. ECM, ExtraCellular Matrix. This model is based on the studies reviewed in (Horvitz and Sternberg 1991) and our results.

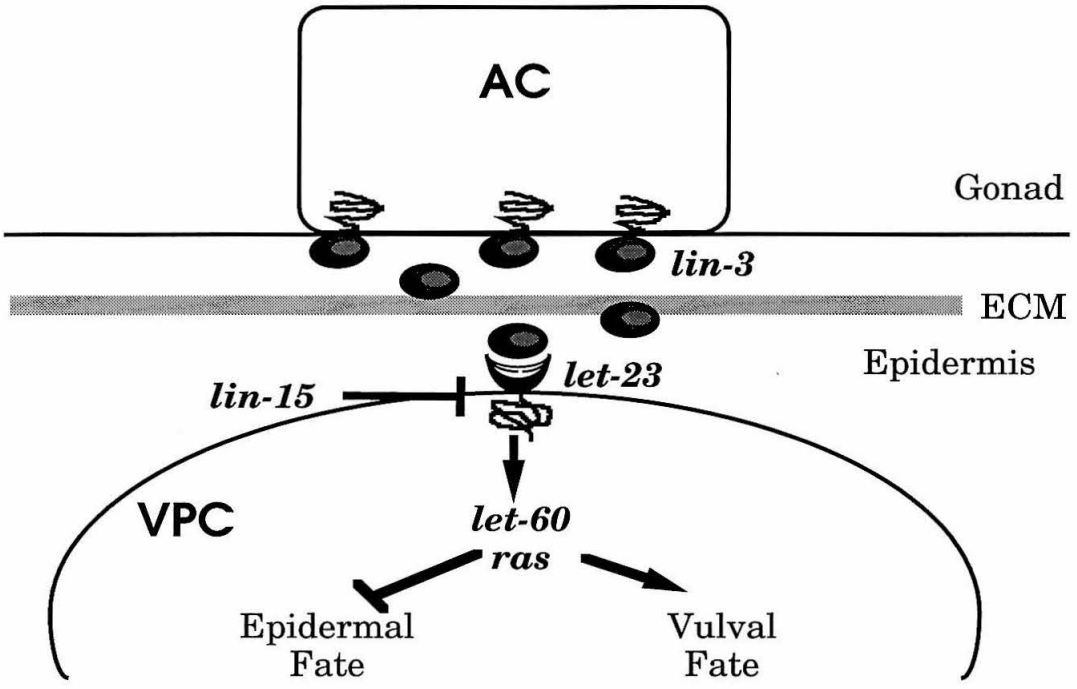



Figure 1b. Schematic model of vulval induction.

**Figure 2. Molecular map of the *lin-3* locus.**

A physical map of the *lin-3* locus which illustrates the position of the two known *lin-3* RFLPs (center), the ability of various genomic clones to provide *lin-3* function (bottom), and an expanded view of part of the *lin-3* transcription unit (top). *syP1* is a Tc1 transposon insertion associated with *lin-3(sy91)*. *syP2* is an additional EcoRV site found in *lin-3(n1058)*. The ability of genomic subclones (below) to rescue a *lin-3* Vul phenotype and to cause a dominant Muv phenotype when introduced as transgenes is indicated in the columns on the right. The 3.3 kb genomic region subcloned as pRH19 is the minimal region known to be sufficient for transgenic rescue. The genomic sequence of this 3.3 kb region has been determined (data not shown). The positions of *lin-3* exons, shown by boxes above, was deduced from the genomic sequence and the sequence of two *lin-3* cDNAs (See Fig. 4). The position of *lin-3* exons outside of this 3.3 kb region are not known. The section of the transcript that encodes the EGF repeat is shown in solid black and the region that encodes the putative membrane spanning domain is stippled. The cosmid F17G6 links the *lin-3* genomic clones to the physical map such that left in Fig. 2 corresponds to left on the genetic map of LG IV. The unique BamHI site is defined as position +1 of *lin-3* genomic DNA. The restriction map of B, H and RV is complete for the entire region. All K, N, S, and Sp sites are only shown for the region from +0 kb to +5 kb. Abbreviations: ND, not determined; Bm, Bam HI; RV, Eco RV; H, Hind III; K, Kpn I; N, Nde I; S, Sac I; Sp, Spe I.

**Methods. Transposon tagging of *lin-3*.** A transposon-induced allele of *lin-3* was found in a non-complementation screen. The screen allows a specific search for *lin-3* mutations, which are rare among the set of mutations that cause an egg-laying defective (Egl) or vulvaless (Vul) phenotype. This

screen also allows the recovery of null alleles of *lin-3,* which confer lethality when homozygous, but which are viable and Vul in *trans* to *lin-3(e1417)* (Ferguson and Horvitz 1985). Three to four PS231 *[lin-3(e1417) dpy-20(e1362)/lin-3(e1417) unc-31(e169) IV; him-5(e1490)V]* males were mated with four or five hermaphrodites of the mutator strain RW7096 *[mut-6(st702) unc-22(st192::Tc1) IV]* (Mori, et al. 1988). The parents were transferred to new plates every 24 hours and the progeny were examined every 12 hours for Egl animals. Hermaphrodite self-progeny are Unc-22 and cross progeny are non-Unc-22. Cross progeny of the mating are non-Egl if they receive a wild-type allele of *lin-3* from the mutator strain or Egl if they receive a transposon-induced allele of *lin-3* from the mutator strain. A single mutation, *lin-3(sy91::Tc1),* was found among 10,000 hermaphrodite cross progeny and was recovered in the strain PS253 *[mut-6(st702) lin-3(sy91) unc-22(st192)]*. *sy91* confers recessive Vul and Egl phenotypes described in Fig. 3b.

**Mapping of *syP1*.** The Tc1 transposon insertion *syP1* was determined by genetic mapping to be inseparable from *lin-3(sy91)*. PS253 was backcrossed 6 times against *N2* to reduce the number of RW7096-derived Tc1 elements that were unlinked to *lin-3*. At the last backcross with *N2,* a F2 segregant that was homozygous for *lin-3(sy91)* called PS388 was selected, and a F2 segregant that was homozygous for the wild-type allele of *lin-3* was selected. Genomic DNAs from these strains were analyzed for the presence of different Tc1 insertions on Southern blots. Genomic DNA (Wood 1988) was digested with XbaI, which does not cut within Tc1 sequence, and run on 20 cm TAE gels for 40-50 hr at 1 V/cm, blotted, and hybridized with labeled Tc1 DNA (pTR14 a gift of Phil Anderson) according to standard methods (Ruvkun et al. 1989, Sambrook et al. 1989). Six Tc1 insertions were identified that are

present in the *lin-3(sy91)* strains PS253 and PS388, but which are absent in N2 and the non-Lin-3 backcrossed derivative of PS253. These six insertions were genetically mapped to *lin-3* by a multipoint mapping strategy described previously (Ruvkun, et al. 1989). N2 males were mated to TU355 *[unc-24(e138) mec-3(e1338) dpy-20(e1282)]* hermaphrodites and F1 males of this mating were mated with PS388 hermaphrodites. non-Unc non-Mec Dpy recombinants were recovered from the heterozygote *unc-24(e138) mec-3(e1338) + dpy-20(e1282) + / + + lin-3(sy91) + unc-22(st192)* and homozygous strains of each recombinant were established. Each recombinant strain was analyzed for its *lin-3* genotype and for the presence of each of the six Tc1 elements using Southern blots as described above. Five of the Tc1 elements are located outside of the *mec-3 dpy-20* interval because they are either present or absent in all recombinants while the sixth element, *syP1,* maps within this interval. *syP1* is a Tc1 insertion of 1.6 kb into a 390 bp Xba1 fragment. *syP1* is present in all 18 recombinant strains that maintained *lin-3(sy91)* and absent in all 14 recombinant strains that lost *lin-3(sy91)*. This places *syP1* with a 95% level of confidence in an interval of 0.046 map units (mu) to the left of *lin-3* and 0.076 mu to the right of *lin-3*. This genetic interval is equivalent to about 120 kb of DNA given correlations between the physical and genetic maps in the vicinity of *lin-3* (Sulston et al. 1992). The mapping indicated that *syP1* was the closest identified physical marker to the *lin-3* locus, and it was therefore used to identify genomic clones in the vicinity of *lin-3* (below). The nucleotide sequence of a subclone containing the *syP1* insertion (generated below) indicates that *syP1* is a typical Tc1 insertion within a target site duplication of the sequence TA (Herman and Shaw 1987) located at positions 243-244 in genomic sequence.

This genomic sequence is an intron located between nucleotides 216 and 217 of the composite cDNA sequence in Fig. 4.

**Isolation of *lin-3* genomic clones.** The sequence flanking the *syP1* Tc1 insertion was amplified from genomic DNA by inverse PCR (Ochman et al. 1988). In this technique, a restriction fragment containing a transposon is circularized by self-ligation. The sequence abutting the transposon is then amplified from the circular fragment by PCR using a single primer complementary to the inverted repeat of the transposon. Parallel reactions were performed on DNAs isolated from two recombinant strains generated in the mapping of *syP1* (above) that differ only in their *syP1* and *lin-3(sy91)* genotype. The genomic DNAs were digested with Xba1, extracted with phenol, and ethanol precipitated. The digested genomic DNAs were then circularized by self-ligation at a concentration of 1.5 μg/ml with T4 DNA ligase (New England Biolabs) at 1 U/μl at 15°C for 22 hr. The DNA was purified after the ligation by phenol extraction and ethanol precipitation and special care was taken to remove excess salt. PCR was performed according to the standard protocol of Perkin Elmer/Cetus (Norwalk, Ct) with the following modifications: circularized genomic DNA was used at 7 μg/ml and a single primer (5'-ACC AAA AGT GGA TAT CTT TTT GGC CAG C-3') specific to the inverted repeat of Tc1 (Rosenzweig et al. 1983) was used at 2 pmoles/μl. Inverse PCR on both DNAs amplified a similar ladder of products except that only the DNA containing the *syP1* Tc1 insertion produced a 400 bp fragment that is the size of the wild-type XbaI fragment in which *syP1* is located. This 400 bp band was digested with EcoRV, which cuts within the sequence of the PCR primer and ligated into pBluescript SK+ to give the plasmid pRH1. The insert of pRH1 detects a 2.0 kb Xba I fragment in *lin-3(sy91)* strains and a 400bp XbaI fragment in non-*lin-3(sy91)* strains, which confirms its identity as

*syP1* flanking DNA. pRH1 was used to isolate the recombinant λ phage λPS#1 and λPS#2 from a λ2001 genomic library (Coulson et al. 1986).

**Identification of *syP2*.** The genomic clones identified by homology to *syP1* were used to search for additional *lin-3* RFLPs. Genomic DNAs were purified from different strains each containing one of the following ten *lin-3* alleles: *e1417* (Horvitz and Sulston 1980); *n378* , *n1058,* and *n1059* (Ferguson and Horvitz 1985); *s751* and *s1263* (Clark et al. 1988); *sy51*, *sy52*, and *sy53* (R.J.H. and P.W.S. unpublished); *sy91* (this study). The DNAs were digested separately with each of six enzymes (BamHI, ClaI, EcoRI, EcoRV, HindIII, XbaI), blotted and hybridized with labeled DNA from λPS#1. DNA from a *lin-3(n1058)* strain was found to possesses a novel EcoRV site at position +2.8 kb (data not shown). We refer to this RFLP as *syP2*. No other polymorphisms were detected.

**DNA mediated transformation and transgenic rescue of *lin-3*.** The ability of genomic clones to rescue the Vul phenotype of *lin-3* mutants and to cause a dominant Muv phenotype was tested by DNA mediated transformation (Mello, et al. 1991). This technique generates an extrachromosomal array containing approximately one hundred copies of the injected DNA. These arrays are heritable, but are lost at an appreciable frequency at both mitosis and meiosis. The presence of the transgene is made observable with a marker plasmid that confers an easily observed dominant phenotype on the transgenic animals. The marker plasmid and the experimental DNA are incorporated together into the transgene. A variety of marker systems were used. For most injections we used pRF4, which causes a dominant Rol phenotype (Mello, et al. 1991). In other experiments, we used pMH86 (Han and Sternberg 1990), which rescues *dpy-20* (D. Clark & D. Baillie, personal communication), or C14G10, which rescues *unc-31* (R.

Hoskins, personal communication). Each genomic clone was tested by injecting 8-20 animals. All of the progeny of each injected animal were examined for the presence of the transgenic marker and their *lin-3* phenotype. All F1 transgenic animals were allowed to self-fertilize to test whether it possessed a heritable transgene. Approximately 1 of 25 F1 transgenic animals possessed a heritable transgene and yielded a stable line. We did not identify any transgenes that were integrated into the chromosomes. We determined the rescuing ability of most genomic clones by the analysis of multiple stable lines, although all stable lines derived from a given clone were qualitatively similar in their ability to rescue *lin-3* and to cause a Muv phenotype, and differed only in their rate of transmission and penetrance. In the initial set of experiments different genomic clones were injected at a concentration of 50 ng/μl with pRF4 at 50 ng/μl into *lin-3(e1417) unc-22(e66)* / + + hermaphrodites. From these injections, Unc-22 Rol animals that should be homozygous for *lin-3(e1417)* and shold possess a transgene, were scored for their inability to lay eggs (Egl) and for the presence of ectopic pseudovulvae (Muv). Injection of λPS#1, λPS#2, pRH9, and pRH19 all yielded stable lines that could rescue *lin-3* and cause a dominant Muv phenotype. F1 transients and stable lines from pRH22 or pRH10 could not rescue *lin-3* or cause a dominant Muv phenotype. These experiments indicate that a 3.3 kb genomic region (pRH19) centered over the location of the *lin-3* RFLPs is sufficient to stimulate vulval induction. Transgenes possessing genomic DNA 5' of the BamH1 site at position 0 kb have the same vulval properties as pRH9 although they possess additional *lin-3* genomic sequence. In other experiments, pRH9 was injected at 50 ng/μl with the cosmid C14G10 at 20 ng/μl into *lin-3(+) unc-31(e169)* animals. This injection yielded the transgene *syEx13*, which has a strong Muv phenotype

and which can rescue the Egl and lethal defects of *lin-3(sy53).* *syEx13* was used in the ablation and epistasis experiments. The ability of pRH9 to stimulate vulval development is thus not dependent upon a particular transgenic marker. For the remainder of the experiments DNA was injected into *lin-3(n378)* animals that had been phenotypically suppressed for their Egl defect by passage through the dauer stage (Ferguson and Horvitz 1985). pRH9 was injected at 50 ng/μl with pRF4 at 50 ng/μl, pRH35 was injected at 75 ng/μl and pRF4 at 50 ng/μl, and pRH54 was injected at 42 ng/μl with pRF4 at 50 ng/μl and pBluescript $SK^+$ at 22.5 ng/μl. All transgenes recovered from these experiments could rescue *lin-3* and cause a strong Muv phenotype. pRH9 contains a 5kb genomic insert cloned into $pSK^+$ and pRH54 was made by subcloning the insert of pRH9 into pMobKS (Strathmann et al. 1991). These two clones both rescue *lin-3* but have different vector sequence adjoining the insert which indicates that adjoining vector sequence is not responsible for the ability of the 5kb genomic region to rescue *lin-3* All other plasmid clones are derived from $pSK^+$ except for pRH36 and pRH56. pRH35 and pRH54 were injected at the same molarity of *lin-3* DNA and the derived transgenes have similar effects on vulval development.

**Figure 2. Molecular map of the *lin-3* locus.**

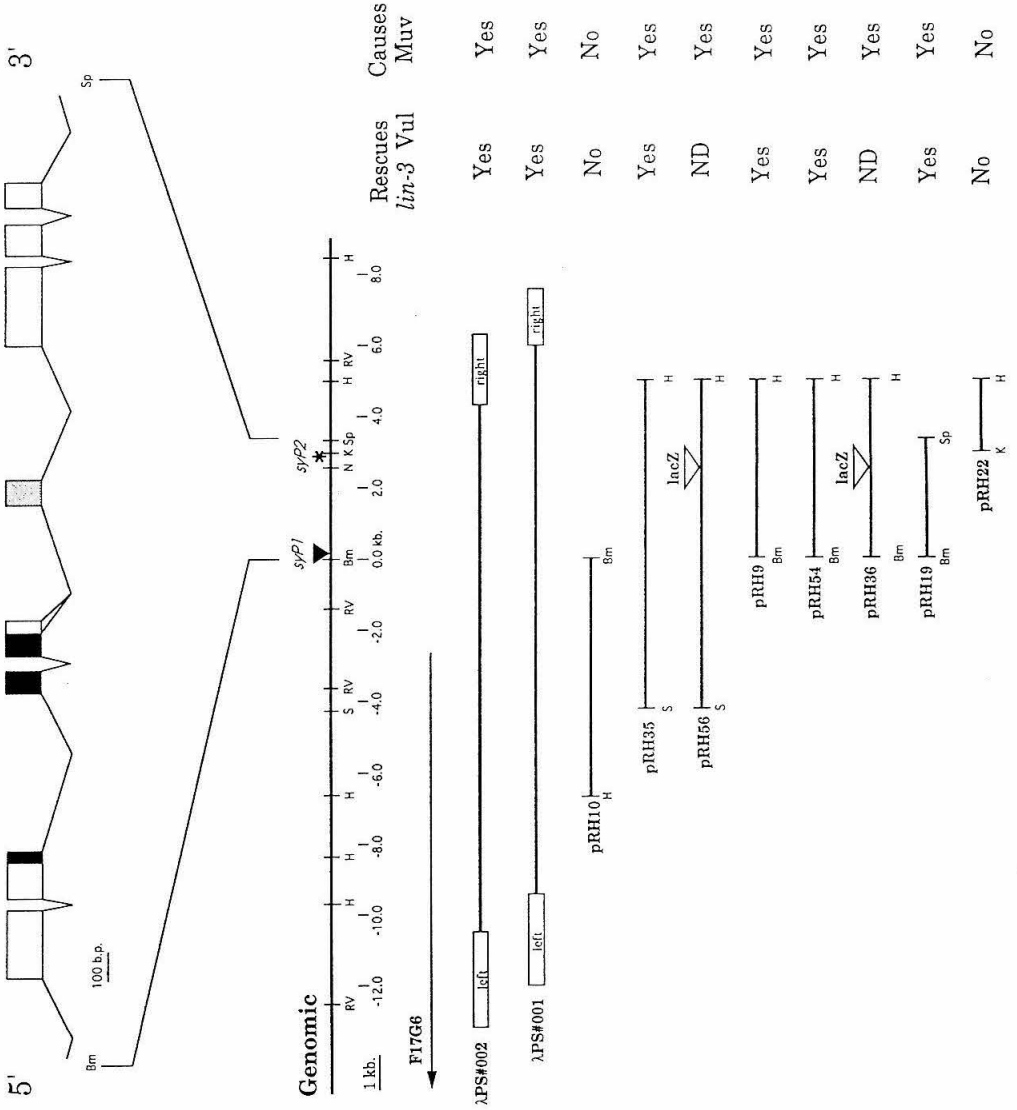


**Fig. 3. Nomarski photomicrographs of *lin-3* phenotypes.**

a. An adult, wild-type *C. elegans* hermaphrodite. The position of the vulva is marked by an arrowhead. The vulva is necessary for copulation with males and for egg laying. Anterior is left. The animal is photographed with a 20x Neofluor objective. The scale bar is equal to 100 μM.

b. An adult, vulvaless *lin-3(sy91::Tc1)* hermaphrodite. Mutations that lower *lin-3* activity, like *sy91*, result in decreased levels of vulval induction. The lack of a vulva prevents egg laying, but does not prevent internal self-fertilization of the eggs (Horvitz and Sulston 1980). The individual shown has no vulva (Vul) and is retaining embryos in the three-fold stage which indicates that it has an egg laying defect (Egl). Anterior is right. The animal is photographed with a 10x Neofluor objective and 1.25x optovar. The scale bar is equal to 100 μM.

c. A young adult, multivulva *syEx13* hermaphrodite. The transgene *syEx13* is an extrachromosomal array containing multiple copies of wild-type *lin-3* DNA. The transgene causes excess vulval induction, which we believe is due to overexpression of *lin-3*. Ectopic pseudovulvae are often visible as bumps on the ventral side of an adult worm when more than the wild-type number of VPCs assume vulval fates (Horvitz and Sulston 1980, Sulston and Horvitz 1981). The animal shown has three pseudovulvae which are marked by arrowheads and has a phenotype common for the transgenic *lin-3* Muv animals. No chromosomal mutations of *lin-3* that confer a Muv phenotype exist. Anterior is left. The animal is photographed with a 20x Neofluor objective. The scale bar is equal to 100 μM.

d. An adult, gonad-ablated, vulvaless *syEx13* animal. Ablation of the four gonad precursors Z1-Z4 prevents the development of the AC, which is the

source of the inductive signal. Ablation of the gonad precursors in *syEx13* animals lowers the ability of the transgenes to stimulate vulval induction such that most ablated animals are Vul like the one pictured. The ablation also destroys the germline. Anterior is left. The animal is photographed with a 10x Neofluor objective. The scale bar is equal to 100 μM.

Control mock-ablated *syEx13* animals had an average of 143% induction. Seven animals had wild-type induction of P[5-7].p, one animal each had P[4-7].p, P[4-7].p and P8.pa, or P[4-8].p forming vulval tissue, and five animals had six VPCs forming vulval tissue. Ablated *syEx13* animals had an average induction of 23%. 12 animals had no induced VPCs, in 2 animals P[3-5].p developed as vulval tissue, and in one animal P4.p, P5.pp, and P[6-8].p developed as vulval tissue.

**Methods.** Photographs were taken using an Axioplan microscope equipped with Nomarski objectives. Ventral is down in all photographs and anterior is left in a., c., and d., and right in b. The scale bar is equal to 100 μm. in all photographs. All animals except for Fig. 3c are anaesthetized on an agar pad containing 1.7 mM Sodium Azide. Ablation of the four gonad precursors Z1-Z4 of *syEx13* L1 hermaphrodites was performed as previously described (Avery and Horvitz 1987, Sternberg 1988, Sulston and White 1980). Animals were observed 12-24 hours after ablation to confirm that no VPCs had been destroyed by the surgery and to confirm the destruction of the gonad. The fate of all six VPCs was determined by direct observation of the anatomy at different timepoints throughout development. VPCs that went through at least two rounds of division or VPC daughters that went through at least one more round of division were scored as having a vulval fate. The extent of induction was defined as in (Aroian and Sternberg 1991) where three induced VPCs per animal is equal to 100% induction. Induction can

range from 0% when no VPCs are induced per animal to 200% when 6 VPCs are induced per animal. Since the *syEx13* transgene can be lost mitotically, only animals that were non-Unc-31 through the L4 stage and that thus presumably still possessed the transgene past the time of normal vulval development were included in the analysis. Any individual non-Unc-31 animal could lack the *syEx13* transgene in the appropriate tissue for vulval induction due to somatic loss of the transgene. The proportion of control, unablated, non-Unc-31 animals that have wild-type patterns of vulval development should represent a maximal estimate for the frequency of such chimeric animals.

The epistasis of *syEx13* and *let-23(sy97)* was tested by constructing the double mutant. Spontaneous *unc-31(e169); syEx13* (non-Unc) males were mated with *let-23(sy97) unc-4(e120) / mnC1 [dpy-10(e128) unc-52(e444)]; unc-31(e169)* hermaphrodites. non-Unc-31 L4 hermaphrodite cross progeny were selected as individuals. Unc-4 non-Unc-31 F2 survivors were picked from F1 mothers that segregated *let-23(sy97)* dead larvae. All such worms were Egl as adults, and segregated no Muv progeny. These worms segregated non-Unc-31 individuals and more than one fourth dead larvae which confirms their genotype as *sy97; syEx13*. Therefore *let-23(sy97)* is epistatic to *syEx13*. To quantify the epistasis, L4 non-Unc-31 hermaphrodites were picked as individuals and rescored as adults for their Muv and Egl phenotypes under a dissecting microscope. 89% of L4 non-Unc-31 progeny from an *unc-31(e169); syEx13* mother were Muv as adults (n= 100). 0% of L4 non-Unc-31 progeny from a *let-23(sy97) unc-4(e120); unc-31(e169); syEx13* mother were Muv as adults and only one was capable of laying eggs (n= 348). All *let-23(sy97)* animals are Egl (Aroian and Sternberg 1991). Routine genetic manipulations

and strains are as described in (Brenner 1974), except for *mnC1* (Herman 1978).

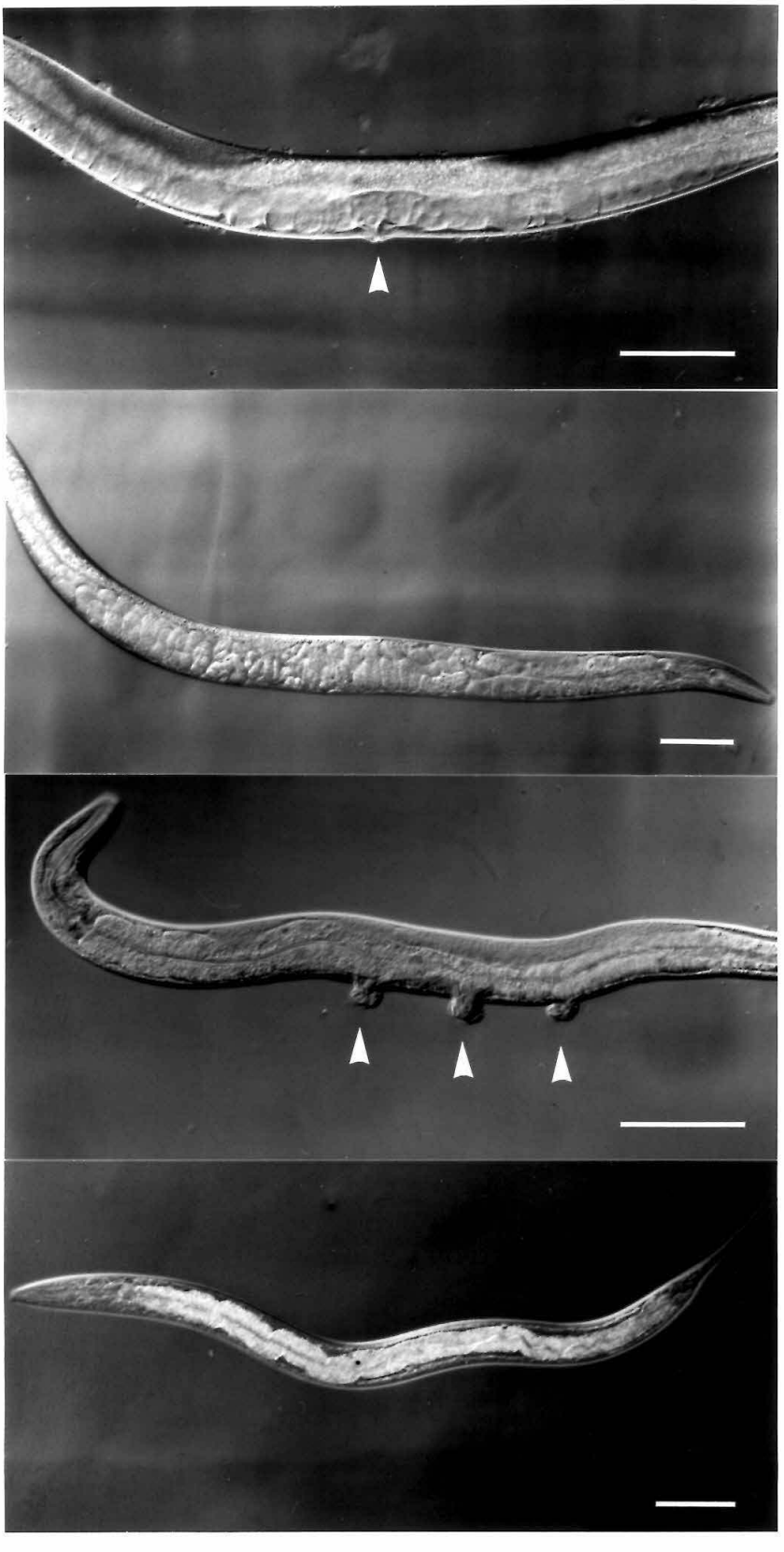


**Fig. 4. Sequence analysis of *lin-3*.** A composite sequence of *lin-3* cDNAs and the deduced *lin-3* protein sequence is shown with key features noted. The two main hydrophobic stretches of the *lin-3* protein are underlined. These two stretches appear to be a signal peptide and a transmembrane domain. The single EGF repeat is boxed and is described in further detail in Fig. 5. The extent of the repeat is chosen arbitrarily as it is not known if the EGF repeat of *lin-3* can be proteolytically processed away from the rest of the protein. Sequences similar to the sites at which EGF and TGF-α are processed (Carpenter and Wahl 1990) are overlined where present in the extracellular domain of *lin-3*. A stretch of 15 amino acids between the EGF repeat and the transmembrane domain which is encoded by an alternatively spliced region of RNA is underlined in bold. A potential N-glycosylation site is located at position 161, which is within the EGF repeat. The region of the *lin-3* protein after the signal peptide and before the EGF repeat is composed of aproximately 50% proline, glutamate, serine, and, threonine. Regions enriched in these amino acids, termed PEST domains, have been proposed to mark a protein for rapid turnover (Rogers et al. 1986). PEST domains have been noted in cytoplasmic and nuclear proteins and the significance of this feature in what would be the extracellular domain of *lin-3* is unclear. Compared to the rest of the *lin-3* protein, however, this region is only enriched in the amino acid glutamate. The presence of a region rich in the amino acids S, T, and P amino terminal to the EGF of the growth factor amphiregulin has been noted (Shoyab, et al. 1989) and may also be present in HB-EGF (Higashiyama, et al. 1991). *lin-3* differs from these two molecules in that this domain is much larger and richer in glutamate in *lin-3* and in that *lin-3* lacks a stretch of basic amino acids amino terminal to the EGF repeat as significant as that seen in amphiregulin and HB-EGF.

Translation is predicted to start at position 162 because of the location of the leader sequence and because of the presence of upstream in frame termination codons. cDNA sequence from position 217 to 1400 is present within the genomic sequence of pRH19 and thus cDNA sequence from +1 to +216 is located to the left of the BamH1 site at genomic position +1 in Figure 1. The signal peptide of the protein predicted above is encoded by this region of the cDNA. Therefore, the clones pRH9, pRH19, and pRH36 could not encode the protein predicted above, although these clones can provide *lin-3* function as transgenes. A possible explanation for the ability of these clones to function is that they include a genomic region called ExB, which if transcribed, could splice in frame to the cDNA and encode a possible signal peptide like sequence. ExB is located at position +196 to +248 of genomic DNA and has the sequence 5'-TAA ATG TTC GGT AAA TCG ATT CCT GAA CGA CTT CTA GTC GCA TTT GGT ATG TC-3'. A splice donor consensus at position 239 to 248 would splice nucleotide 241 of ExB to nucleotide 217 of the cDNA sequence (located at position +299 in genomic DNA). This splice would encode OchMetPheGlyLysSerIleProGluArgLeuLeuValAlaPheVal and translation would continue with Ser20 of the protein predicted above.

The nucleotide sequence is a composite of the cDNAs pRH39 and pRH40. pRH39 starts at position 117 and ends at position 1844. pRH39 contains the alternatively spliced region of 792 to 836 while pRH40 lacks this region such that nucleotide 791 is spliced next to nucleotide 837.

**Methods. Sequencing**. We determined genomic sequence from the BamH1 site at position 1 to position +3734, which is inclusive of the minimal region necessary for transgenic rescue (data not shown). pRH9 and derivative subclones were sequenced using the T3 and T7 primers specific to the vector and several gaps were filled by primer walking. Synthetic

oligonucleotides designed from the genomic sequence were used for the sequencing of cDNA clones. Sequencing of the cDNA clone pRH39 has been determined for both strands except for nucleotide 353 which is only determined for the sense strand. Only the unique regions of pRH40 have been sequenced. We found no discrepencies between the sequence of the cDNAs and the sequence of the genomic exons. Double stranded DNA sequencing reactions were performed on CsCl-banded plasmid DNA using Sequenase v2.0 and other reagents from U.S. Biochemical (Cleveland, Ohio) and standard protocols (Ausubel et al. 1992, Sambrook, et al. 1989).

**Sequence analysis.** Sequence was assembled using the MacVector software package of IBI (New Haven CT). Analysis was performed using MacVector and the software of the Genetics Computer Group v7.0 (Devereux et al. 1984). The signal peptide is predicted by a GCG program using the method of von Heijne (von Heijne 1986). Database searches of Genbank v70.0 were performed using the BLAST program (Altschul et al. 1990) and were run at the National Center for Biotechnology Information. The EGF repeat was first detected with the program MacPattern (Rainer Fuchs, EMBL data library, Postfach 10.2209, D-6900 Heidelberg, FRG) and is also detected by BLAST. The statistical significance of regions of sequence similarity found by database searches was assessed by RDF2 analysis (Pearson and Lipman 1988).

**Site-directed mutagenesis.** To confirm that the protein predicted above is responsible for the ability of the transgenes to rescue *lin-3*, point mutations were introduced into the transgenes using the method of Kunkel (Kunkel 1985) and reagents from Promega (Madison, Wisconsin). Mutations were introduced into the transgene pRH9 that would change the third and fifth cysteine residues of the EGF repeat to serine residues. This would block the

formation of two of the three predicted disulfide bonds of the EGF repeat. The introduction of these mutations into the transgene completely blocked the ability of the transgene to rescue the Egl phenotype of *lin-3* and the ability of the transgene to cause a Muv phenotype (below). This observation strongly suggests that the transgenes produce an EGF repeat protein that is providing the *lin-3* function. Oligo Cys3 (5' AC TTC CAC GTG GGA CGT CGC GTT GTG A 3') changes nucleotide 650 to G and nucleotide 652 to C, which introduces a novel AatII site, causes a silent change in Thr163, and changes Cys164 to Ser. Oligo Cys5 (5' TTC CCA ACC CTG CGG AGA ACT GCA AAA AG 3') changes nucleotide 709 to C and nucleotide 713 to G, which destroys a BstXI site, causes a silent change in Pro185 and changes Cys184 to Ser. The plasmid pRH43 contains the 5 kb *lin-3* region with both sets of mutations. Transgenes were created as described in Fig. 2 by microinjecting a *lin-3* plasmid at 50 ng/μl and pRF4 at 50 ng/μl into *lin-3(n378)*. 20% of *n378* animals bearing the transgene derived from pRH9 were Egl and 72% were Muv (n= 50) while 99% of *n378* animals bearing the transgene derived from pRH43 were Egl and 0% were Muv (n= 76). 97% of *lin-3(n378)* hermaphrodites are Egl without any transgenes (Ferguson and Horvitz 1985).

**Figure 4. cDNA and predicted protein sequence of *lin-3*.**

```
                                                                  AAGTTTG

  8  AGATTGTACATAGCCATCGGCACCACATCGCCACTCAACGGGTATTCAATGCTGAAAAT

 67  TTAATAACGGATCGTCGGCTTGTCGGTGTTCTTCCCACCCCTTATGCATCAATTTTTCC

126  TGTTTCATAAGATATCGTCGCCCACAAGTTTCCAAAATG CGG AAA ATG CTA CTT
                                          Met Arg Lys Met Leu Leu

180  TTT TGC ATC CTT CTA CTC TTT ATG CCT CAA TTT ACA GTT TCA GAA
  7  Phe Cys Ile Leu Leu Leu Phe Met Pro Gln Phe Thr Val Ser Glu

225  TCG TGT CTC CCT TCG TGG TTT CGT CAA GAA CGT AGT GCT CCC GAA
 22  Ser Cys Leu Pro Ser Trp Phe Arg Gln Glu Arg Ser Ala Pro Glu

270  CAG CTT CAA TCT GCA GAG AAT GCA GCT GAA AAT AGT GGC TCT GTA
 37  Gln Leu Gln Ser Ala Glu Asn Ala Ala Glu Asn Ser Gly Ser Val

315  CCA CCC GAT ACT TCT CGA AAT TCT CTA GAA ACA AAC GAA ATA GGT
 52  Pro Pro Asp Thr Ser Arg Asn Ser Leu Glu Thr Asn Glu Ile Gly

360  GAT GCA CCG TCG TCG ACT TCG ACA CCT GAA ACA CCT ACT GAA ACT
 67  Asp Ala Pro Ser Ser Thr Ser Thr Pro Glu Thr Pro Thr Glu Thr

405  ACG ATT TCC GAA GCT GGA GAC GAT GAA AAA CGA ACT GAA GAG GTT
 82  Thr Ile Ser Glu Ala Gly Asp Asp Glu Lys Arg Thr Glu Glu Val

450  GCA AAA GAA TTA ATC GAG AAA GAA GCA GAA TAT GAG GGT GAA TAT
 97  Ala Lys Glu Leu Ile Glu Lys Glu Ala Glu Tyr Glu Gly Glu Tyr

495  GAA GAT GAA AAG GTT GAT GAA GAA GTA GAA GAA GCG TTA AAA TAT
112  Glu Asp Glu Lys Val Asp Glu Glu Val Glu Glu Ala Leu Lys Tyr

540  AAT GAA GAT GCC ACT CAA GAT GCC ACG TCG ACT CTT AAA CCG GCG
127  Asn Glu Asp Ala Thr Gln Asp Ala Thr Ser Thr Leu Lys Pro Ala

585  GTT CGG AAG GAA ATC GAG AAG TTG AAA GAA GCA AAA TGC AAA GAC
142  Val Arg Lys Glu Ile Glu Lys Leu Lys Glu Ala Lys Cys Lys Asp

630  TAC TGT CAT CAC AAC GCG ACA TGC CAC GTG GAA GTG ATA TTC CGT
157  Tyr Cys His His Asn Ala Thr Cys His Val Glu Val Ile Phe Arg

675  GAA GAT AGA GTT TCA GCA GTT GTT CCT TCT TGC CAT TGT CCA CAG
172  Glu Asp Arg Val Ser Ala Val Val Pro Ser Cys His Cys Pro Gln

720  GGT TGG GAA GGC ACT CGT TGT GAT CGT CAC TAC GTT CAG GCG TTC
187  Gly Trp Glu Gly Thr Arg Cys Asp Arg His Tyr Val Gln Ala Phe

765  TAT GCC CCA ATC AAC GGC AGA TAT AAT GTA CGT TTG AGC ACG ATG
202  Tyr Ala Pro Ile Asn Gly Arg Tyr Asn Val Arg Leu Ser Thr Met

810  AGC AGC ACG GCG CAA CTC CTC GTT CAA CAA TCT TCA ACA TCA GCT
217  Ser Ser Thr Ala Gln Leu Leu Val Gln Gln Ser Ser Thr Ser Ala
```

```
 855  ATT CCT GCG TTC GCA TTT CTC ATT GTC ATG CTC ATC ATG TTT ATA
 232  Ile Pro Ala Phe Ala Phe Leu Ile Val Met Leu Ile Met Phe Ile

 900  ACA ATT GTT GTT TAT GCT TAT AGA AGA ATG TCT AAA CGA TCG GAT
 247  Thr Ile Val Val Tyr Ala Tyr Arg Arg Met Ser Lys Arg Ser Asp

 945  GAT ATG ACA TAT ACA ATG AGT CAT ATG TGC CCA CCA GAA GCA TTC
 262  Asp Met Thr Tyr Thr Met Ser His Met Cys Pro Pro Glu Ala Phe

 990  AAT GTC CTC AAA ACA CCA AAT GGA CGA CAT ATT CCA GTT CAT CAA
 277  Asn Val Leu Lys Thr Pro Asn Gly Arg His Ile Pro Val His Gln

1035  ATT CCA TCA TGT TCT TAT ACT ATC CCA ACA CCG GGT ACA GTA CCT
 292  Ile Pro Ser Cys Ser Tyr Thr Ile Pro Thr Pro Gly Thr Val Pro

1080  CCA AAT ATA TCA TCA ACT CCT GGA TCA AGA ATA CCC ACT CGT CAA
 307  Pro Asn Ile Ser Ser Thr Pro Gly Ser Arg Ile Pro Thr Arg Gln

1125  CAA GCT ATT CGA AAT AAT GAA CAA GCA CGG AAC AAC TTT TTC AGC
 322  Gln Ala Ile Arg Asn Asn Glu Gln Ala Arg Asn Asn Phe Phe Ser

1170  ATT CTC AGA AGT CAA GGT ACC ATT CCA TCC AGG AGT ATC AAT GAC
 337  Ile Leu Arg Ser Gln Gly Thr Ile Pro Ser Arg Ser Ile Asn Asp

1215  GAC GAC ACG CCG AAG CAC TAC AAG TCA GTG CCA CGT GTT GAA GTT
 352  Asp Asp Thr Pro Lys His Tyr Lys Ser Val Pro Arg Val Glu Val

1260  TCA GCA ATT AAT TAC TCT GGC CAC ATT GAT TTT TCA ACA GTA TCA
 367  Ser Ala Ile Asn Tyr Ser Gly His Ile Asp Phe Ser Thr Val Ser

1305  TAT CAG TCG ACT GAA TCA GAA GTT TCA AAA GCA TCA GTA ACA TGT
 382  Tyr Gln Ser Thr Glu Ser Glu Val Ser Lys Ala Ser Val Thr Cys

1350  CCA CCA CCG GCG CAC ACT GTA ATT AAT ATC GAG TTG GAT TCT GCA
 397  Pro Pro Pro Ala His Thr Val Ile Asn Ile Glu Leu Asp Ser Ala

1395  GAT ACG AAT TTT CGA TCC CCG TCT CGA AGT TCT GGA GAA CAA GGA
 412  Asp Thr Asn Phe Arg Ser Pro Ser Arg Ser Ser Gly Glu Gln Gly

1440  TCA CCA GCA ACA TGT GAA CCA ATG ATT CGA CAC ACA TGAAATTATAA
      Ser Pro Ala Thr Cys Glu Pro Met Ile Arg His Thr End

1487  TTTGTTTCCTTATTTTGTCTTATCTTTTTCTTTTTTCATCAACTTCTCTGTGCTATAAT

1546  TGTGATTTACCACTGCTCAAACATCTCTGGTCTCTTCAAATTTTTTCGCTTTCTCTTTT

1605  TTCCTCTATTTTCATATCGTTCTACATGTTTTATAGATTGCATTTGGCACCCTATTATA

1664  TCCCACCCAAATGTTCTATAGTTCTCTCACTCTCCCTCTCAGTTCCTGTGACAATTTGT

1723  ATAATTTTGCCAGTTCAACTCCTCGATTTCCAACGGGTACAATATTTGCATCGTTTTTG

1782  TAATTTCCTTTGATCCCTAGTCTCTTCAGGTCCTCGTTGAAAAAACTAAACATTTATTT

1841  CATGTTTTTATTAATGGAAAATTGAGAAAAATCATATAGAAAAAATATATATTTAGATA

1900  GTCAATAAAATCCAGGAAGTT
```

**Fig. 5. Comparison of the EGF repeat of *lin-3* to ligand and non-ligand EGF repeats.**

The EGF repeat of *lin-3* is aligned with EGF repeats known or believed to be used as soluble ligands of growth factor receptors. *lin-3* matches the consensus sequence C-X-C-X5-G-X-R-C of EGF repeats. *lin-3* shows conservation or conservative substitutions of many of the residues conserved in EGF repeats including: tyrosine 13, glycine 39, arginine 41, alanine for glycine 18, tryptophan for tyrosine 37, and valine for leucine 47. The residues Tyr13 and Arg41 are conserved preferentially among the EGF repeats that are growth factors. The spacings between the last three cysteines is conserved between *lin-3* and the ligand EGF repeats. The spacing between the first and 2nd, and the 3rd and 4th cysteines of *lin-3* is unique. *lin-3* lacks the consensus for $Ca^{++}$ binding by EGF repeats which is Asp/Asn2, Asp/Asn4, Gln/Glu5, Asp/Asn22, Tyr/Phe29 and Glu38 (Handford et al. 1991, Rees et al. 1988). None of the EGF repeats used as growth factors contain this consensus. EGF, TGF-α, HB-EGF, SDGF, Amphiregulin, and VVGF are all soluble factors derived from precursors with putative transmembrane domains, and which inhibit the binding of EGF to the EGF receptor. SVGF and MVGF are viral genes with a structure similar to VVGF. An analysis with the program RDF2 (Pearson and Lipman 1988) indicates there is statistically significant sequence similarity between the EGF repeat of *lin-3* and some of the EGF repeats of other proteins. This and other statistical analyses do not clearly indicate that the EGF repeat of *lin-3* is most similar to the EGF repeats of a particular functional class of molecule. The results of the RDF2 analysis, using 500 shuffles, a ktup of 1, and uniform shuffling are tabulated with the repeats: *z* is the ( optimal score - the mean of the optimal alignment of shuffled sequences) / standard deviation of the

optimal alignment of shuffled sequences; R is the ratio of the optimal alignment to the best optimal alignment of a shuffled sequence. Suggested minimal values for significance (Pearson and Lipman 1988) are z = 3 and R = 1. Some EGF repeats found in non-growth factors have good matches to the EGF repeat of *lin-3* including residues 426-465 of *crumbs* ($z$ = 4.6 R = 62/60) (Tepass et al. 1990) and residues 406-447 of Lin-12 ($z$ = 6.3. R = 73/63) (Yochem et al. 1988). References and abbreviations: amino acid sequence of hEGF (human Epidermal Growth Factor) (Gregory 1975), amino acid sequence of mEGF (murine EGF) (Savage, et al. 1972), deduced amino acid sequence of hTGF-α, (human TGF-α) (Derynck, et al. 1984), direct amino acid sequence of HB-EGF, (Heparin-Binding EGF) (Higashiyama, et al. 1991); amino acid sequence of ampi, (human amphiregulin) (Shoyab, et al. 1989); deduced amino acid sequence of VVGF (Vaccinia Virus Growth Factor) (Venkatesan et al. 1982); deduced amino acid sequence of SFGF (Shope Fibroma virus Growth Factor) (Chang, et al. 1987); deduced amino acid sequence of MVGF (Myxoma Virus Growth Factor) (Upton, et al. 1987). Amino acid residues that are known to be the amino terminal or carboxyl terminal of processed factors are underlined. If the amino terminal residue is not underlined, then its position within the deduced amino acid sequence of the precursor is given. The EGF repeats are aligned manually. For purposes of comparison, the numbering system of processed mEGF is used.

## Fig 5. Comparison of the EGF repeat of *lin-3* to other EGF repeats.

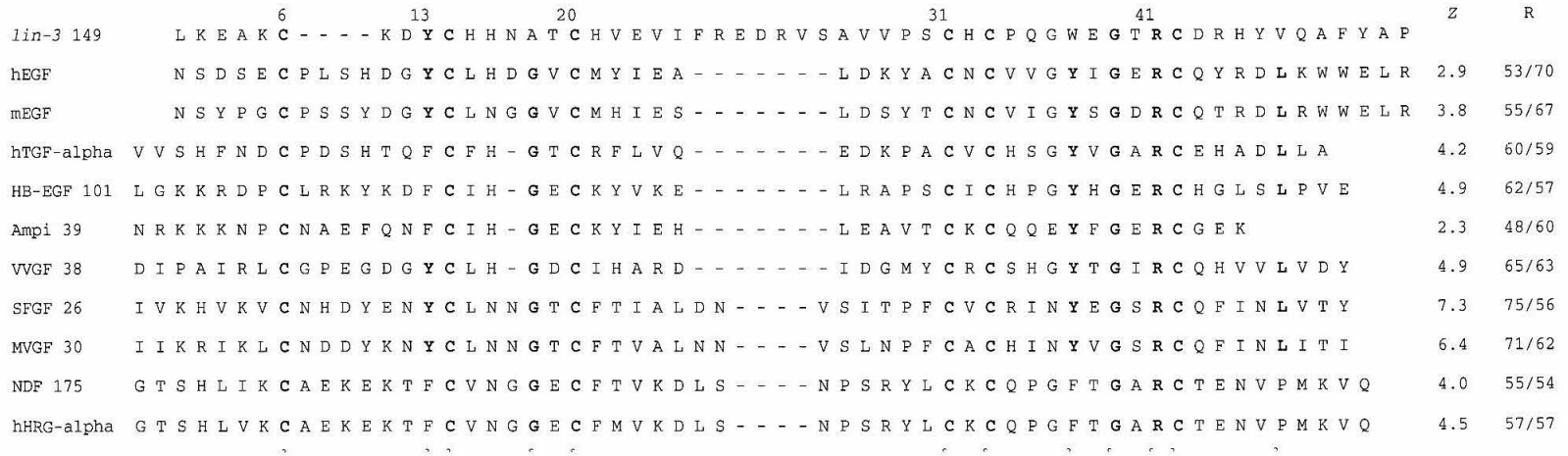


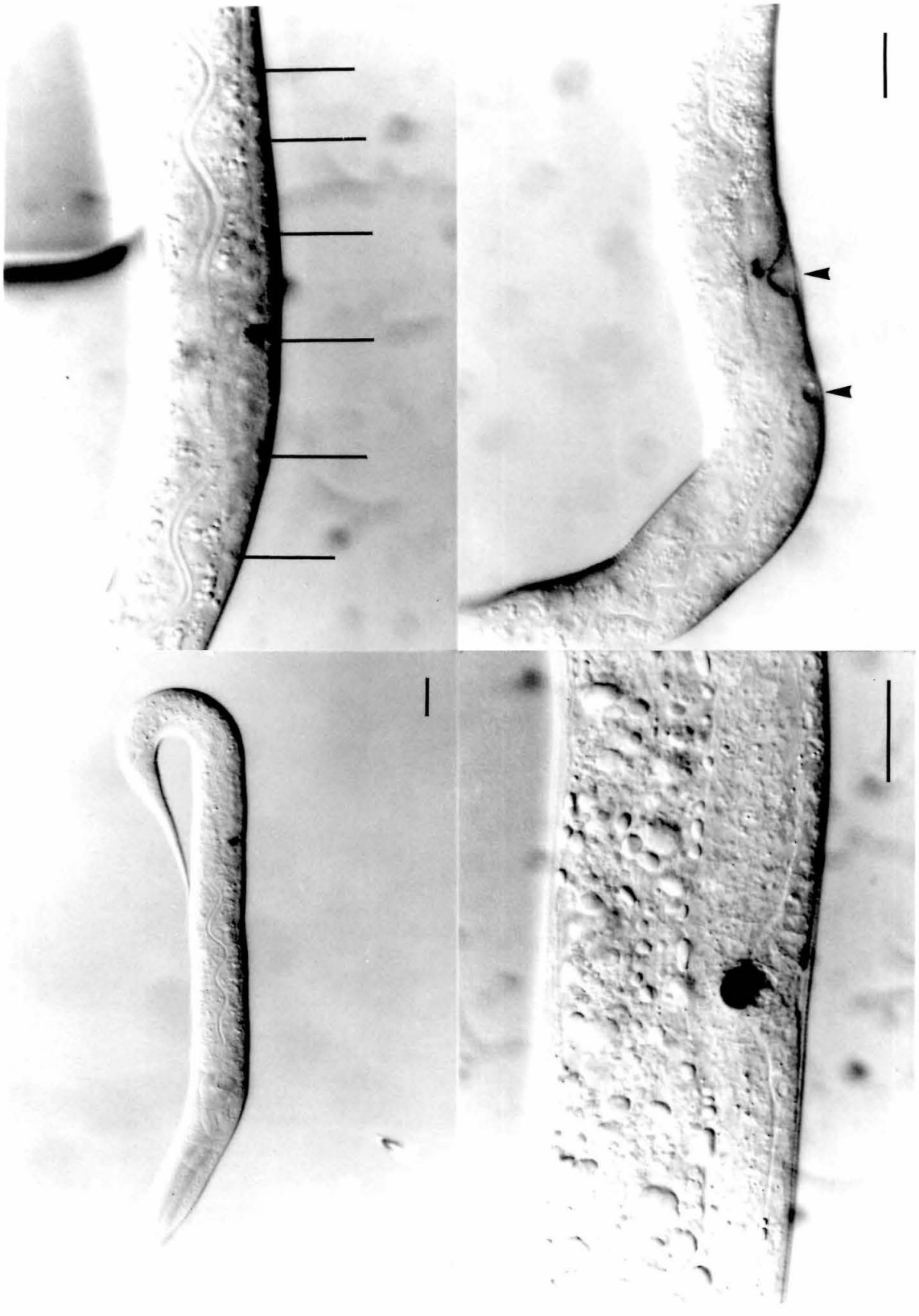
**Fig. 6. Expression patterns of *lin-3::lacZ* transgenes.**

Nomarski photomicrographs of animals carrying a *lin-3::lacZ* pRH36 transgene that have stained for β-galactosidase (blue color). **A.** An L2 lethargus animal showing specific expression of β-galactosidase in the anchor cell. The old cuticle is apparent near the head and tail thus identifying this worm as a late molt. 20 minute glutaraldehyde fixation. Anterior is left. 40x Neofluor objective. The scale bar is equal to 20 μM. **B.** A L2 molt animal with all six VPCs in focus and expression of β-galactosidase in the AC. This expression of β-galactosidase is detected prior to any divisions of the VPCs and is before or during the time of vulval induction. The six VPCs (P[3-8].p) are marked by vertical lines. 20 minute glutaraldehyde fixation. Anterior is right. 100x Neofluor objective. The scale is same as Fig6.c. **C.** β-galactosidase activity is still detected after VPC fates have been determined. In this animal the VPCs are in the third and final round of division. The AC is closely associated with the vulva at this stage and has a distinctive morphology and can be unambigiously identified. The animal is fixed and permeabilized by standard methods (Fire et al. 1990). Anterior is right. 100x Neofluor objective. The scale bar is equal to 20 μM. **D.** A Muv L4 hermaphrodite with staining of the AC. All vulval divisions have been completed and vulval morphogenesis has started. The presence of two invaginations (marked by arrowheads) indicates that extra vulval induction has occured. 30 minute glutaraldehyde fixation. Anterior is left. 40x Neofluor objective and 1.6x optovar. The scale bar is equal to 20 μM.

**Methods.** All animals were photographed on an Axioplan microscope equipped with Nomarski optics on Ektar 25 film. Ventral is down in all photographs. Transgenic animals were made using either pRH36, a *lacZ*

cognate of pRH9, or pRH56, a *lacZ* cognate of pRH35. Both transgenes contain the same *lin-3::lacZ* fusion and differ in the amount of upstream genomic DNA present (diagrammed in Fig. 2). *lacZ* is inserted as a translational fusion into the first cytoplasmic exon of *lin-3*. The *lacZ* cassette is from the vector pPD16.51(Fire, et al. 1990) and contains a nuclear localization signal, the coding region of *lacZ*, and a region containing 3' untranslated sequence from the *unc-54* gene. The fusion transgenes would make a protein containing the extracellular domain, including the EGF repeat, the transmembrane domain, and the first 12 amino acids of the cytoplasmic domain of *lin-3*. The protein would continue with an arginine encoded at the junction, protein encoded by the synthetic linker sequence of pPD16.51 including the nuclear localization signal, followed by β-galactosidase. Translation of the fusion transgene should stop within the *lacZ* cassette and polyadenylation of the transcript should occur within the *unc-54* region. The fusion deletes no *lin-3* DNA present in the orginal transgenes so that internal or 3' enhancer-like sequences, if they exist, would still be present in the *lacZ* fusion. pRH36 was made as follows. The 5.0 kb *lin-3* genomic BamHI-HindIII insert of pRH9 was subcloned into pMob KS (a pBluescript derivative lacking the *lacZ* and F1 sequence (Strathmann, et al. 1991)) to create pRH54. pRH54 was digested with NdeI, which cuts at a unique site within the first cytoplasmic exon of *lin-3,* and endfilled. This fragment was ligated to an endfilled 4.5 kb Sma1Spe1 fragment of pPD16.51 to create pRH36. pRH56 was constructed as follows. An upstream genomic fragment from SacI(-4.2 kb) to BamHI(0.0 kb) was subcloned as pRH55 into pMob KS. The *lin-3::lacZ* fusion of pRH36 was excised intact as a 9.5 kb BamHI HindIII fragment and subcloned into a HindIII BamHI fragment of pRH55 to create pRH56.

Transgenic animals bearing the short (pRH36) construct were generated by injection of pRH36 at 65 ng/μl, pSK+ at 25 ng/μl and pMH86 at 10 ng/μl into *dpy-20(e1282)* using the method described in Fig. 2. Transgenic animals bearing the long (pRH56) construct were generated by injection of pRH56 at 90 ng/μl and pMH86 at 10 ng/μl into *dpy-20*. Worms were permeabilized and fixed using either a lyophilization protocol (Fire, et al. 1990), which gave stronger staining but poorer tissue preservation, or a novel glutaraldehyde protocol suggested by Lynn Carta, which gave weaker staining but better tissue preservation. To fix worms with glutaraldehyde, the worms were washed twice in M9 and were then resuspended in a pre-warmed solution of 2-3.5% EM grade glutaraldehyde (8% solution from Polysciences Inc. Warrington, PA), 100 mM sodium phosphate buffer (pH7.2), and 4 mM MgCl2, and incubated for 15-30 minutes at 40°C followed by two washes in M9. Longer fixation times left all worms impermeable and shorter fixation times would be necessary to achieve staining of L1s. Worms were stained with the regular staining solution of Fire (Fire, et al. 1990) for 1/2 to 48 hour at room temperature for lyophilized worms or 40°C for glutaradehyde-fixed worms. Staining of the anchor cell was readily evident under a dissecting microscope within 30 minutes.



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# Chapter 3

## Lin-3 is sufficient to induce vulval development

Prepared as a submission to Nature

Note: Wendy Katz and I collaborated on this work. I constructed the hsp::Lin-3EGF vectors, generated the transgenic strains, and performed the sufficiency experiments in Table 1. Wendy Katz performed the VPC isolation experiments in Table 2.

# The EGF Domain of Lin-3 is Sufficient to Induce Vulval Differentiation in *C. elegans*.

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**During the development of the *C. elegans* hermaphrodite, the anchor cell of the somatic gonad induces the three more proximal of six tripotent vulval precursor cells to assume the 1° and 2° vulval fates (Kimble 1981). The *lin-3* gene encodes a putative nematode member of the Epidermal Growth Factor (EGF) family that genetic evidence suggests is the vulval-inducing signal made by the anchor cell (AC) (Hill and Sternberg 1992). The EGF-like growth factors can act as secreted factors that consists of a single EGF domain. We show that a transgene that ectopically expresses an obligate secreted version of the EGF domain of the Lin-3 protein can induce vulval differentiation, which suggests that Lin-3 can act as a secreted factor in a manner analogous to the EGF growth factors. The EGF domain of Lin-3 can induce a single VPC, isolated by the ablation of the gonad and the other VPCs, to assume either the 1° or the 2° fate. Thus, Lin-3 may be the sole vulval-inducing signal made by the anchor cell.**

The vulva of the nematode *Caenorhabditis elegans* develops post-embryonically from ectodermal blast cells (P[3-8].p) called Vulval Precursor Cells (VPCs) that are located in the ventral epidermis (Sulston and Horvitz 1977). Each of the VPCs is equivalent in its ability to assume either of three developmental fates (Sternberg and Horvitz 1986, Sulston and White 1980). The 1° and 2° fates both produce vulval tissue, although they differ in the types of vulval cells produced. The 3° fate produces non-vulval epidermis. In wild-type development, P6.p, which is the VPC closest to the AC, assumes the 1° fate, P5.p and P7.p, which are the two lateral neighbors of P6.p, assume the 2° fate, and P3.p, P4.p, and P8.p, which are more distal to the AC, assume the 3° fate. The fate choice of a VPC is regulated by three intercellular

signals (reviewed in (Horvitz and Sternberg 1991)). The AC of the somatic gonad is necessary and sufficient to induce the three more proximal VPCs to assume vulval fates: if the AC or its precursors are ablated sufficiently early in development, then all six of the VPCs assume the 3° fate (Kimble 1981). The vulval-inducing signal made by the AC is proposed to be a secreted factor because induction occurs without apparent direct cell contact between the AC and the VPCs in wild type (Sulston and White 1980). Vulval induction also occurs in *dig-1* mutant animals in which the AC is displaced to the dorsal side of the body away from the VPCs (Thomas et al. 1990). A lateral signal among the VPCs regulates the pattern of fates assumed by the VPCs induced by the inductive signal (Sternberg 1988). The lateral signal is likely mediated by the gene *lin-12* which promotes the 2° fate (Greenwald et al. 1983). An inhibitory signal (Herman and Hedgecock 1990) mediated by a pathway of genes that includes *lin-15* (Ferguson and Horvitz 1985, Ferguson and Horvitz 1989) inhibits the VPCs from assuming vulval fates in the absence of the inductive signal.

Vulval induction is mediated by an Epidermal Growth Factor (EGF) signalling pathway. This pathway includes the gene *let-23* that is required for vulval induction (Aroian and Sternberg 1991, Ferguson and Horvitz 1985) and that encodes a nematode homologue of the EGF-Receptor (Aroian et al. 1990). *let-23* is proposed to act in the VPCs and to encode the receptor for the inductive signal. A number of genes that encode homologues of signal transduction molecules and that act genetically downstream of *let-23* have been characterized, including: *sem-5*, which encodes an adapter protein containing 2 SH3 domains and a SH2 domain (Clark et al. 1992); *let-60*, which encodes a *ras* protein (Han and Sternberg 1990), and *lin-45*, which encodes a raf serine-threonine kinase (Han et al. 1993). The gene *lin-3* is

proposed to encode the vulval inducing signal made by the anchor cell (Hill and Sternberg 1992). Wild-type activity of *lin-3* is required for induction of vulval fates (Ferguson and Horvitz 1985). *lin-3* encodes a membrane-spanning protein with a single EGF domain that is a putative nematode member of the EGF family of growth factors (Hill and Sternberg 1992). *lin-3* acts genetically upstream of *let-23*, consistent with the model that *lin-3* encodes a ligand for the receptor encoded by *let-23*. In contrast to the phenotype of reduction-of-function *lin-3* alleles, *lin-3* transgenes that contain multiple copies of wild-type *lin-3* genomic DNA clones can cause greater than the normal number of VPCs to assume vulval fates. The transgenes apparently act in the AC to induce the VPCs to assume vulval fates since ablation of the gonad, which prevents the development of the AC, strongly reduces the ability of the transgenes to induce vulva development. Indeed, *lin-3::lacZ* fusion transgenes that retain the ability to induce vulval development express β-galactosidase specifically in the AC at the time of vulval induction.

The EGF family of growth factors is a large family of intercellular signalling molecules that is mitogenic for many cell types and that induces diverse physiological effects on many other cell types (reviewed in (Carpenter and Wahl 1990)). This family includes EGF (Gregory 1975, Savage et al. 1972), Transforming Growth Factor-alpha (TGF-α) (Derynck et al. 1984), amphiregulin (Shoyab et al. 1989), Heparin-Binding EGF (HB-EGF) (Higashiyama et al. 1991), Neu Differentiation Factor (Wen et al. 1992)/ heregulin (Holmes et al. 1992), and the predicted protein product of the *spitz* locus of *Drosophila* (Rutledge et al. 1992). These proteins are structurally diverse, but are usually made as membrane spanning proteins, and have in common the presence of at least one EGF domain. EGF domains are a

sequence motif found in a wide variety of proteins that consists of six cysteine residues with semi-conserved spacing. In some cases, the cysteine residues are known to form a particular pattern of disulfide bonding. The EGF domain is the portion of the growth factor that binds and activates the receptor. In many cases the EGF domain is released from the membrane by proteolytic processing to produce a secreted factor. For example, the EGF domain of TGF-α is processed and released as a 50 amino acid factor. These factors may not always act as secreted factors, since membrane-bound TGF-α can activate the EGF-R (Brachmann et al. 1989, Wong et al. 1989).

Based on the prediction that the vulval-inducing signal is a secreted factor, and the similarity of Lin-3 to the EGF growth factors, we decided to test whether the EGF domain of Lin-3 would be sufficient to induce vulval differentiation. Transgenes were produced that express the 63 amino acid EGF domain of Lin-3 under the control of an inducible, and tissue-general heat shock promoter (Fig. 1). The transgenes also include a signal peptide sequence to help ensure that the EGF domain of Lin-3 is secreted. These transgenes are known as the Lin-3EGF sense transgenes. Another set of transgenes called the Lin-3EGF antisense transgenes contain the same DNA sequences, although the DNA encoding the EGF domain of Lin-3 is present in the antisense orientation with respect to the translation initiation site. The Lin-3EGF antisense transgenes should produce no functional Lin-3 protein and thus serve as a control for whether there are any affects on vulval development caused by the heat shock procedure that are not due to the EGF domain of Lin-3.

The Lin-3EGF sense transgenes are able to induce vulval differentiation whereas the Lin-3EGF antisense transgenes are not able to do so (Table 1). In this experiment the transgenes are present in animals that

are wildtype at the chromosomal *lin-3* locus. Animals bearing the Lin-3EGF sense transgenes have 192% of the wild-type level of vulval differentiation upon mock-ablation of the gonad and heat shock at the time of vulval induction (Fig 2C). Specifically, in 9 animals all six VPCs assumed vulval fates, and in three animals five of six VPCs assumed vulval fates. In these animals the inductive signal produced endogenously by the AC is sufficient to induce P5.p, P6.p, and P7.p. The induction of P3.p, P4.p and P8.p in these animals, however, must be due to the presence of the Lin-3EGF sense transgene. Ablation of the gonadal precursor cells Z[1-4] at hatching prevents the development of the AC and in wildtype results in all six VPCs assuming the 3° non-vulval fate. Vulval differentiation, however, can occur independently of the AC in animals bearing the Lin-3EGF sense transgenes. In 15 gonad-ablated, heat-shocked animals bearing the Lin-3EGF sense transgenes, the level of vulval differentiation was 198% of the wild-type level (Fig. 2D). Animals bearing the Lin-3EGF antisense transgenes behave in a manner equivalent to the behavior of wildtype. In 11 mock-ablated, heat-shocked animals bearing the Lin-3EGF antisense transgenes, P5.p, P6.p, and P7.p assume a normal pattern of vulval fates in response to the inductive signal produced by the AC, and the three other VPCs assumed 3° fates (Fig. 2A). This indicates that neither the antisense transgene nor the heat shock conditions interfere with vulval induction. In 12 gonad-ablated, heat-shocked animals bearing the Lin-3EGF antisense transgenes, no VPCs assumed vulval fates (Fig 2B). This indicates that the ablation protocol is sufficient to remove all of the endogenous inductive signal, and that the Lin-3EGF antisense transgenes have no ability to induce vulval differentiation. Because only the Lin-3EGF sense transgene is able to induce vulval development, we believe that it is the production of Lin-3 protein by the

transgenes that is responsible for their ability to induce vulval development. These results indicate that the 63 amino acid EGF domain of Lin-3 is sufficient to induce vulval differentiation in the absence of the normal source of the vulval-inducing signal.

To determine the fate choice of a single VPC in response to the EGF domain of Lin-3, and in the absence of lateral signalling between different VPCs, single VPCs were isolated by the ablation of the gonadal precursor cells and the 5 other VPCs. In response to activation of the Lin-3EGF sense transgenes by a mild stress stimulus, isolated VPCs assumed the 3° fate in 9 animals, the 2° fate in 3 animals, the 1° fate in 2 animals, and intermediate fates with character of both 1° and 2° fates in 4 animals (Table 2). It was previously known that the anchor cell could induce an isolated VPC to assume either the 1° or the 2° fate (Sternberg and Horvitz 1986), and thus that lateral signals between different VPCs were not essential to get either vulval fate. Our results confirm this observation and indicate that the EGF domain of Lin-3 is sufficient to induce both vulval fates. This indicates that separate signals from the anchor cell are not required to get the different vulval fates and thus, that *lin-3* may be the sole vulval-inducing signal made by the anchor cell.

We have made a transgene that should produce an obligate secreted form of the EGF domain of Lin-3, which can stimulate vulval differentiation. This result suggests that *lin-3*, like the EGF growth factors, can produce a secreted factor that consists of an EGF domain. It has been predicted that the vulval-inducing signal made by the AC is a secreted factor, and our results support this prediction. However, it is possible that the transgene does not act by producing a secreted product. For example, the transgene could produce Lin-3 protein in the VPCs that associates with Let-23

intracellularly. It has not yet been determined whether the Lin-3 protein produced endogenously by the AC is processed to release a secreted factor. Expression of the EGF domain of Lin-3 can induce vulval fates in the absence of the gonad. It remains possible that there are other factors or accessory proteins necessary to induce the VPCs to assume vulval fates, although these factors could not be made uniquely by the gonad. It is also possible that there are other factors made by the gonad that are normally necessary for vulval induction but that they are dispensable for vulval induction if Lin-3 is overexpressed. The EGF domain of Lin-3 can induce a single VPC, isolated by the cell ablation of the gonad and the other VPCs to assume either the 1° or the 2° fate. Thus both the 1° and the 2° fate can be specified by Lin-3 without the action of another signal from the gonad or a second VPC. These results suggest that Lin-3 may be the sole vulval-inducing signal made by the anchor cell.

**Tables and Figures.**

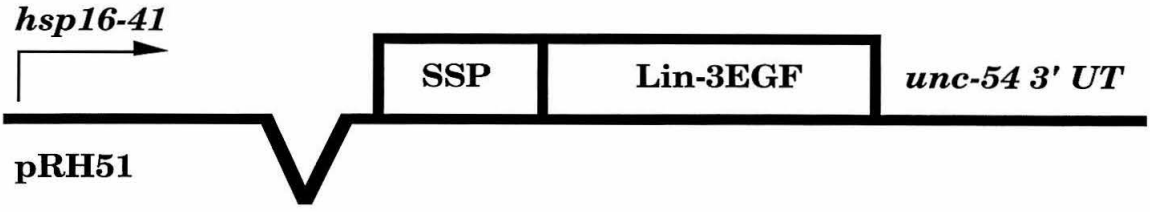
Figure 1. Inducible Lin-3EGF transgenes. The vector pRH51 (sense) expresses the EGF domain of Lin-3 under the control of the heat shock promoter *hsp16-41*. This promoter directs expression in most tissues and expresses strongly in the pharynx and the intestine (Stringham et al. 1992). Translation initiates in a synthetic sequence that encodes a signal peptide (Perry et al. 1993) and thus the EGF domain of Lin-3 should be secreted. The vector also contains the 3' untranslated region of the *unc-54* gene and a synthetic intron to ensure that the message is stable and is properly processed (Fire et al. 1990). The vector pRH52 (antisense) contains the same sequences but the DNA encoding the EGF domain of Lin-3 is cloned in the antisense orientation with respect to the synthetic signal peptide and should produce no functional Lin-3 protein. The sequence of the predicted protein produced by pRH51 is shown. The EGF domain of Lin-3 (amino acids 34-96) is overlined in bold. The signal peptide is underlined. SSP: Synthetic Signal Peptide. 3' UT: 3' untranslated region. Arrowhead, possible cleavage site of the signal peptide (von Heijne 1986).

Methods. The EGF domain of Lin-3 from Val142 to Pro204 was directly amplified from a *lin-3* cDNA by PCR using the primers V123: 5'-GG CAT ATG GTT CGG AAG GAA ATC GAG-3', which adds a NdeI site and initiation codon to the 5' end of the product, and P185: 5'- T AGG ATC CTA TGG GGC ATA GAA CGC CTG-3', which adds a termination codon and BamHI site to the 3' end. The PCR product was endfilled and subcloned into pSK+. The vector pRH45 that places the synthetic signal peptide cassette of pPD52.63 (Perry, et al. 1993) under the transcriptional control of the promoter *hsp16-41* was constructed by subcloning a 970 bp BamHI-SpeI fragment of

pPD52.63 into a 2.9 kb BamHI-SpeI vector fragment of pPD49.83 (A. Fire, personal communication). The EGF domain was subcloned as a translational fusion into pRH45 by digesting the EGF vector with NdeI and BamHI, end filling the product, and gel purifying the insert fragment. The insert was subcloned into an end-filled NcoI digest of pRH45 to yield pRH51 with the EGF insert in the sense orientation and pRH52 with the EGF insert in the antisense orientation. The sequence of the translational fusion of pRH51 and the EGF insert has been checked by Sanger dideoxy sequencing. Standard molecular biological techniques were performed as described (Sambrook et al. 1989).

The transgenes *syEx20* and *syEx24* were obtained by microinjection of pRH52 at 50 ng/µl, C14G10(*unc-31(+)*) at 20 ng/µl, and pMob (Strathmann et al. 1991) at 30 ng/µl, into the syncytial germline (Mello et al. 1991) of *unc-31(e169)* mutant animals and examining the progeny for non-Unc-31 animals. The transgenes *syEx21* and *syEx23* were obtained by microinjection of pRH51 at 50 ng/µl, C14G10(*unc-31(+)*) at 20 ng/µl, and pMob at 30 ng/µl, into *unc-31(e169)*. The transgene *syEx42* was obtained by microinjection of pRH51 at 7 ng/µl, C14G10(*unc-31(+)*) at 20 ng/µl, and pMob at 73 ng/µl, into *lin-3(n378) unc-31(e169)*.

**Figure 1. Inducible Lin-3EGF transgenes.**

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  1  ATG CAT AAG GTT TTG CTG GCA CTG TTC TTT ATC TTT CTG GCA CCA
  1  Met His Lys Val Leu Leu Ala Leu Phe Phe Ile Phe Leu Ala Pro

 46  GCA TCC GCA CTG GCA GTC TCC GAA CCG GTC CTA GCG TCG ACG GTA
 16  Ala Ser Ala Leu Ala Val Ser Glu Pro Val Leu Ala Ser Thr Val

 91  CCA TGT ATG GTT CGG AAG GAA ATC GAG AAG TTG AAA GAA GCA AAA
 31  Pro Cys Met Val Arg Lys Glu Ile Glu Lys Leu Lys Glu Ala Lys

136  TGC AAA GAC TAC TGT CAT CAC AAC GCG ACA TGC CAC GTG GAA GTG
 46  Cys Lys Asp Tyr Cys His His Asn Ala Thr Cys His Val Glu Val

181  ATA TTC CGT GAA GAT AGA GTT TCA GCA GTT GTT CCT TCT TGC CAT
 61  Ile Phe Arg Glu Asp Arg Val Ser Ala Val Val Pro Ser Cys His

226  TGT CCA CAG GGT TGG GAA GGC ACT CGT TGT GAT CGT CAC TAC GTT
 76  Cys Pro Gln Gly Trp Glu Gly Thr Arg Cys Asp Arg His Tyr Val

271  CAG GCG TTC TAT GCC CCA TAG
 91  Gln Ala Phe Tyr Ala Pro End
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Figure 2. Nomarski photomicrographs of animals from Table 1. **a**. A heat-shocked animal bearing a Lin-3EGF antisense transgene with an intact gonad. The pattern of vulval fates is wild-type with P3.p, P4.p and P8.p assuming the 3° fate and P5.p, P6.p and P7.p assuming vulval fates. The AC is out of the plane of focus. **b**. A heat-shocked, gonad-ablated animal bearing a Lin-3EGF antisense transgene. All six VPCs have assumed the 3° fate (P3.p and P4.p out of the field of view). **c**. A heat-shocked animal bearing a Lin-3EGF sense transgene with the gonad intact. All six VPCs have assumed vulval fates (P8.p out of the field of view). **d**. A heat-shocked, gonad-ablated animal bearing a Lin-3EGF sense transgene. One cell of the P5/6(L/R) pair was also ablated. The remaining 5 VPCs have all assumed vulval fates.

The fate of the VPCs was determined by examination of the anatomy near the time of the L3 lethargus. At this time VPCs that have assumed the 3° fate will have produced two large epidermal nuclei that have fused to the main body epidermis hyp7, and the VPCs that have assumed the 1° and 2° vulval fates will have undergone two to three rounds of cell division. The syncytial epidermal nuclei of the 3° fate are denoted with a squares, and the smaller vulval nuclei (at the 4 cell stage) are marked by the small arrowheads. The line drawings illustrate the inferred lineal history of the cells. One round of cell division indicates the 3° fate and 2 rounds of cell division indicate a 1° or 2° vulval fate. The large arrowhead marks the position of the AC. Scale bar 20 μm. Ventral to the bottom, anterior to the left in B, C, D. AC: anchor cell.

AC
P3.p
P4.p
P5.p
P6.p
P7.p
P8.p
AC
P3.p
P4.p
P5/6.p
P7.p
P8.p
AC
P3.p
P4.p
P5.p
P6.p
P7.p
P8.p
P5.p
P6.p
P7.p
P8.p

Table 1. Sufficiency of the EGF domain of Lin-3 to induce vulval differentiation. Expression of the EGF domain of Lin-3 from a heat shock promoter generally induces all six of the VPCs to differentiate as vulval tissue and can do so when the the development of the tissue that normally induces the vulva has been prevented by the ablation of the gonadal precursor cells. The Lin-3EGF antisense transgenes can not induce vulval differentiation. When the gonad is present in animals bearing Lin-3EGF antisense transgenes, the AC induces the three more proximal VPCs to assume vulval fates as in wildtype. Thus, the Lin-3EGF antisense transgenes do not dominantly interfere with vulval induction, for example by the production of antisense RNA. Three VPCs did not assume vulval fates in the mock-ablated, heat-shocked animals bearing the Lin-3EGF sense transgenes. In each case this VPC was P3.p and it fused with the syncytial epidermis without dividing. P3.p fuses with the syncytial epidermis instead of assuming the 3° fate in approximately 50% of wild-type animals. A single VPC did not assume a vulval fate in the gonad-ablated, heat-shocked animals bearing the Lin-3EGF sense transgenes. This VPC was P8.p and it assumed the 3° fate.

Methods. The gonadal precursor cells Z(1-4) of non-Unc-31 hatchlings were ablated with a laser microbeam by standard methods (Avery and Horvitz 1987, Sulston and White 1980). The gonad ablations were performed stringently and as a consequence a P cell was incidentally ablated in some animals. The P cells give rise to the VPCs, so that some animals had 5 VPCs instead of 6 VPCs. The success of the gonadal ablation was assayed 12-19 hours after ablation. Worms were heat shocked in the late L2 to L2 lethargus, generally 20 hours after ablation of the gonad. Worms were heat

shocked for 2 hr at 33° C in sealed worm culture plates suspended in a water bath. Induction was measured as described (Aroian and Sternberg 1991) such that the percent of induction of an experimental population is equal to the (number of VPCs assuming vulval fates per animal) divided by (3, which is the number of VPCs that assume vulval fates in wildtype,) times 100. Animals bearing the Lin-3EGF sense transgenes grew slowly after heat shock compared to animals bearing the Lin-3EGF antisense transgenes. The Lin-3EGF sense transgenes occasionally cause excess levels of vulval differentiation when maintained at 20° C. Strains were maintained at 20°C as described (Brenner 1974).

Table 1. Sufficiency of the EGF domain of Lin-3 to induce vulval differentiation.

| EGF Orientation | Transgene | Gonad | n | Induced VPCs / Total VPCs | Total | Induction |
|---|---|---|---|---|---|---|
| Antisense | *syEx20* | + | 8 | 3/6 | | |
| | *syEx24* | + | 3 | 3/6 | | |
| | | | | | 11 | 100% |
| Antisense | *syEx20* | - | 4 | 0/5 | | |
| | *syEx20* | - | 4 | 0/6 | | |
| | *syEx24* | - | 2 | 0/5 | | |
| | *syEx24* | - | 2 | 0/6 | | |
| | | | | | 12 | 0% |
| Sense | *syEx21* | + | 5 | 6/6 | | |
| | *syEx23* | + | 3 | 5/6 | | |
| | *syEx23* | + | 4 | 6/6 | | |
| | | | | | 12 | 192% |
| Sense | *syEx21* | - | 4 | 5/5 | | |
| | *syEx21* | - | 3 | 6/6 | | |
| | *syEx23* | - | 1 | 4/5 | | |
| | *syEx23* | - | 3 | 5/5 | | |
| | *syEx23* | - | 4 | 6/6 | | |
| | | | | | 15 | 198% |

Table 2. Fate choice of isolated VPCs.

Single VPCs were isolated in animals bearing Lin-3EGF sense transgenes by ablation of the gonadal precursors and the 5 other VPCs. In response to a mild induction of the transgene, isolated VPCs can assume the 1°, the 2°, the 3°, or intermediate 1°/2° fates. This indicates that the EGF domain of Lin-3 can induce a VPC to assume either the 1° or the 2° fate in the absence of lateral signals from other VPCs.

Methods. The gonads of non-Unc-31 animals were ablated in the early L1 when there were 6-8 gonadal cells and the VPCs were ablated at 19-22 hours of development. It was noted in previous experiments that the Lin-3EGF sense transgenes are weakly activated in response to sodium azide (data not shown). Thus the transgenes are activated in all the animals during the ablation of the VPCs since this procedure exposes the worms to 5mM sodium azide for 10-15 minutes. The transgene was additionally activated in other animals by a 10 minute heat shock of 33°C, at ±2 hours of the time of the VPC ablation. Fate is assigned according to the cell lineage of a VPC and the morphogenesis of the descendents of the VPC in the L4 stage. A wild-type 2° lineage is LLTN and a wild-type 1° lineage is TTTT. Any lineage that had progeny that adhered to the ventral cuticle is considered to have 2° character. The morphogenesis of a 1° lineage is a symmetrical invagination and the morphogenesis of a 2° lineage is an asymmetrical invagination. Abbreviations. N3: activation of the transgene by 5 mM sodium azide. 10' hs: activation of the transgene by 10 minute exposure to 33°C. Morph: The morphogenesis of the VPC progeny in the L4 stage. NA: Not Applicable. ND: Not Determined. int: intermediate vulval fate with 1° and 2° vulval properties. Lineage abbreviations as in (Sternberg and Horvitz 1986), L: longitudinal division of PN.pxx nuclei; O: oblique division of PN.pxx

nuclei; T: transverse division of PN.pxx nuclei; D: PN.pxx nuclei divided, axis unknown; N: PN.pxx nuclei did not divide; S: PN.p or PN.px nucelus did not divide and fused with synchytial epidermis; Underline: progeny cells adhered to the ventral cuticle.

Table 2. Fate choice of isolated VPCs.

| Key | Transgene | Stimulus | Lineage | Morph | Fate |
|---|---|---|---|---|---|
| 130 | *syEx23* | 10' hs | LLLL | 2° | 2° |
| 172 | *syEx23* | 10' hs | TTLL | 2° | 2° |
| 173 | *syEx23* | 10' hs | LTNO | 2° | 2° |
| 171 | *syEx42* | N3 | LTTL | 2° | int |
| 174 | *syEx23* | 10' hs | LLTL | ND | int |
| 175 | *syEx23* | 10' hs | LTTL | ND | int |
| 127 | *syEx23* | 10' hs | LTDD | hyb | int |
| 128 | *syEx23* | 10' hs | LTTL | ND | 1° |
| 166 | *syEx42* | N3 | TONL | 1° | 1° |
| n=7 | syEx42 | N3 | SS | NA | 3° |
| n=2 | syEx23 | 10' hs | SS | NA | 3° |

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# Chapter 4

## The role of *lin-3* in vulval precursor cell fate patterning.

**The *C. elegans* gene *lin-3* encodes the signal made by the anchor cell that induces the vulval precursor cells (VPCs) to assume vulval fates. The inductive signal has been proposed to be distributed in a gradient centered on the anchor cell that establishes the graded pattern of fates assumed by the VPCs (Sternberg and Horvitz 1986). We examine the pattern and extent of vulval induction in hypomorphic *lin-3* mutant animals. We find that when vulval induction occurs in these animals, that it is usually centered beneath the anchor cell. This observation is consistent with the model that the inductive signal is distributed in a gradient. We also observe non-graded fate patterns, hybrid vulval/non-vulval fates, and evidence for cooperativity in the extent of induction. Thus, other signals may be modifying the fate choice of the VPCs.**

**We also examine the pattern of fates assumed by the VPCs in animals bearing *lin-3* transgenes that likely overexpress the Lin-3 protein from the anchor cell. In these animals adjacent VPCs near the anchor cell can adopt the 1° fate. Therefore, high doses of the inductive signal can override the action of the lateral signal, mediated by *lin-12*, which normally prevents adjacent VPCs from assuming the 1° fate. Thus, the dose of *lin-3* activity is important in establishing the proper pattern of vulval fates.**

The vulva of the nematode *C. elegans* develops post-embryonically from a subset of ectodermal blast cells (P[3-8].p) called the Vulval Precursor Cells (VPCs), which are located in a longitudinal row within the ventral epidermis (Sulston 1976). A variety of experiments suggest that each of the six VPCs is equivalent in its ability to assume any of three developmental fates: the 1°,

the 2°, and the 3° fates (Sternberg and Horvitz 1986, Sulston and White 1980). Each fate consists of a unique pattern of cell division and produces a unique set of cell types (see figure 2 of chapter 1). The 1° and 2° fates both produce vulval tissue and are called the vulval fates; the 3° fate produces non-vulval epidermis and is called the epidermal fate. In wild-type development the six VPCs reproducibly assume a 3° 3° 2° 1° 2° 3° pattern of fates. This pattern of fates is spatially graded beneath the anchor cell as only the VPC closest to the anchor cell, P6.p, assumes the 1° fate.

The current model for the fate determination of the VPCs, called the "three-signal model," is shown in figure 1 (reviewed in chapter 1 and in (Horvitz and Sternberg 1991)). This model proposes that the number of VPCs induced to assume vulval fates is set by the level of *lin-3* activity produced by the anchor cell. The 2° 1° 2° pattern of fates assumed by the three induced VPCs is established by a gradient of *lin-3* activity centered on the anchor cell. High levels of *lin-3* activity promote P6.p to assume the 1° fate and lower levels of *lin-3* activity promote P5.p and P7.p to assume the 2° fate. Lateral signalling among the VPCs mediated by the gene *lin-12* promotes the 2° fate and initially acts in proportion to the level of *lin-3* activity that each VPC receives. Thus P6.p, which receives the most inductive signal, is the VPC best able to laterally signal its neighbors to be 2°. A signal mediated by a pathway that includes *lin-15*, and which may arise from the main body epidermis inhibits the VPCs from assuming vulval fates in the absence of the inductive signal.

Some features of the "three-signal model" are uncertain. First, the relative contribution of the graded distribution of the inductive signal, versus the action of the lateral signal, in establishing the 2° 1° 2° pattern of fates assumed by the induced VPCs is unclear. It is possible either that P5.p and

P7.p assume the 2° fate because they receive a low dose of the inductive signal, or that they assume the 2° fate as the result of lateral interactions among the VPCs. Second, it is not clear from the "three-signal model" whether lateral signalling would prevent adjacent VPCs from assuming the 1° fate if they were both challenged with a high dose of inductive signal. The "three-signal model" also makes several predictions about the function of the inductive signal that can be tested by examining *lin-3* mutations. If the inductive signal is distributed in a gradient, then most hypomorphic mutations of *lin-3* should lower *lin-3* activity without disrupting its graded distribution. Thus, in hypomorphic *lin-3* mutant animals, the residual induction of vulval fates should occur preferentially beneath the anchor cell. If different doses of *lin-3* activity specify the 1° and 2° fates, then in some hypomorphic *lin-3* mutant animals, P6.p, which usually assumes the 1° fate, should assume the 2° fate. This chapter presents the current data on VPC fate choice in different *lin-3* genotypes and discusses the implications of these results for vulval fate patterning.

## Results.

VPC fate patterning in *lin-3* hypomorphs.

It was previously demonstrated that mutations in the gene *lin-3* cause a defect in the fate determination of the VPCs such that P5.p, P6.p, and P7.p, which in wildtype assume the 1° and 2° fates, instead often assume the 3° epidermal fate (Ferguson and Horvitz 1985, Ferguson et al. 1987, Horvitz and Sulston 1980, Sternberg and Horvitz 1989, Sulston and Horvitz 1981). As a result, *lin-3* mutant animals often lack vulval tissue (the vulvaless (Vul) phenotype), which results in an inability to lay eggs (the Egl phenotype). We have examined the number of VPCs that assume vulval fates in a variety of

hypomorphic *lin-3* genotypes (Table 1). Our results confirm and extend the previous results. The *lin-3* mutations *n378*, *n1058*, *sy53*, and *sy91* cause a vulvaless phenotype that is recessive to wildtype. In all hypomorphic *lin-3* genotypes examined, the number of VPCs induced to assume vulval fates is equal to, or less than, the number of VPCs induced in wild-type. Thus reduction of *lin-3* activity does not result in a Hyper-Induced (Hin) phenotype. Some of the larval lethal alleles of *lin-3* confer very little or no ability to induce vulval fates as measured by their failure to complement the hypomorphic alleles. For example, approximately only one in 600 animals of the genotype *n378/n1059* is able to lay eggs. Thus, the null phenotype of *lin-3* is likely to be a complete absence of vulval induction. Since putative null mutations in *lin-3* confer lethality prior to the time of vulval development, the extent of vulval induction in animals homozygous for null mutations of *lin-3* has not been examined. The smallest genetic deficiency of the *lin-3* region, *sDf64*, causes a slightly dominant defect in vulval induction. Thus, it can not be used to determine the phenotype of null alleles of *lin-3* in complementation tests.

We have examined the pattern of vulval induction in different *lin-3* hypomorphic genotypes. Figure 2 shows different possible patterns of VPCs assuming vulval and non-vulval fates and a histogram that shows the per centage of animals of a particular *lin-3* genotype with that pattern of fates. A pattern of fates is classified as a graded pattern if the VPCs assuming vulval fates are centered beneath the anchor cell, or as a non-graded pattern if there are VPCs assuming non-vulval fates closer to the anchor cell than other VPCs assuming vulval fates. In *n1058*, *sy91*, and *n378* mutant animals vulval induction, when it occurs, often occurs in a graded pattern (19 of 22 *n1058* homozygotes, 29 of 31 *sy91* homozygotes and 15 of 18 *n378* homozygotes).

Since the VPCs respond in a graded manner in the *lin-3* mutant animals, *lin-3* activity may be distributed in a gradient in these animals.

In some hypomorphic *lin-3* genotypes, the distribution of VPC fate patterns is bimodal such that there is an under-representation of animals with an intermediate number of VPCs induced to assume vulval fates. In homozygous *sy91* mutant animals, the average level of vulval induction is 38%. This is equivalent to an average of one induced VPC per animal, yet there are no observed *sy91* animals with exactly one induced VPC. Instead 59% of *sy91* mutant animals have induction of 0-0.5 VPCs, and 24% of *sy91* mutants have induction of three VPCs. The distribution of VPC fate patterns is also bimodal in *n1058* mutant animals which have an average of 46% induction and in *n378/sy53* animals that have an average level of 55% induction. In all of these genotypes there is a high fraction of animals with three cells assuming vulval fates. In *n378* mutant animals the average level of vulval induction is 11% and the fate patterns form a simple distribution in which animals with increasing levels of induction are increasingly rare. The current data suggest that bimodal distributions of VPC fate patterns are not an allele specific effect but result from certain dose ranges of *lin-3* activity. Thus, for example, *n378* homozygotes do not show a bimodal distribution of fates but *n378/sy53* animals have a complex distribution of fate patterns. The bimodal distributions of fate patterns suggests that there is cooperativity in the extent of vulval induction.

For some of the animals in figure 2 it could be determined whether an induced VPC was assuming the 1° or the 2° fate. The data for VPC fate choice in the hypomorphic *lin-3* mutant animals is summarized below and in Figure 3. In animals in which only P6.p assumes a vulval fate, P6.p assumes either a 1° fate or a hybrid vulval/non-vulval fate. In animals in which P6.p

and a lateral neighbor are induced, P6.p also assumes either a 1° fate or a hybrid vulval/non-vulval fate. Although the fate of P6.p could not be determined in all animals, there are no animals in which P6.p clearly assumed the 2° fate. P5.p and P7.p have not been observed to assume the 1° fate in *lin-3* hypomorphic mutant animals. When P6.p is induced P5.p and P7.p assume either the 3°, the 2° or hybrid vulval/non-vulval fates. In animals in which only P5.p or P7.p is induced, that cell has assumed the 2° fate in six cases and hybrid vulval/non-vulval fates in other animals. It is thus possible for a single VPC to assume the 2° fate in *lin-3* hypomorphic mutant animals, and thus there may be a dose of *lin-3* activity that specifies the 2° fate. P5.p and P7.p may be assuming the 2° fate in these animals in response to lateral signals.

P5.p, P6.p, and P7.p have been observed to undergo hybrid vulval/non-vulval lineages in *lin-3* hypomorphic mutant animals. As discussed in the methods, it is hard to assign 1° or 2° fates to the vulval half of the hybrid vulval/non-vulval lineages. Hybrid lineages have been previously observed in reduction-of-function mutant animals for other genes required for vulval induction (Aroian and Sternberg 1991, Sternberg and Horvitz 1986, Sternberg and Horvitz 1989). The existence of hybrid vulval/non-vulval lineages is an anomaly in the current model of vulval development in which the VPC chooses between one of three discrete fates and the descendants of the VPCs are committed to executing the fate chosen by the VPC. Models for the existence of hybrid fates are presented in the discussion.

VPC fate pattern in *lin-3* transgenic animals.

Transgenes that are made up of multiple copies of wild-type *lin-3* genomic DNA clones, which are believed to over-express the Lin-3 protein in

the anchor cell, cause up to all six of the VPCs to assume vulval fates (Hill and Sternberg 1992). The lineages assumed by the VPCs in *lin-3* transgenic animals are shown in Table 2A. In 4 of 12 animals bearing the *lin-3* transgene *syEx13*, adjacent VPCs have assumed the 1° fate, and in two of these animals more than 2 adjacent VPCs have assumed the 1° fate. The fates are judged to be 1° because the VPC granddaughters divide in the transverse axis, because the great-granddaughters undergo a morphogenic invagination similar to a wild-type 1° lineage, and because none of the great-granddaughter nuclei adhere to the ventral cuticle, which is the key property of the 2° fate. This result indicates that the *lin-3* transgenes can override, or interfere with, the lateral signalling among the VPCs that normally prevents adjacent VPCs from assuming the 1° fate. Thus, high levels of *lin-3* activity may specify the 1° fate. Adjacent VPCs with the 1° fate are only seen proximal to the anchor cell, which is consistent with the model that adjacent 1° fates are specified only by high levels of *lin-3* activity. In approximately half of the animals, there is a 1° fate farther from the anchor cell than a 2° fate: thus, the VPCs do not always assume a graded pattern of fates in the *lin-3* transgenic animals.

The normal pattern of early and late VPC granddaughter divisions is sometimes disrupted in the adjacent 1° lineages. In a wild-type 1° lineage, the granddaughters of the VPC divide in a temporal pattern of "early, late, late, early" whereas in *lin-3* mutant animals the VPC granddaughters can divide in an alternating pattern of "early, late" that runs from one 1° fate to another. An example of this is shown in figure 4B in which there is an alternating pattern of early and late divisions that runs from P4.p to P6.p. This same disruption has been noted in the adjacent 1° fates found in amorphic *lin-12* mutant animals (P. Sternberg, personal communication).

These results could indicate that cell interactions affect the timing of divisions of the VPC granddaughters in the 1° lineage.

The lineages of the VPCs in animals bearing *lin-3* transgenes and hypermorphic *lin-12* mutations (*lin-12(d)*)were followed to test if the ability of the *lin-3* transgenes to promote adjacent 1° fates is epistatic to the ability of *lin-12(d)* mutations to promote the 2° fate. In the lineages of 9 such animals, there are no examples in which adjacent VPCs have assumed normal 1° lineages (Table 2B). There are, however, cases in which P6.p is 1° and P5.p or P7.p has assumed a lineage that has properties of both the 1° and the 2° lineages. For example, in animal 5G3, P5.p assumed a lineage which is 1°-like with the exception that P5.paa(a/p) adhered to the ventral cuticle. This result could mean that it is more difficult to specify adjacent VPCs to be 1° in *lin-12(d)* mutant animals than in animals wild-type for *lin-12*. Alternatively, the absence of animals with clear adjacent 1° fates could be due to chance, since the *lin-3* transgenes provide a variable dose of *lin-3* activity. Some *lin-12(d)* mutant animals bearing *lin-3* transgenes display phenotypes that correlate with the presence of adjacent VPCs with the 1° fate. For example, adult animals sometimes have large, broad ventral protrusions that can result from adjacent VPCs assuming the 1° fate. Also, some L3 molt animals were observed in which the VPC grandprogeny were staggered in the left-right or dorsal-ventral planes beneath the anchor cell. In wild-type development, the VPC grandprogeny remain in a longitudinal row. Staggering of the VPC grandprogeny has been previously observed in *lin-3* transgenic animals and in amorphic *lin-12* mutant animals in which adjacent VPCs are assuming the 1° fate. These observations suggest that adjacent 1° fates may be present in this strain.

# Discussion.

## Fate patterning in *lin-3* hypomorphs.

Graded patterns of induction

In most hypomorphic *lin-3* mutant animals, there is either no vulval induction or the pattern of vulval induction is graded such that only the VPCs closer to the anchor cell have assumed vulval fates. Thus, in response to lowered levels of *lin-3* activity, the three VPCs that are induced in wildtype do not have an equal chance of assuming vulval fates. P5.p and P7.p are less likely to assume a vulval fate than P6.p and usually only assume vulval fates in animals in which P6.p has assumed a vulval fate. This observation is consistent with the model that *lin-3* activity is distributed in a gradient in wildtype and that the hypomorphic *lin-3* mutations result in a graded distribution of *lin-3* activity that is reduced in amplitude and extent. Thus, in animals with low levels of *lin-3* activity there is either no vulval induction, or only the VPC closest to the anchor cell receives enough *lin-3* activity to assume a vulval fate. In those animals with enough *lin-3* activity to induce either P5.p or P7.p, it is very likely that P6.p will receive enough *lin-3* activity to assume a vulval fate because it is in the center of the gradient. The graded patterns of vulval induction could also be explained by a "two-step" model of vulval induction in which Lin-3 made by the anchor cell induces P6.p to be 1°, and P6.p induces P5.p and P7.p to be 2°. In this case P5.p and P7.p would only assume vulval fates when enough *lin-3* activity is present to induce P6.p to be 1°. As is discussed later, the bulk of experimental evidence is against the "two-step" model of vulval induction, so this explanation is likely to be incorrect.

Non-graded patterns of induction

There are examples of non-graded patterns of induction in most hypomorphic *lin-3* genotypes. The presence of these non-graded patterns in *lin-3* mutant animals could be due to several effects. First, it is possible that *lin-3* activity is occasionally distributed in a non-graded manner in the *lin-3* mutant animals and thus that each of the VPCs is responding in proportion to the level of *lin-3* activity that it receives. Second, it is possible that in some animals the level of *lin-3* activity is near the threshold for induction of vulval fates. If VPC induction is stochastic at threshold levels of *lin-3* activity, then in some cases P6.p may, by chance, not assume a vulval fate while its lateral neighbors do assume vulval fates, even though P6.p received a slightly higher level of *lin-3* activity. Third, the non-graded patterns of vulval induction could result from lateral signalling among the VPCs that regulates the boundary between cells assuming vulval fates and cells assuming non-vulval fates. This "boundary" signal could arise from VPCs assuming the 2° fate, and direct VPCs receiving even lower levels of *lin-3* activity to adopt the 3° fate. Thus, in some cases in which P[5-7].p are receiving low levels of *lin-3* activity, the combined action of the boundary signal arising from P5.p and P7.p could direct P6.p to assume the 3° fate. It might be possible to test whether lateral signalling among the VPCs is responsible for the non-graded patterns of fate by comparing, for a given *lin-3* genotype, the frequency at which P6.p assumes a vulval fate when it has lateral neighbors to the frequency at which P6.p assumes vulval fates when it does not have neighbors. If the frequency at which P6.p assumes a vulval fate is greater when it lacks lateral neighbors, then this might indicate that the neighbors were inhibiting P6.p from assuming vulval fates. This experiment would be

subject to the caveat that P6.p might receive more inductive signal upon isolation because it lacks neighbors that absorb the inductive signal.

Cooperativity in induction.

In some hypomorphic *lin-3* genotypes the distribution of VPC fate patterns does not form a Poisson distribution around a single average value. This effect does not seem to be allele-specific and appears to be associated with a particular dose range of *lin-3* activity. The bimodal distribution of fate patterns could indicate that the mechanisms that control the extent of vulval induction display cooperativity. This cooperativity could be explained be several models. First, the VPCs could decide as a group whether they should assume vulval fates. Second, there could be cooperativity in the production of the inductive signal. For example, the anchor cell may initially release a low level of the inductive signal that is only sufficient to induce the closest VPC. When a VPC receives the inductive signal, it signals back to the anchor cell and either it or the anchor cell moves to center the anchor cell above the induced VPC. Once centered, the anchor cell increases production of the inductive signal to a level sufficient to induce three VPCs. Thus, in a hypomorphic *lin-3* mutant animal, the anchor cell either does not make enough *lin-3* activity to fully induce P6.p, in which case the level of induction stays below one cell, or the anchor cell makes enough *lin-3* activity to fully induce P6.p, in which case the anchor cell increases its production of *lin-3* activity in response to induction of P6.p and additional VPCs are induced. The third model that could explain cooperativity is that there is a novel signal from a cell assuming the 1° fate that promotes its lateral neighbors to assume a vulval fate. In this model, anytime there was enough *lin-3* activity to fully induce P6.p, then P6.p would instruct its lateral neighbors to assume

vulval fates. This model is essentially a variant of the "two-step" model of vulval induction discussed later.

It should be possible to distinguish among these three models experimentally. In the first model in which the VPCs make a cooperative decision as a group to respond to the inductive signal, the decision of a particular VPC to assume a vulval fate is influenced by its lateral neighbors. If this model is correct, then in a *lin-3* genotype that displays cooperativity, P6.p should assume a vulval fate more often when it has lateral neighbors than when its lateral neighbors have been ablated with a laser microbeam. In the second model in which there is cooperativity in the production of the inductive signal, then cooperativity should be dependent upon the anchor cell. Thus, if the VPCs were induced by a mechanism independent of the anchor cell, the extent of their induction should not display cooperativity. Thus, for example, if the transgenes that express the EGF domain of Lin-3 under the control of heat shock promoter (hsp::Lin-3EGF) were activated in a single cell (Stringham and Candido 1993), then the extent of vulval induction should increase in a non-cooperative manner as the intensity of the laser activation is increased. In contrast in the third model in which the cooperativity results from lateral signals among the VPCs, then the extent of vulval induction should show cooperativity regardless of the source of the inductive signal. In this case when the hsp::Lin-3EGF transgenes are activated in a single cell, then the extent of induction should increase cooperatively in response to increasing doses of the laser activation.

VPC fate choice in *lin-3* hypomorphs

If different doses of *lin-3* activity specify a VPC to assume either the 1° or the 2° fate, then a strong prediction is that P6.p, the only VPC induced to

assume the 1° fate in wild-type, should assume the 2° fate in some *lin-3* hypomorphic mutant animals in response to the lowered level of *lin-3* activity. In the hypomorphic mutant animals, P6.p has been observed to assume the 3° fate, the 1° fate, and hybrid vulval/non-vulval fates, but not the 2° fate (Fig. 3). Thus, the *lin-3* hypomorphic mutations have not been observed to provide a dose of *lin-3* activity that specifies P6.p to assume the 2° fate, although it is possible that this would be observed if a greater number of animals was examined. This result can be interpreted in several ways. First, the current model that different doses of *lin-3* activity specify the 1° and the 2° fates could be wrong. In this case, *lin-3* activity would likely only promote a VPC to assume a vulval fate. The decision between the 1° and the 2° vulval fate would be made by lateral signalling and in the absence of lateral signalling a VPC assumes the 1° fate. This model does not explain why isolated VPCs have assumed the 2° fate in other experiments (Hill et al. In preparation, Sternberg and Horvitz 1986). A second possibility is that it is hard for technical reasons to achieve the dose of *lin-3* activity that specifies the 2° fate in the *lin-3* mutant animals. For example, the dose of *lin-3* activity that promotes the 2° fate could be narrow and it may be difficult for P6.p to receive this dose since it is close to the source of Lin-3. Alternatively, a feedback mechanism between the anchor cell and P6.p may act to move the production of *lin-3* activity out of the dose range that specifies the 2° fate. Thus, in a *lin-3* mutant animal, the anchor cell either does not make enough inductive signal to induce P6.p, in which case P6.p assumes the 3° fate. Or the anchor cell produces enough inductive signal to induce P6.p, P6.p signals the anchor cell that it is induced, and the anchor cell up-regulates production of the inductive signal such that P6.p is induced to assume the 1° fate. If the lack of P6.p assuming the 2° fate in *lin-3* hypomorphic mutants is due to

feedback mechanisms that involve the anchor cell, then it should be possible to achieve the dose of *lin-3* activity that specifies the 2° fate from a transgene that expresses Lin-3 under the control of a heat shock promoter. There are animals in which P5.p or P7.p is the only induced cell and this cell assumes the 2° fate. This situation is difficult to interpret, since if the inductive signal is distributed in a gradient, then P5.p and P7.p should only assume a vulval fate when P6.p is also induced. In this case the VPC may be responding to the dose of *lin-3* that specifies the 2° fate or it may assume the 2° fate in response to *lin-3* and lateral signalling among the VPCs.

The failure to observe P6.p assuming the 2° fate in hypomorphic *lin-3* mutant animals could also be due to the mechanism by which the 2° fate is specified. Genetic evidence suggests that activity of the gene *lin-12* is required to specify the 2° fate (Greenwald et al. 1983). In the current model of lateral signalling, *lin-12* is normally activated in one cell by a lateral signal from a neighboring cell and that the neighboring cell produces the lateral signal in response to *lin-3* activity. Given these two points, it is unclear how an isolated VPC assumes the 2° fate since the 2° fate requires *lin-12* activity and there is no lateral neighbor to activate *lin-12*. Although it is relatively certain that an isolated VPC is not receiving lateral signal from other VPCs, the status of the molecules involved in lateral signalling in the isolated VPC is unclear. Thus, an isolated VPC may assume the 2° fate by engaging in autocrine signalling between the ligand for Lin-12 and Lin-12, or by interacting with cells other than the VPCs. Still this apparent paradox of how an isolated VPC assumes the 2° fate could be the problem in specifying P6.p to assume the 2° fate in the *lin-3* hypomorphic mutant animals. Thus, in a particular animal, P6.p may receive a dose of the *lin-3* activity that normally promotes the 2° fate. However, there is not enough *lin-3* activity to

induce either P5.p or P7.p to assume a vulval fate. Therefore P6.p receives no lateral signal and thus assumes the 1° fate by default. In this model there are doses of *lin-3* activity that specify the 2° fate, but the specification of the 2° fate is a two step mechanism.

hybrid fates

Hybrid vulval/non-vulval lineages are seen in many *lin-3* hypomorphic mutant animals. The existence of these hybrid lineages can not be simply explained in the current model of vulval development that a VPC chooses one of three fates and that its descendants are committed to executing this fate. Hybrid vulval/non-vulval lineages are observed in many other genotypes that lower the activity of the vulval inductive pathway and are thus not a unique feature of *lin-3* mutants. As discussed in the methods section it is not always possible to conclude with certainty whether the vulval half of a hybrid lineage is half of a 1° or of a 2° lineage because there is a limited number of cell divisions and progeny to examine. The most common hybrid lineage in the hypomorphic *lin-3* mutant animals is a hybrid lineage of P6.p that may be a 1°/3° hybrid. It is not clear from the current data whether the hybrid fates are specified by particular dose ranges of *lin-3* activity. Nor is it clear how the hybrid fates, especially the 1°/3° hybrid, should be classified.

These hybrid lineages could be explained by several models. First, in response to intermediate or low levels of the inductive signal, a VPC could choose a fate that is not maintained by its daughters. Second, when there is a low level of *lin-3* activity, the process of fate determination may not always occur before the VPC divides and thus the daughters of the VPC can make independent fate choices. Third, the hybrid fates could be generated by a hypothetical "boundary" signal that refines the border between vulval and

non-vulval cells by instructing cells that receive low amounts of the inductive signal to assume the 3° fate. If this boundary signal can act on the daughters of the VPCs, then a hybrid lineage would result when one of the daughters of an induced VPC was specified to assume an epidermal fate by the boundary signal. If the hybrid lineages consistently appear in locations that are proper for the vulval half of the lineage such that P6.p undergoes hybrid 1°/3° lineages and P5.p undergoes 2°/3° lineages, then this would suggest that the hybrid lineages are created by mechanisms that act after the VPCs have chosen their fate in response to the inductive and lateral signals.

summary of patterns in hypomorphs

If the inductive signal is distributed in a gradient, then when *lin-3* activity is lowered genetically the inductive signal should be distributed in a gradient that is reduced in amplitude and extent. A population of VPCs should respond to the residual inductive signal in a spatially graded manner. The action of other signals among the VPCs, however, could mask the graded response of the VPCs to the inductive signal. Four features of the patterns of VPC fate choice in hypomorphic *lin-3* mutants are difficult to explain in the "three-signal" model: the presence of non-graded fate patterns, the presence of hybrid vulval/non-vulval fates, cooperativity in the extent of induction, and the lack of a dose range of *lin-3* activity that results in P6.p assuming the 2° fate. A hypothetical "boundary signal" that causes some of the VPC daughters to assume an epidermal fate after initiating a vulval fate could explain both the non-graded patterns of fate and the presence of hybrid vulval/non-vulval fates. Positive feedback in the production of the inductive signal could explain both the cooperativity in the extent of induction and the failure of P6.p to assume the 2° fate.

**Consequences of Lin-3 overexpression on fate pattern**

Transgenes made up of multiple copies of *lin-3* genomic DNA clones are proposed to overexpress the Lin-3 protein from the AC and can cause all six of the VPCs to assume vulval fates (Hill and Sternberg 1992). A cell lineage analysis of the VPCs in the *lin-3* transgenic animals indicates that adjacent VPCs close to the anchor cell can both assume the 1° fate. Thus, the *lin-3* transgenes overcome the lateral signalling among VPCs that is normally sufficient to prevent adjacent VPCs from assuming the 1° fate. This result can be interpreted in two ways. First, a high level of *lin-3* activity could direct a VPC to assume the 1° fate, even if the VPC is receiving the lateral signal. If so, then the dose of *lin-3* activity made by the anchor cell is important in establishing the proper 2° 1° 2° pattern of fates because lateral signalling among the VPCs is not sufficient to ensure that only one VPC assumes a 1° fate in response to high levels of *lin-3* activity. Second, the *lin-3* transgenes could interfere with the action of the lateral signal. For example, when Lin-3 is overexpressed, Lin-3 may bind to Lin-12 and prevent Lin-12 from being activated by the lateral signal. Thus, adjacent VPCs assume the 1° fate in response to *lin-3* activity because they do not receive the lateral signal It would be possible to test the model that *lin-3* transgenes interfere with the transmission of the lateral signal by examining the epistasis of *lin-3* transgenes with *lin-12* mutations that are active in the absence of the lateral signal. Transgenes that express the cytoplasmic domain of the Lin-12 protein promote the VPCs to assume 2° fate and it is believed that these truncated Lin-12 proteins are constituitively active (Roehl and Kimble 1993, Struhl et al. 1993). If a high level of *lin-3* activity specifies the 1° fate, then the *lin-3* transgenes should promote adjacent 1° fates in animals also bearing the *lin-*

*12* transgenes. If the *lin-3* transgenes interfere with the transmission of the lateral signal, then the *lin-3* transgenes should not cause adjacent 1° fates in animals also bearing the *lin-12* transgenes.

It was previously known that the inductive signal could specify a single VPC to assume the 1° fate in animals bearing *lin-12(d)* mutations since when an anchor cell is present in a *lin-12(d)* mutant animal, P6.p adopts the 1° fate (Sternberg and Horvitz 1989). Thus for P6.p, the action of the inductive signal in specifying the 1° fate is epistatic to the *lin-12(d)* mutation in specifying the 2° fate. In this experiment the VPCs are presumably exposed to a wild-type dose of *lin-3* activity and only a single VPC assumes the 1° fate. Thus, this result does not indicate if the inductive signal is epistatic to the action of the lateral signal, i.e., whether the inductive signal could cause two adjacent VPCs to assume the 1° fate. The interpretation of this experiment depends upon the nature of the *lin-12(d)* mutation. If the *lin-12(d)* mutation causes constitutive activation of the lateral signalling pathway, then each *lin-12(d)* mutant VPC should act as if its neighbors are assuming the 1° fate and should not be sensitive to the actual state of its neighbors. Since a single *lin-12(d)* mutant VPC assumes the 1° fate in response to a high dose of inductive signal, each member of a group of *lin-12(d)* mutant VPCs should assume the 1° fate if they are exposed to a high dose of inductive signal. However, if the *lin-12(d)* mutant VPCs can still engage in lateral signalling, then lateral signalling may be responsible for P6.p assuming the 1° fate in the *lin-12(d)* mutant animals with anchor cells. In this case it could not be predicted how two adjacent VPCs would respond to high levels of the inductive signal. Thus, the ability of *lin-3* transgenes to specify adjacent 1° fates in a *lin-12(+)* animal is an important confirmation that the inductive signal is epistatic to the lateral signal.

Since *lin-3* transgenes can cause adjacent 1° fates in animals wild-type for *lin-12*, and since *lin-3* activity can cause P6.p to assume the 1° fate in *lin-12(d)* mutant animals, *lin-3* transgenes should be able to promote adjacent 1° fates in a *lin-12(d)* mutant animal. No adjacent 1° fates have been seen in *lin-12(d)* mutant animals bearing a *lin-3* transgene although, in some animals, adjacent VPCs both have 1°-like character. This could suggest that it is more difficult for *lin-3* transgenes to specify adjacent 1° fates in *lin-12(d)* mutant animals than in *lin-12(+)* animals. Alternatively, since the *lin-3* transgenes provide variable doses of *lin-3* activity, it is possible two VPCs were not exposed to a high dose of *lin-3* activity in any of the examined animals. If it is more difficult for the *lin-3* transgenes to specify the 1° fate in *lin-12(d)* mutant animals than in *lin-12(+)* animals, this would suggest that the activity of the *lin-12(d)* mutation is not equivalent to the activity of wild-type *lin-12* that is activated by the lateral signal. For example, perhaps the dominant *lin-12* mutations result in stronger activation of the 2° fate than wild-type *lin-12* activated by the lateral signal. Alternatively, the *lin-12(d)* mutations may be less susceptible to negative regulation in VPCs receiving the dose of *lin-3* activity that specifies the 1° fate.

The adjacent 1° fates observed in *lin-3* transgenic animals suggests that high doses of *lin-3* activity specify the 1° fate and make a cell unresponsive to the lateral signal. This does not rule out the possibility that at moderate doses of *lin-3* activity a VPC can choose either the 1° or the 2° fate in response to the action of the lateral signal. This appears to happen in *lin-15* mutant animals. Such animals all six VPCs assume vulval fates due to a defect in a presumed inhibitory signal that negatively regulates the basal activity of *let-23*. Since the inhibitory signal is believed to arise in a spatially general manner, the *let-23* pathway should be activated at an equivalent level

in all of the VPCs in a *lin-15* mutant in which the inductive signal is absent. In such animals the VPCs assume alternating patterns of 1° and 2° fates(Sternberg 1988). Thus the lateral signal can specify a VPC that has a moderate level of activity in the *let-23* pathway to assume either the 1° or 2° fate. Although adjacent 1° fates are observed in *lin-3* transgenic animals, the adjacent 1° fates are seen only proximal to the anchor cell. The fate patterns of the VPCs distal to the anchor cell in *lin-3* transgenic animals can resemble the patterns of fates in *lin-15* mutant animals such that in about half of the *lin-3* transgenic animals there is a VPC with the 2° fate closer to the anchor cell then the furthest VPC assuming the 1° fate. Since it is extremely unlikely that these alternating patterns of 1° and 2° fates reflect alternating high and low levels of *lin-3* activity, it is likely that the lateral signal can specify a VPC receiving intermediate amounts of *lin-3* to assume either the 1° or the 2° fate. This indicates that vulval fate choice is not being set only by the absolute amount of inductive signal that the VPCs are receiving.

Insights into the Hyper-Induced Phenotype.

Mutations that cause a Hyper-INduced (Hin) phenotype in which greater than the wild-type number of VPCs assume vulval fates have been described (Aroian and Sternberg 1991, Ferguson and Horvitz 1985). Several properties of Hin mutations distinguish them from multivulva mutations in genes such as *lin-15*. First, Hin mutations are a distinct class of hypomorphic mutations in genes such as *let-23, lin-2, lin-7* and *lin-10* that are believed to have an amorphic vulvaless phenotype (Aroian and Sternberg 1991, G. Jongeward, personal communication ). Multivulval mutations, however, are amorphic as in the case of *lin-15*, (Ferguson and Horvitz 1989, L. Huang, personal communication) or are hypermorphic as in the case of *let-60* (Beitel

et al. 1990, Han et al. 1990). Second, the ability of Hin mutations to cause VPCs to assume vulval fates is dependent upon the inductive signal, whereas multivulva mutations can confer vulval fates in the absence of the inductive signal. Third, adjacent VPCs can assume the 1° fate in Hin mutant animals and this is infrequently observed in *lin-15* multivulva mutant animals. A simple interpretation of the Hin phenotype is that it is caused by a defect in the negative regulation of the *let-23* pathway that renders the VPCs hyper-sensitive to the inductive signal (G. Jongeward and P. Sternberg, personal communication). Thus, additional VPCs assume vulval fates in Hin mutant animals because they can respond to a level of the inductive signal that is beneath the threshold for induction in wildtype. However, the presence of adjacent 1° fates in Hin mutant animals was problematic because it was not known if hyperactivity of the response pathway could cause adjacent VPCs to assume the 1° fate. The presence of adjacent 1° fates also suggested that there might be a defect in the lateral signal in Hin mutant animals. Our results indicate that high levels of the inductive signal can cause adjacent VPCs to assume the 1° fate. Thus, both the increased extent of vulval induction and the presence of adjacent 1° fates in Hin mutant animals can be explained by the model that Hin mutant animals are hyper-sensitive to the inductive signal and are responding as if there is too much inductive signal. We also note that in all hypomorphic *lin-3* genotypes examined, that induction of vulval fates is limited to the cells P5.p, P6.p, and P7.p. This suggests, as expected, that there is no dose of *lin-3* lower than wild-type that results in a Hin phenotype. Thus the Hin mutations do not cause the response pathway to act as if it is receiving too little inductive signal.

**Is *lin-3* a morphogen?**

A key issue for explaining VPC fate patterning is whether there are distinct doses of *lin-3* activity that specify each of the three VPC fates. The current "three-signal" model of vulval induction is ambiguous as to the relative contribution of the inductive signal and the lateral signal in establishing the 2° 1° 2° pattern of fates assumed by the three induced VPCs. It is possible that the induced VPCs choose their fate primarily in response to the absolute concentration of Lin-3 that they receive, the morphogen model. Alternatively, it is possible that the induced VPCs choose their fate primarily through lateral signalling that measures which VPC is receiving more inductive signal and which VPC is receiving less inductive signal, the "lateral comparison" model. Two experiments support the morphogen model. First, high levels of *lin-3* activity can cause adjacent cells to adopt the 1° fate, which suggests that there is a dose of *lin-3* activity that instructs a VPC to assume the 1° fate and to ignore input from the lateral signal. Second, isolated VPCs that have no neighboring VPCs with whom they can signal, can assume the 2° fate. This suggests that there is a dose of the inductive signal that can specify the 2° fate without input from the lateral signal stating that another VPC is receiving more inductive signal. Two observations, however, support the "lateral comparison model." First, in hypomorphic *lin-3* mutant animals, P6.p has not been observed to assume the 2° fate. If an intermediate dose of *lin-3* activity promotes the 2° fate, it would be predicted that certain hypomorphic *lin-3* genotypes would commonly expose P6.p to this dose. Second, in some cases such as in *lin-15* mutant animals, VPCs appear to choose between the 1° and the 2° fates in response to lateral signalling and not in response to the absolute concentration of inductive signal they receive.

The relative contribution of the level of the inductive signal and the action of the lateral signal in controlling VPC fate choice could be determined

by measuring the fate choice of an isolated VPC in response to different doses of *lin-3* activity and comparing this to the fate choices of groups of VPCs in response to the same doses of *lin-3* activity. The current *lin-3* genetic reagents have limitations for establishing a dose response curve of VPC fate as a function of *lin-3* activity. The hypomorphic *lin-3* mutations have the disadvantage that they do not provide a means of challenging any VPC other than P6.p with a high dose of *lin-3*, or a means of controlling *lin-3* dose independently of VPC position. Thus for example, it is not possible to challenge P5.p and P6.p with the same level of inductive signal to see if the lateral signal causes them to assume different fates. The gain-of-function *lin-3* transgenes, which are created by a method that generates high copy number extrachromosomal arrays (Mello et al. 1991), also present technical difficulties. The existing transgenes do not provide different effective doses of *lin-3* activity. They are also genetically unstable and do not provide a consistent dose of *lin-3* activity from one animal to the next. As was seen earlier, this complicated comparison of the action of *lin-3* transgenes in animals of different *lin-12* genotypes.

It should be possible to design *lin-3* transgenes that avoid these limitations. The transgenes that express the EGF domain of Lin-3 under the control of a heat shock promoter (hspLin3) provide a means of controlling the dose of *lin-3* activity independently of the position of a VPC. These transgenes can be activated in a spatially general manner by heat shock of the entire animal, or possibly, in a single cell by activation in response to an attenuated laser microbeam (Stringham and Candido 1993). Low copy-number, chromosomally integrated transgenes would provide doses of *lin-3* activity that are lower, and which are more consistent from animal to animal. Chromosomally integrated transgenes can be obtained in an oocyte injection

protocol (Fire 1986) and low copy number transgenes can be obtained by coinjection of a plasmid containing the *sup-7* gene, which is lethal at high copy number (Fire et al. 1990).

Low copy number, chromosomally integrated, hspLin3 transgenes could be used in experiments that test VPC fate choice in response to different doses of *lin-3* activity. These transgenes could be tested in gonad-ablated, reduction-of-function *lin-3* mutant animals so that there was no source of *lin-3* activity other than the transgene. The fate of an isolated VPC could be examined upon different amounts of heat shock activation of the transgene. Such experiments could define stimulation conditions for reference *lin-3* transgenes that specify different fates. In particular, these experiments would determine whether there is a distinct dose of *lin-3* activity that consistently specifies the 2° fate. It would then be possible to examine the fates assumed by pairs of VPCs in response to the same stimulation conditions to see if lateral signalling among the VPCs modifies fate choice. For example, if a pair of VPCs is challenged with the minimal dose of *lin-3* activity that induces an isolated VPC to assume the 1° fate, will both VPCs assume the 1° fate, or will one of them assume the 2° fate? Likewise, if two VPCs are challenged with the dose of *lin-3* activity that causes an isolated VPC to assume the 2° fate, will both VPCs assume the 2° fate, or will one VPC now assume the 1° fate? The generation of reference transgenes that provide consistent doses of *lin-3* activity would also be useful in examining how different mutations affect the fate choice of the VPCs in response to defined induction conditions.

<u>How do the VPCs respond in a graded manner?</u>

In the " three-signal" model of VPC fate determination, an inductive signal is distributed in a gradient around the anchor cell and the VPCs choose different fates in response to the amount of inductive signal they receive. An interesting problem is how the graded information provided by the inductive signal is transmitted within the responding VPCs. The graded response of the VPCs could be explained by two models. First, the graded information provided by the inductive signal could be conveyed by the graded activation of a single response pathway. Thus, each component of the *let-23*, *sem-5*, *let-60*, *lin-45* pathway would need to activated in proportion to the amount of inductive signal that the VPC received. At the end of this pathway, high levels of activity would cause the VPC to assume the 1° fate and intermediate amounts of activity would cause the VPC to assume the 2° fate. This model makes two predictions about the patterns of VPC fates in different mutant animals. First, if the response pathway carries graded information at each step, then hypomorphic mutations that lower the activity of the pathway at any step should result in a low average level of vulval induction that is preferentially centered around the anchor cell. The fate patterns conferred by most mutations that disrupt the response pathway has not been extensively examined. In *let-23(sy1)* homozygotes, however, approximately one third of the animals that have vulval induction have induction in a non-graded pattern (P. Sternberg, R. Aroian, G. Jongeward, personal communication). This could indicate that the *let-23* pathway is not carrying graded information, or that other interactions among the VPCs is disrupting this pattern. Second, since this model implies that high levels of activity in the response pathway specify the 1° fate, gain-of-function mutations in any step of the pathway should cause adjacent VPCs to assume the 1° fate. This means that there should be adjacent 1° fates in *let-60* gain-

of-function mutant animals. The alternative model is that the graded information provided by the inductive signal results in the combinatorial activation of parallel response pathways. For example, *let-23* is activated in proportion to the amount of *lin-3* activity present and high levels of *let-23* activity in P6.p stimulates two pathways and intermediate levels of *let-23* activity in P5.p and P7.p stimulates a single pathway. Input from both pathways in the nucleus of P6.p would specify the 1° fate; input from one pathway in P5.p and P7.p would specify the 2° fate. One advantage of this model is that it avoids the prediction that each member of the *let-23* pathway acts in a graded fashion. Another advantage is that the second signalling pathway activated in response to high levels of the inductive signal could be the lateral signalling pathway mediated by *lin-12*. In this case high amounts of *let-23* activity in P6.p cause activation of the lateral signal which promotes P5.p and P7.p to assume the 2° fate. It is unknown how the lateral signalling pathway mediated by *lin-12* and the inductive signalling pathway of *let-23, sem-5, let-60*, and *lin-45* interact. Thus another way of examining how the graded information is carried within the VPCs may be to determine at what step the *let-23* pathway affects the lateral signalling pathway.

**Other Models, Other Signals.**

Two-step models

The main alternative to the "three-signal model" is a sequential "two-step model" (Sternberg and Horvitz 1986). In the "two-step model" the AC induces P6.p to be 1° and then P6.p sends a signal that induces P5.p and P7.p to be 2°. The strongest point of evidence against the two step model is that an isolated VPC can assume the 2° fate, indicating that a signal from a VPC assuming the 1° fate is not required for another VPC to assume the 2° fate

(Hill, et al. in preparation, Sternberg and Horvitz 1986). The only characterized lateral signal that acts between the VPCs is the lateral signalling pathway that involves *lin-12*. This lateral signalling pathway is believed to regulate whether VPCs induced by *lin-3* activity assume the 1° or 2° fate and is not believed to be a signal from a 1° VPC that induces its neighbors to assume the 2° fate since in animals homozygous for amorphic *lin-12* mutations, the number of VPCs that assume vulval fates does not appear to be decreased (Sternberg and Horvitz 1989). This experiment is complicated by the fact that there are multiple anchor cells in amorphic *lin-12* mutant animals. So it remains possible that a decrease in the extent of vulval induction due to loss of the lateral signal is counteracted by increased production of *lin-3* activity. The model that *lin-12* has no role in controlling the number of VPCs that assume vulval fates could be confirmed by two related experiments. First, the extent of vulval induction could be examined in amorphic *lin-12* mutant animals in which the development of more than one anchor cell has been prevented by ablation of gonadal precursor cells. If *lin-12* has no role in controlling the extent of vulval induction then exactly three VPCs should be induced in amorphic *lin-12* mutant animals with only one anchor cell. Second, these experiments could be repeated in animals also bearing hypomorphic *lin-3* mutations. Since these animals produce low amounts of the inductive signal, any change in the extent of induction caused by the loss of *lin-12* activity would be easy to detect. The fact that P5.p and P7.p can assume vulval fates in amorphic *lin-12* mutant animals indicates that they can assume vulval fates without the action of the lateral signalling pathway. It remains possible that in wild-type development, P5.p and P7.p assume the 2° fate not in response to *lin-3* but in response to a lateral signal from P6.p. This lateral signal could be mediated by *lin-12* or could be a novel

signal. It is possible to explicitly test whether P5.p assumes a vulval fate due to any lateral signals from P6.p by examining animals that are genetically mosaic for *let-23*. An animal that has wild-type *let-23* activity in P6.p and which has no activity of *let-23* in P5.p serves as a test case. If P5.p assumes a vulval fate in response to the inductive signal, then P5.p will assume a 3° fate in this mosaic animal because P5.p will not be able to respond to the inductive signal. If P5.p assumes a vulval fate in response to lateral signals, then P5.p will assume the 2° fate in this mosaic animal, because P6.p will be induced by the inductive signal and P6.p will be able to laterally signal P5.p to assume a vulval fate.

Boundary signals

The existence of a "boundary" signal that regulates whether a VPC should assume a 3° or 2° fate in response to low levels of *lin-3* activity has been invoked to explain both the non-graded patterns of vulval induction seen in hypomorphic *lin-3* mutant animals and the existence of hybrid vulval/non-vulval fates. The existence of a boundary signal is appealing for theoretical reasons, since it would have a similar function in the 2°/3° fate choice that the *lin-12* lateral signal has in the 1°/2° fate choice. For P6.p and P5.p the graded distribution of *lin-3* activity should promote P6.p to assume the 1° fate and P5.p to assume the 2° fate. The *lin-12* lateral signal measures differences in the amount of inductive signal received between P5.p and P6.p to ensure that P5.p assumes the 2° fate and P6.p assumes the 1° fate. Likewise, for P5.p and P4.p the low levels of *lin-3* received by P4.p should cause P4.p to assume the 3° fate, but the action of a boundary signal that measures that P4.p is receiving less signal than P5.p would ensure that P4.p assumes the 3° fate. Some experimental evidence for the existence of a

boundary signal has been obtained (J. Simske, and S. Kim, personal communication). In animals heterozygous for hypermorphic *let-60* mutations, there is a low level of constitutive activity in the response pathway of the VPCs such that when the inductive signal has been removed by ablation of the gonad, an intermediate number of VPCs assume vulval fates. In such animals, P8.p often assumes a vulval fate when it has been isolated by ablation of its lateral neighbors, but rarely assumes a vulval fate when it has lateral neighbors. This suggests that when a VPC has low levels of activity in the *let-23* pathway, the decision to assume a vulval fate is influenced by its neighbors. An experimental test of whether a boundary signal is responsible for hybrid fate patterns in hypomorphic *lin-3* mutant animals could be a useful confirmation that the boundary signal exists.

**Materials and Methods.**

Vulval induction.

The extent of vulval induction was determined by examining the number and size of nuclei in the ventral epidermis at single time point per animal from the late-L3 to mid-L4 developmental stages as in (Aroian and Sternberg 1991). At this time the 3° lineage has produced two nuclei and the 1° and the 2° lineages are undergoing or have completed the third round of nuclear divisions. The progeny of the 3° lineage are larger and syncytial and the progeny of the 1° and the 2° lineages are smaller and cellular. The number of VPCs that have assumed vulval fates can usually be determined by the number and sizes of the nuclei present. In some cases, it can be determined with some certainty whether a VPC has assumed the 1° or the 2° fate.

A given *lin-3* heterozygous genotype was examined by setting up a mating in which the desired genotype was the only genotype present with a wildtype (non-Unc non-Dpy ) phenotype. The general mating is *lin-3(allele1) / ( lin-3(allele1) OR ( unc-24(e138) mec-3(e1338) dpy-20(e1282) ) )* males by *lin-3(allele2) dpy-20(e1282) / nT1[ unc(n754d)let ]; + / nT1* hermaphrodites. *nT1[unc(n754d)let]* is a chromosomal translocation that suppresses recombination in the *lin-3* region and which confers dominant Unc and recessive lethal phenotypes. Among the F1 self and cross progeny of this mating the only worms with a nonUnc non-Dpy-20 phenotype are of the genotype *lin-3(allele1) / lin-3(allele2)*. Each *lin-3* allele was tested for whether it caused a dominant defect in vulval induction by examining the F1 non-Unc non-Dpy cross progeny of the mating N2 males by *lin-3(allele1) dpy-20(e1282) / nT1[unc(754d) let]* hermaphrodites. Hemizygous *lin-3* genotypes were examined by using the same genetic strategy but with hermaphrodites

of the genotype *sDf64 unc-31/ nT1[unc(n754d)let].* sDf64 uncovers genetic markers that flank *lin-3* on the left and right including *dpy-20* and *let-60* and fails to complement *lin-3* mutations.

Lineage analysis.

Lineages were obtained by direct observation of the nuclear divisions in living animals under Nomarski optics as described (Sulston and Horvitz 1977). Lineages of *lin-3* transgenic strains were typically started in the L3 stage before the second round of cell divisions in the VPC lineages has started. At this point the 1° and 2° lineages are indistinguishable and thus a population of worms selected for lineage analysis at this stage should not be biased for particular patterns of 1° and 2° lineages. Starting the lineages at the two cell stage is also necessary to ensure that the lineal history of the vulval progeny is assigned correctly, since in some *lin-3* transgenic animals the grandprogeny of the VPCs proximal to the AC intermingle and thus their lineal history can only be assigned by direct observation of the second round of cell division.

Lineages of heterozygous *lin-12* mutant animals bearing *lin-3* transgenes were obtained by examining non-Unc-31 non-Unc-32 non-Dpy-19 segregants from the strain PS1274 for the presence of an AC. The lineages of the VPCs were followed in animals that had an AC and which could thus express the *lin-3* transgenes.

Fate assignments of VPC lineages.

The wild-type 2° lineage is LLTN and the progeny undergo an asymmetric invagination in which the daughters of the L cells adhere to the ventral cuticle. The wild-type 1° lineage is TTTT and the progeny undergo a

symmetric invagination. The following lineage features are considered to characteristics of the 2° lineage: i) progeny cells that adhere to the cuticle; ii) an invagination that initiates beneath one lateral half of the lineage; iii) VPC progeny dispersed at the 4-cell stage; iv) presence of a N-like nucleus that remains small, moves dorsal and either does not divide or divides late; v) proper division axes. The following lineage features are considered to be characteristics of a 1° lineage: i) lack of adherence to the ventral cuticle; ii) an invagination that initiates beneath the two central grandprogeny, iii) VPC progeny spaced closely at the 4-cell stage; iv) timing pattern in which two outside cells divide well before the two inside cells; v) proper division axes. The division axis is not an absolute indicator of fate. For example, there are a number of examples in which P3.p or P8.p assumed a fate that is typical of a 1° fate except that the lineage is LTTL. The most important feature for assigning the 2° fate is the presence of adherent progeny. Otherwise the most important feature for distinguishing 1° from 2° fates is the morphogenesis displayed by the progeny. Progeny of a 1° lineage should invaginate symmetrically and early compared to progeny of a 2° lineage. Cells from adjacent 1° lineages should crowd together and invaginate as a group with no attachment to the cuticle. Morphogenesis is sometimes ambiguous in multivulva mutant animals because the timing and extent with which the progeny of a VPC invaginate is affected by its environment. The progeny of a 1° lineage may invaginate more fully if it is beneath the AC. The progeny of a 2° lineage may invaginate more quickly if it is cooperating with a 1° fate.

It is often ambiguous whether the vulval half of a hybrid vulval/non-vulval fate is 2°-like or 1°-like fate. Both halves of a 1° lineage produce 4 vulval nuclei that do not adhere to the ventral cuticle. The half of the 2°

lineage proximal to the anchor cell in wildtype produces 3 non-adherent vulval nuclei and the half of the 2° lineage distal to the anchor cell in wildtype produces 4 nuclei that adhere to the vulval cuticle. In some instances, for example in wildtype at 25°C or in *lin-3* transgenic animals the half of the 2° lineage proximal to the anchor cell produces 4 vulval nuclei and it is not yet evident whether this happens in hypomorphic *lin-3* mutant animals. Hybrid vulval/non-vulval P6.p lineages often produce 1 syncytial nucleus and 4 non-adherent vulval nuclei and thus appear to be hybrid 3°/1° fates. They could, however, be hybrid 3°/2° hybrids in which there is an extra division in the 2° part of the lineage. If the vulval cells adhered to the anchor cell then this would indicate that they had 1° character. Hybrid lineages of P5.p and P7.p often include 1 syncytial nucleus and three non-adherent vulval nuclei and thus appear to be 3°/2° fates. Hybrid fates with 1 syncytial nucleus and 4 adherent nuclei have not been noted in hypomorphic *lin-3* mutant animals.

Strains.

Strains were maintained at 20° C as described in (Brenner 1974).

BC3203 *sDf64 unc-31(e169) / nT1 IV; + / nT1 V*

CB169 *unc-31(e169)*

CB1417 *lin-3(e1417)*

MT2375 *dpy-19(e1259) lin-12(n137) /unc-32(e189) lin-12(n676n909); him5(e1467*

N2

PS97 *lin-3(e1417); him-5(e1490)*

PS98 *lin-3(n378)*

PS100 *lin-3(n378); him-5(e1490)*

PS377 *nT1[unc(n754d) let] /unc-22(s7) unc31(e169) IV; nT1/+ V*

PS472 *nT1[unc(n754d)let] /lin-3(n1058) IV; nT1/+ V*

PS484 *nT1[unc(n754d) let] /unc-24(e138) lin-3(n1059) dpy-20(e1282) IV; nT1/+ V*

PS519 *lin-3(sy91)*

PS877 *nT1[unc(n754d)let] /mec-3(e1338) lin-3(sy53) dpy-20(e1282) V; nT1/+ V*

PS1167 *lin-3(sy91); him-5(e1490)*

PS1213 *unc-31(e169); syEx13*

PS1232 *nT1[unc(n754d)let] /lin-3(n1058) dpy-20(e1282) V; nT1/+ V*

PS1244 *nT1[unc(n754d) let] /lin-3(sy91) dpy-20(e1282) IV; nT1/+ V*

PS1245 *dpy-19(e1259) lin-12(n137) /unc-32(e189) lin-12(n676n909); unc-31(e169)*

PS1274 *dpy-19(e1259) lin-12(n137) / unc-32(e189) lin-12(n676n909); unc-31(e169); syEx13*

PS *nT1[unc(n754d) let] /lin-3(n378) dpy-20(e1282) IV; nT1/+ V*

PS *nT1[unc(n754d)let] /sDf64 unc-31(e169) V; nT1/+ V*

PS *unc-31(e169); syIs* aka VII, putative integrant of *syEx13*

**Figures and Tables.**

Figure 1. Three-signal model of VPC fate determination.

The anchor cell of the somatic gonad is proposed to release the Lin-3 inductive signal in a gradient. In response, the three more proximal of the six Vulval Precursor Cells (VPCs) are induced to assume vulval fates. A lateral signal among the induced VPCs promotes the 2° fate and inhibits adjacent VPCs from assuming the 1° fate. The three induced VPCs assume a pattern of fates of 2° 1° 2°. An induced VPC may choose between the 1° fate or the 2° fate either in response to the absolute level of Lin-3 that it receives or in response to lateral interactions whose outcome is biased by the graded distribution of Lin-3. An inhibitory signal that may arise from the hyp7 syncytial epidermis prevents the VPCs from assuming vulval fates in the absence of the inductive signal.

**Figure 1. Three-signal model of VPC fate determination.**

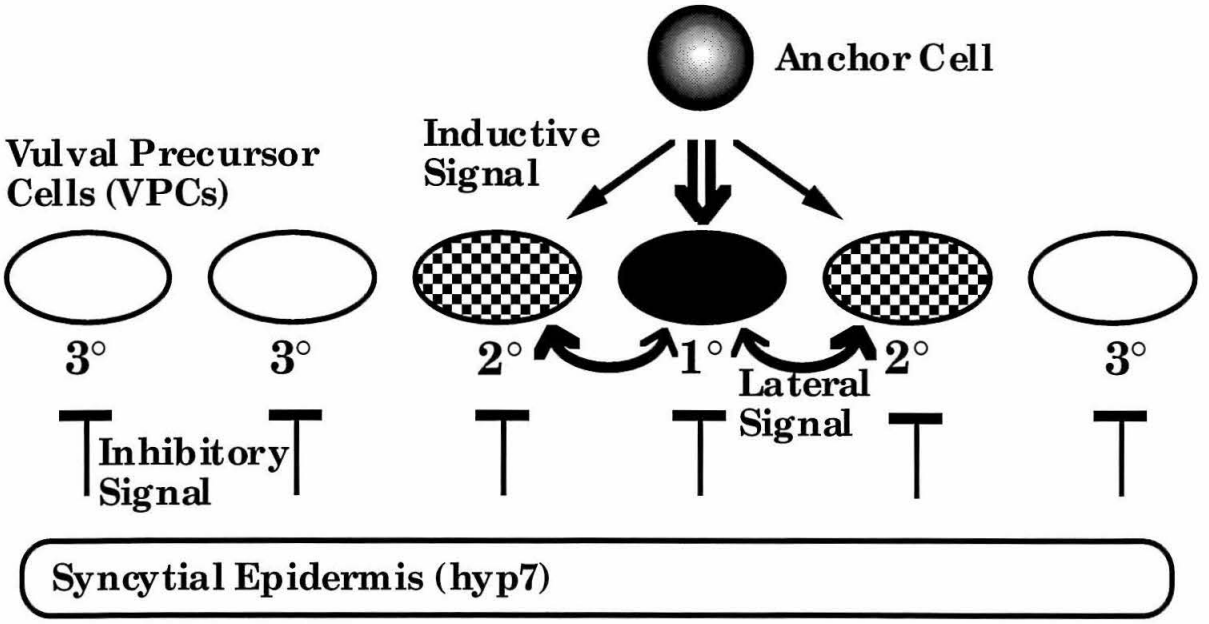


Figure 2. Patterns of vulval induction in *lin-3* hypomorphic mutant animals.

In the pattern box on the left, each row represents a different pattern of VPC fate choice. Each circle represents one of the six VPCs with the circle on the left being P3.p and the circle on the right being P8.p. An empty circle represent a VPC that has assumed the 3° epidermal fate and a black circle represents a VPC that has assumed a 1° or 2° vulval fate. Circles that are half-filled represent hybrid vulval fates. Asymmetric patterns that are mirror images of each other are represented as one pattern. For example, an animal in which P5.p and P6.p have assumed vulval fates is considered to be equivalent in pattern to an animal in which P6.p and P7.p have assumed vulval fates. A pattern is considered to be graded when only the VPCs closer to the AC have assumed vulval fates. Any pattern that has a VPC with an epidermal fate closer to the AC than a VPC with a vulval fate is considered to be non-graded. The graded patterns are placed as a series in the top of the left box and the non-graded patterns are placed in the bottom of the left box. Each box to the right of the pattern box represents a particular *lin-3* genotype. The percentage of animals of that genotype with a given pattern of fate choice is represented as a bar on the same line as the given pattern in the pattern box. For example, in 66% of *n378* homozygote animals no VPCs assumed vulval fates. This graphic representation allows a rapid visual analysis of the trends in patterning in different *lin-3* genotypes. In *n378* mutant animals most induction patterns are graded. The distribution of fate patterns is simple with most animals having little of no vulval induction and only rare animals having more than 1 induced VPC. In *sy91, n1058,* and *n378/sy53* mutant animals the distribution of fate patterns is complex and is not clustered on a single average value. There is a high fraction of animals with three induced VPCs in each of these genotypes.

**Figure 2A.**
**Induction patterns in n378 self progeny**

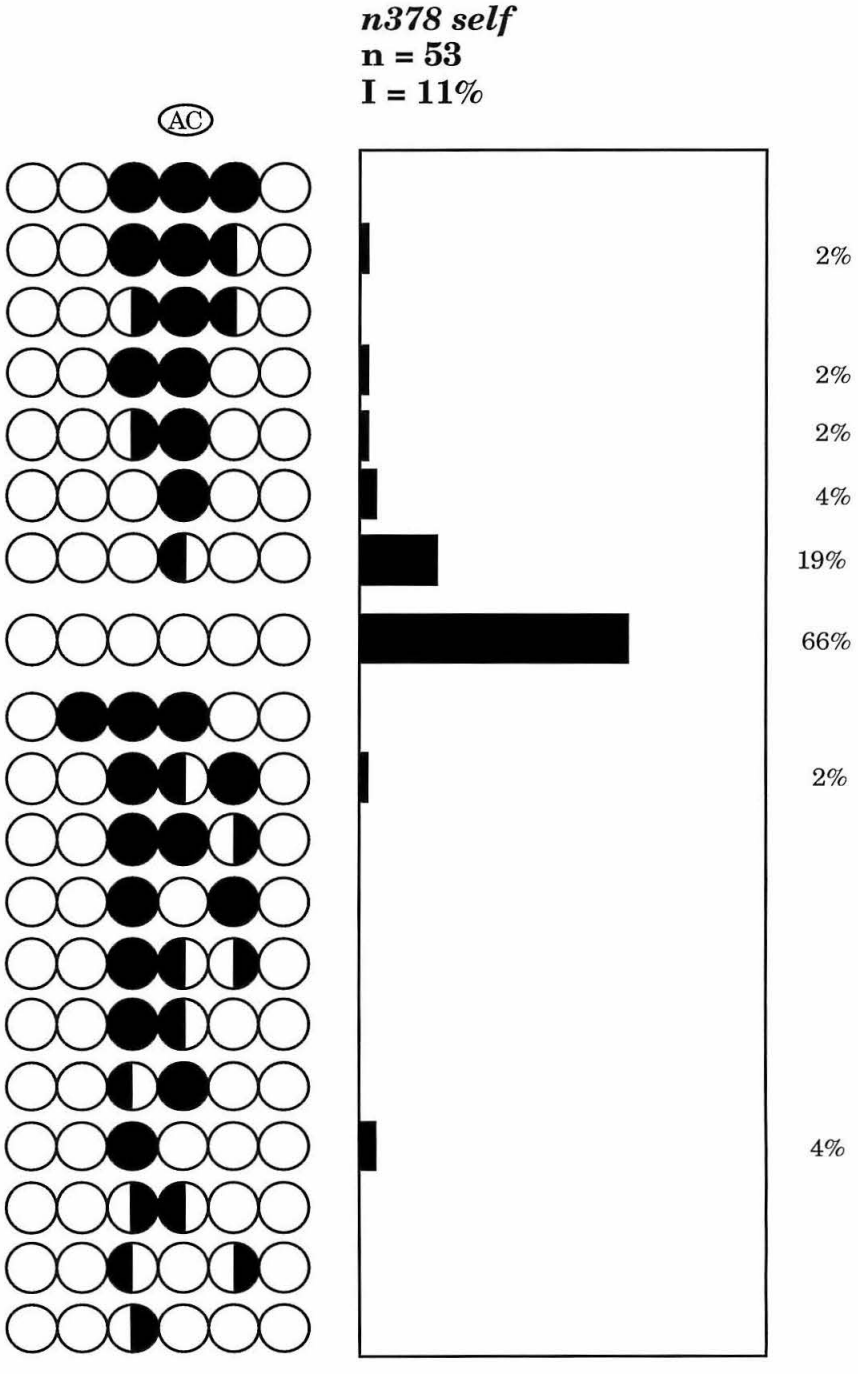


**Figure 2B.**
**Induction patterns in *sy91* self progeny.**

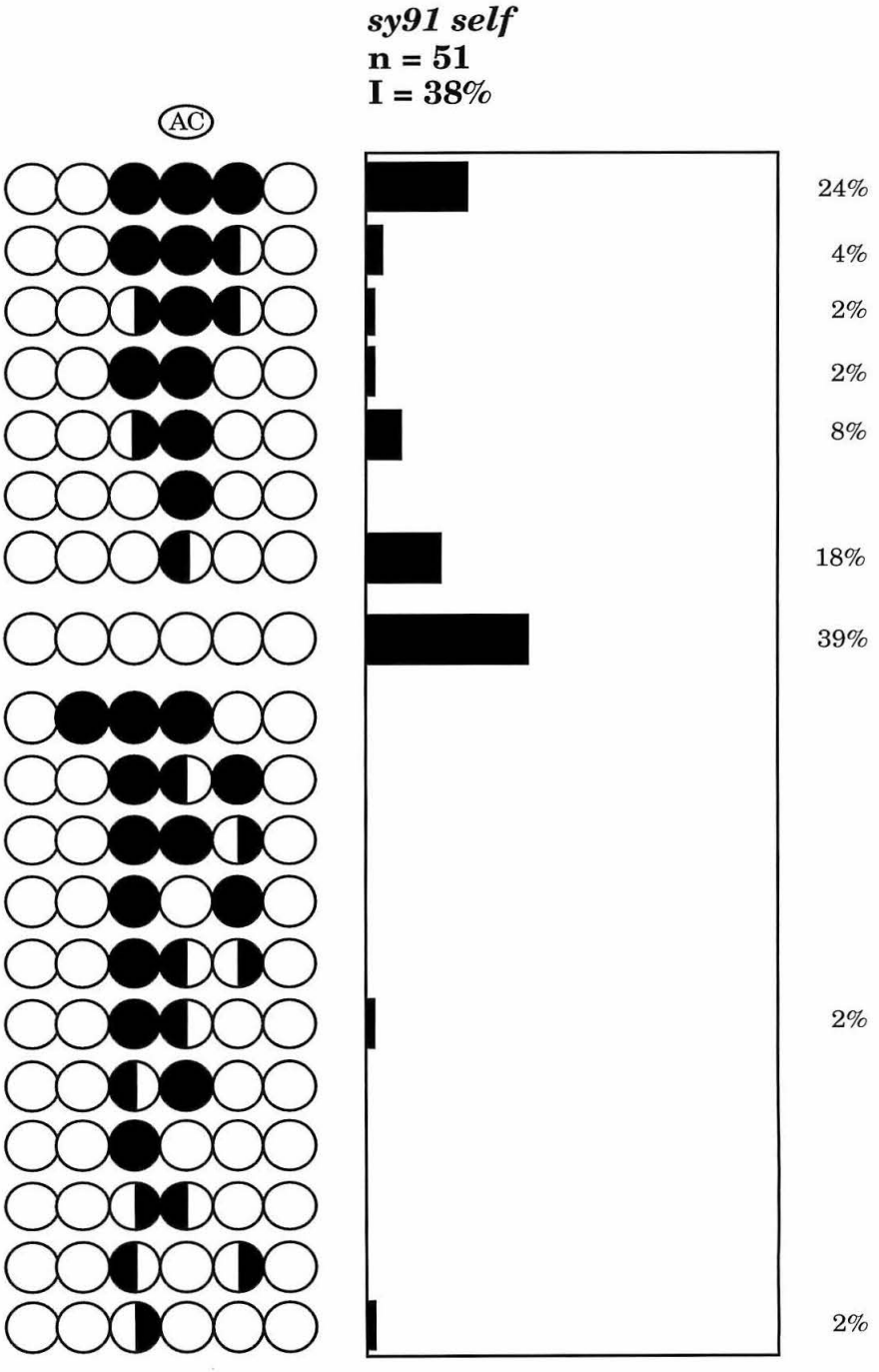


**Figure 2C.**
**Induction patterns in *n1058*.**

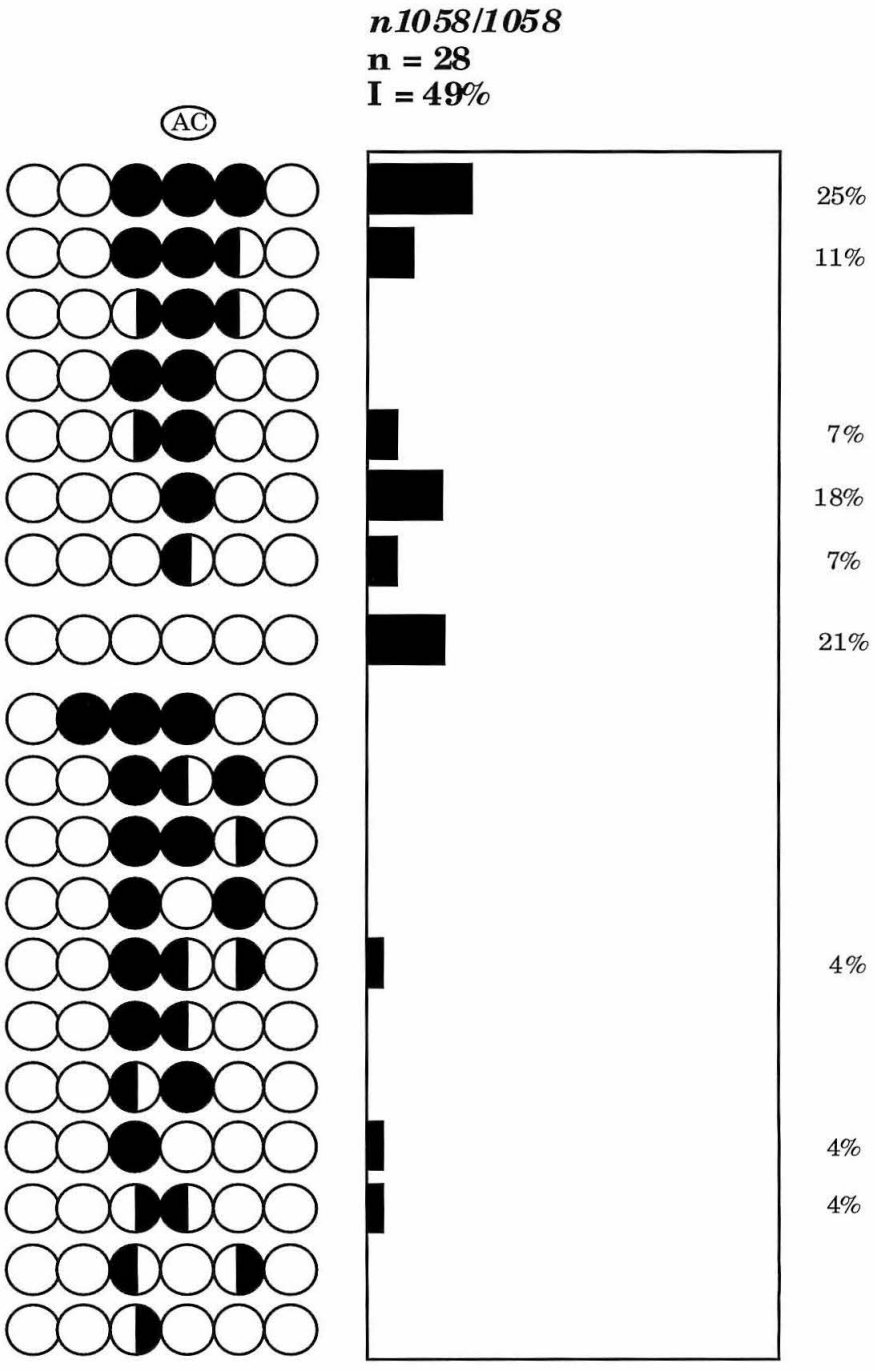


**Figure 2D.**
**Induction patterns in *n378/sy53*.**

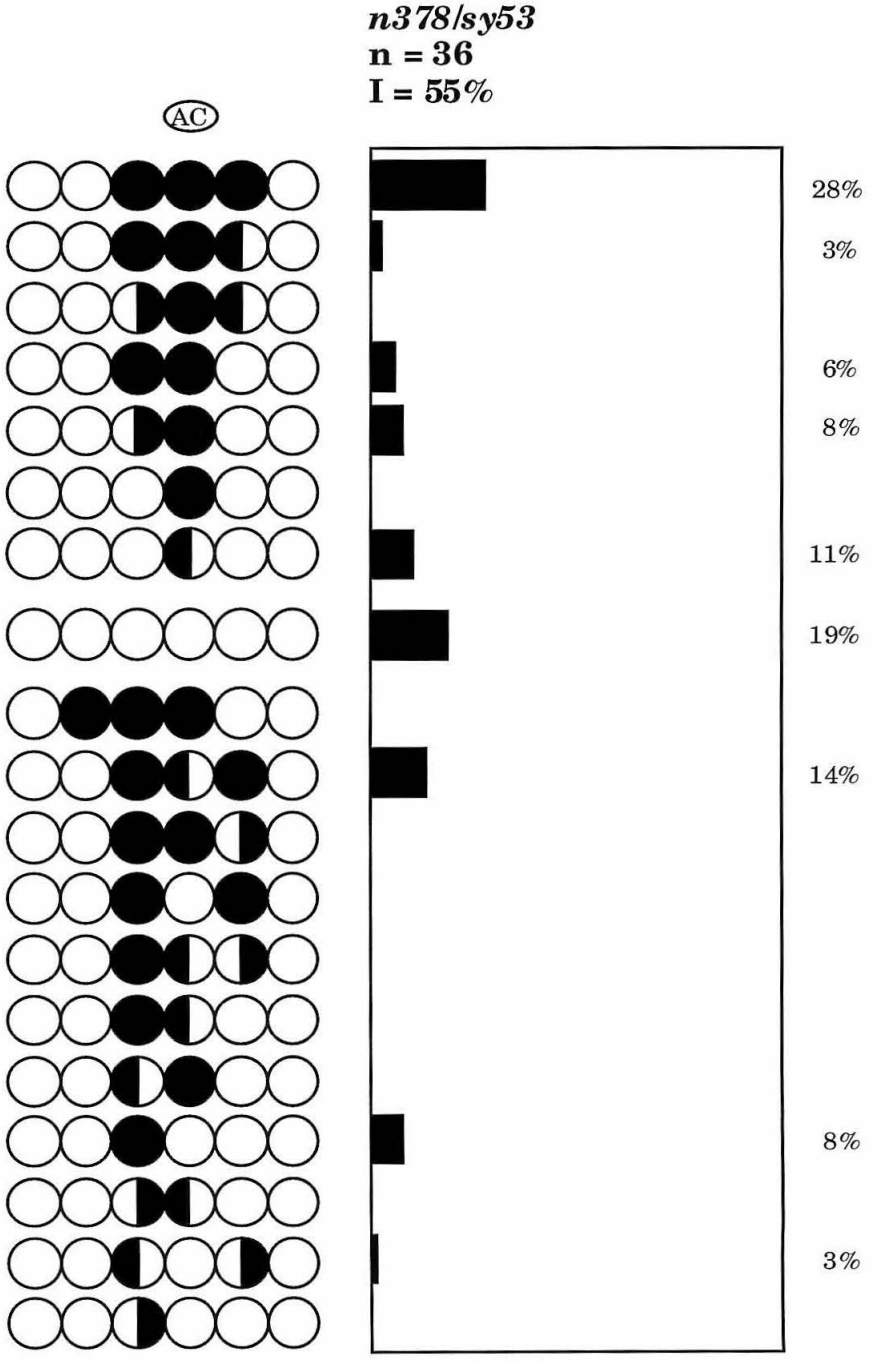
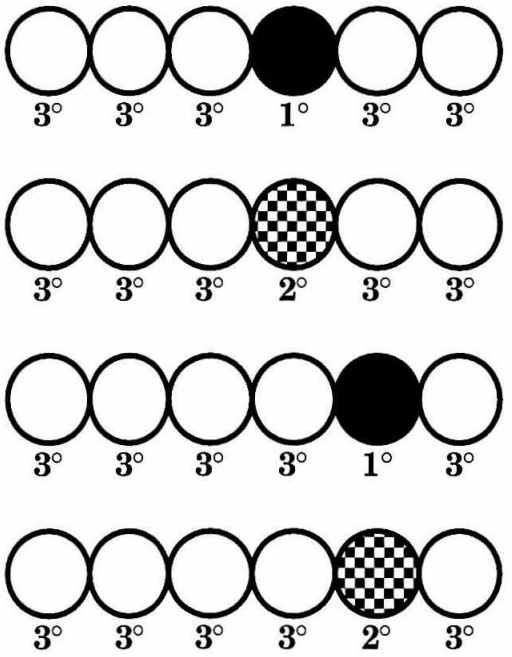


Figure 3. VPC fate choice in *lin-3* hypomorphic mutants. For some of the *lin-3* mutant animals in Figure 2, it is possible to determine if an induced VPC assumed a 1° or 2° fate. In general P6.p assumes either the 1° fate, the 3° fate or a hybrid vulval/non-vulval fate, but not the 2° fate; and P5.p and P7.p assume either the 2° fate, the 3° fate or a hybrid vulval/non-vulval fate but not the 1° fate. Shown in the figure are the fate choices of VPCs that are the only VPC to assume a vulval fate in an animal, and which have not assumed a hybrid vulval/non-vulval fate. P6.p assumed the 1° fate in the single case in which it was the only induced VPC. In the 6 cases in which P5.p or P7.p was the only induced VPC it assumed the 2° fate in all six cases. Notably absent are cases in which P6.p has assumed the 2° fate. It was not possible to determine VPC fate choice in all animals and it is easier to analyze some fate patterns then others. Thus there is an observational bias for the frequency of some fates if the entire panel of *lin-3* mutant animals is considered. The fate choice of a single responding VPC though is easy to determine so the population of single responding cell animals should not be subject to such observational biases. In some *lin-3* mutant animals the number and position of the vulval nuclei are abnormal.

**Figure 3. VPC fate choice in *lin-3* hypomorphic mutant animals.**




| *n378* | *n1058* | *sy91* | *n378/sy53* |
|---|---|---|---|
| | 1 | | |
| | | | |
| | | | |
| 2 | 2 | | 2 |

Figure 4. Lineage diagrams of *lin-3* transgenic animals.

The lineage diagrams of two animals from Table2A is shown. In animal 3B, P4.p, P5.p, and P6.p have 1° fates. In animal 3D, P4.p, P5.p P6.p and P7.p have 1° fates. The pattern of grandprogeny subfates often appears to be altered in *lin-3* transgenic animals when there are adjacent 1° fates. In wild-type the two outer granddaughters are considered to be of one cell type and the two inner granddaughters are considered to be of a second cell type. The outer granddaughters divide early and the inner granddaughters divide late and associate with the AC. In animal 3D, the grandprogeny of P4.p, P5.p and P6.p divide in an alternating pattern of early and late divisions. Alternating patterns of early and late divisions have been seen in amorphic *lin-12* mutant animals in which there are also adjacent 1° fates (P. Sternberg, personal communication). The lineage is represented according to convention (Sulston and Horvitz 1977). Vertical lines represent cells and a horizontal line represents a cell division The dotted lines indicate condensed time. In animal 3B, P7.paa ultimately divided in a transverse axis 45 minutes after ecdysis. The lineage of animal 3D was started at the 2-cell stage. The lineage of animal 3D was stopped for technical reasons.

Table 1 Extent of vulval induction as a function of *lin-3* genotype.

The number of VPCs assuming vulval fates in different *lin-3* genotypes is examined. Abbreviations: self: self progeny of a strain homozygous for a viable *lin-3* allele. The level of vulval induction in these strains could be subject to maternal affects. I: percentage induction as defined in (Aroian and Sternberg 1991), such that I = ( ( number of VPCs assuming vulval fates per animal in an experimental population) divided by 3, the number of VPCs assuming vulval fates per animal in wildtype) times 100. Induction can range from 0% when no VPCs assume vulval fates to 200% when all six VPCs

assume vulval fates. Wild-type induction of three VPCs is equal to 100% induction. Animals heterozygous for the genetic deficiency *sDf64* that uncovers *lin-3* and *let-60* have a slight defect in the extent of vulval induction, and a defect in the location of induction. In three of these animals P[4-6].p assumed vulval fates. It remains to be established whether a defect in the positioning of the anchor cell in these animals is responsible for the defect in induction. *sDf64* also uncovers the *let-60* locus which is also required for vulval induction. The pattern of fates of the *lin-3(-) /lin-3(+)* animals are all wildtype. n: number of animals examined. let: the given genotype confers larval lethality prior to the time of vulval induction.

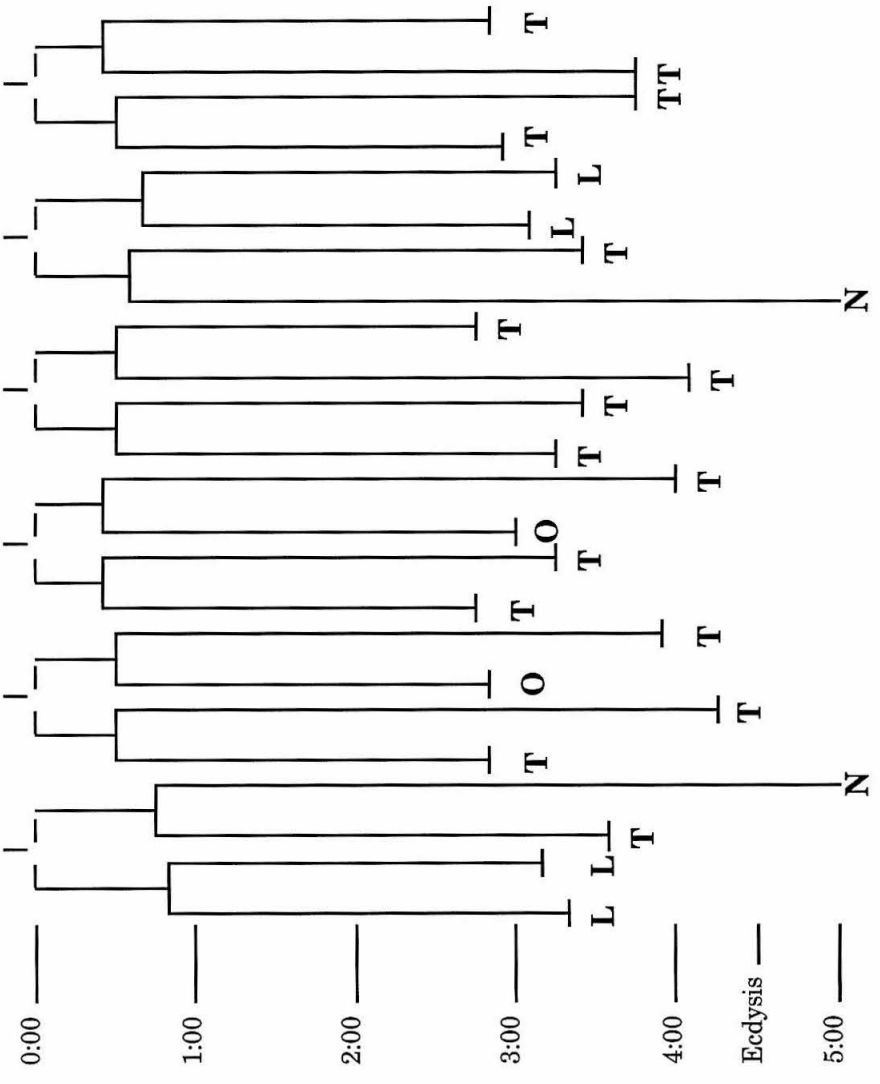



**Figure 4.A Lineage of *e169; syEx13* key 3B**

**Figure 4.B Lineage of *e169; syEx13*key 3D**

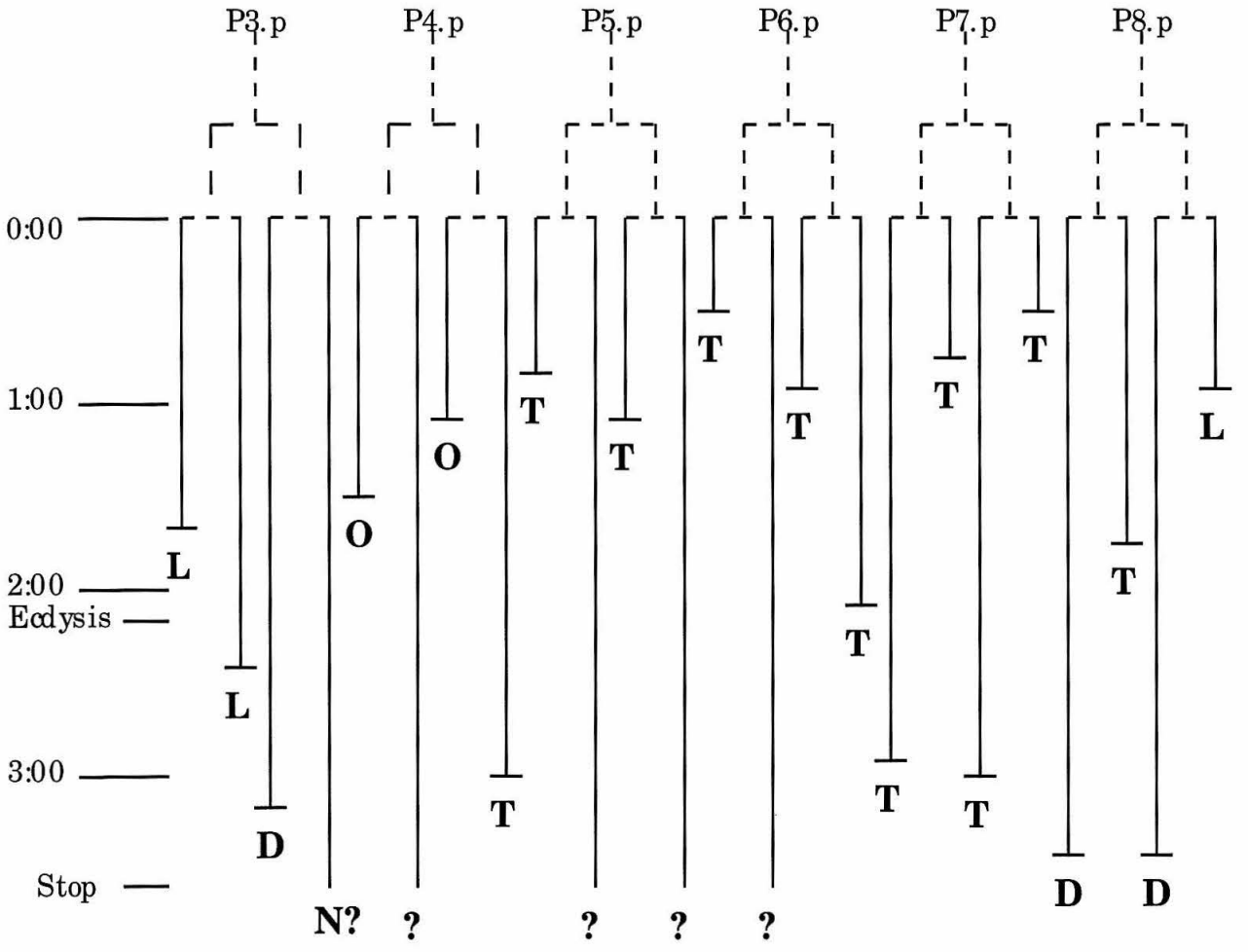


Table 1. Extent of vulval induction as a function of *lin-3* genotype.

| | Paternal | | | |
|---|---|---|---|---|
| | wildtype | | self | |
| Maternal | I | n | I | n |
| sy91 | 100 | 25 | 38 | 51 |
| n378 | 100 | 25 | 11 | 53 |
| n1058 | 100 | 25 | 28 | 49% |
| sy53 | 100 | 25 | | let |
| sDf64 | 26 | 98 | | |

Table 2. Vulval lineages of *lin-3* transgenic animals.

A. The lineages assumed by animals bearing the transgene *syEx13* which contains multiple copies of a 5-kb *lin-3* genomic clone(Hill and Sternberg 1992) are examined. *syEx13* can cause all six of the VPCs to assume vulval fates and is believed to over-express Lin-3 from the AC. Adjacent VPCs have 1° like fates in 4 of 12 *syEx13* mutant animals. In two of these animals there are at least three contiguous VPCs with the 1° fate such that there is a 1° VPC which has 1° VPCs for both lateral neighbors. This result suggests that the response pathway of the VPCs can not prevent adjacent VPCs from assuming 1° fates in response to high levels of *lin-3* activity.

B. The fate choice of VPCs in animals bearing the *lin-3* transgene *syEx13* and a hypermorphic *lin-12* mutation (*lin-12(d)*)are examined. *lin-12(d )* mutations cause all of the VPCs to assume the 2° fate, even in the absence of the inductive signal. Animals heterozygous for a *lin-12(d)* and a putative null allele of *lin-12* were examined because they can have an AC whereas *lin-12(d)* homozygotes never have an AC more infrequently (Greenwald, et al. 1983). The ability of the inductive signal produced endogenously by the AC to promote P6.p to assume the 1° fate is epistatic to the action of *lin-12(d)* mutations in promoting the 2° fate (Sternberg and Horvitz 1989). In all 9 of the *lin-12* heterozygous animals that have an AC, P6.p assumed the 1° fate. In six of the animals a VPC in addition to P6.p assumed a 1° or 1° like lineage, which indicates that *syEx13* can override the action of the *lin-12(d)* mutation to promote the 1° fate. There are no cases in which adjacent VPCs assumed 1° fates in the 9 animals, although in 2 cases, (key numbers 5G3, 5D4 ) P6.p assumed a 1° fate and a lateral neighbor of

P6.p assumed a fate with features of 1° and 2° fates. These lineages are 1° like in their lineage and morphogenesis except that a single grandprogeny adhered to the cuticle. The number of animals lineaged is too small to determine if the ability of *syEx13* to promote adjacent 1° fates in a *lin-12(d)* mutant animal is lower that its ability to promote adjacent 1° fates in an animal wild-type for *lin-12*. In three animals only P6.p assumed the 1° fate. *syEx13* is lost at a high frequency during mitosis and these animals could be chimeras that lack *syEx13* in the AC.

Abbreviations. Lineages are represented as by a single letter code for the behavior of each cell (Sternberg and Horvitz 1986). T: PN.pxx nucleus divided in a transverse axis. L: PN.pxx nucleus divided in a longitudinal axis. O: PN.pxx nucleus divided in an oblique axis. D: PN.pxx nuclei divided but the axis of division was unobserved. N: PN.pxx nuclei did not divide or divided more that 30 minutes after ecdysis. S: PN.p or PN.px nucleus joined epidermal syncytium. ?: Behavior of cell could not be determined, for example, animal lost during lineage. Underline: Progeny of cell adhered to ventral cuticle.

Table 2. Vulval lineages of *lin-3* transgenic animals.
A. *syEx13*

| Genotype | Key | Lineage | | | | | | Fate | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | P3.p | P4.p | P5.p | P6.p | P7.p | P8.p | | | | | | |
| *e169; syEx13* | 3A | S | LDT? | LTT? | T??T | TTTT | ?LTL | 3 | 2 | 2 | 1 | 1 | 2 |
| *e169; syEx13* | 3B | LLTN | TTOT | TTOT | TTTT | NTLL | TTTT | 2 | 1 | 1 | 1 | 2 | 1 |
| *e169; syEx13* | 3C | LLT? | LDT? | ODL? | TDDT | DO?L | ?TLL | 2 | 2 | 2 | 1 | 2 | 2 |
| *e169; syEx13* | 3D | LLD? | O?OT | T?T? | T?TT | TTTT | DTDL | 2 | 1 | 1 | 1 | 1 | 2 |
| *e169; syEx13* | 3E | S | LLON | TTTD | TTTT | TTLL | LLTL | S | 2 | 1 | 1 | 2 | 2 |
| *e169; syEx13* | 3F | S S | S D? | L?T? | T??T | ?TLL | ?D?? | 3 | 3/1 | 2 | 1 | 2 | ? |
| *e169; syEx13* | 3G | LLON | LLOD | LLOT | TTTT | TLLO | DTDL | 2 | 2 | ? | 1 | 2 | 2? |
| *e169; syEx13* | 3H | LLTN | LTOT | LLLL | TTTT | NLLL | LTLL | 2 | ? | 2 | 1 | 2 | 2? |
| *e169; syEx13* | 3I | OTTL | LLLL | TLLL | TTTT | NTLL | OTTO | 1 | 2 | 2? | 1 | 2 | 1 |
| *e169; syEx13* | 3J | TTTT | LLTN | TDTO | LTTT | NLLT | TTTL | 1 | 2 | ? | 1 | 2 | 1 |
| *e169; syEx13* | 3K | LTTO | LLTD | TDDT | OOOT | DLLL | DTDL | 1 | 2 | ? | 1 | 2 | 1 |
| *e169; syEx13* | 3L | S | LLLL | LOTT | TTTT | OTLO | TTOT | S | 2 | 2 | 1 | 2? | 1 |
| *syIs_* (aka VII) | 4A | L??? | LLTT | LLLT | TTTT | NLLL | TLTL | 1 | 2 | 2 | 1 | 2 | 2 |

B. *lin-12(d)/lin-12(0); syEx13*

| Genotype | | Key | Lineage P3.p | P4.p | P5.p | P6.p | P7.p | P8.p | Fate | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *lin-12(d)/(0); syEx13* | AC+ | 5A | LLTT | TTTO | OTLL | OOOT | LLLL | LLLL | 2 | 1 | 2 | 1 | 2 | 1? |
| *lin-12(d)/(0); syEx13* | AC+ | 5B | LTTT | LOOL | LOTT | TTTT | LLLL | OTTL | 1 | 2 | 2 | 1 | 2 | 1 |
| *lin-12(d)/(0); syEx13* | AC+ | 5C20 | LLTN | OLLO | OLTL | TTTT | TLLT | TTOT | 2 | 2 | ? | 1 | 2? | 1 |
| *lin-12(d)/(0); syEx13* | AC+ | 5D4 | LLLL | LLLO | TLLN | TTTT | LTTO | OTTL | 2 | 2 | 2 | 1 | ? | 1? |
| *lin-12(d)/(0); syEx13* | AC+ | 5D6 | LLON | LLTN | LLTN | TTTT | NTLL | NOLL | 2 | 2 | 2 | 1 | 2 | 2 |
| *lin-12(d)/(0); syEx13* | AC+ | 5E6 | LLTN | LLTD | LLTD | TTTT | NTLL | LLLO | 2 | 2 | 2 | 1 | 2 | 2 |
| *lin-12(d)/(0); syEx13* | AC+ | 5F | LLLL | LTTT | LTTD | TTTT | TOLL | TLLL | 2 | 2 | 2 | 1 | 2 | 2 |
| *lin-12(d)/(0); syEx13* | AC+ | 5G18 | LLTN | TOOD | TOTL | TOTL | LLLL | OTLL | 2 | 2 | 2 | 1 | 2 | 1 |
| *lin-12(d)/(0); syEx13* | AC+ | 5G3 | LLTN | TOOO | OTTL | TTTT | NTLL | TTTL | 2 | 2? | 1? | 1 | 2 | 2 |
| *lin-12(d);syEx13* | No AC | 5C4 | LLTL | LLTN | LLON | LLTN | NOLL | NTLL | 2 | 2 | 2 | 2 | 2 | 2 |

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# Chapter 5: Summary

The gene *lin-3* of *C. elegans* encodes an EGF-like growth factor that acts as an inductive signal during development. The gene *lin-3* was identified in the initial screens for mutants that affected the cell lineage (Horvitz and Sulston 1980, Sulston and Horvitz 1981). It was shown that reduction of function mutations of *lin-3* resulted in a developmental defect in which the Vulval Precursor Cells (VPCs) did not assume vulval fates. The VPCs normally assume vulval fates in response to an inductive signal from the anchor cell of the somatic gonad, and thus *lin-3* mutations phenocopy the effect of removing the inductive signal by ablation of the anchor cell with a laser microbeam (Kimble 1981). Subsequent genetic analysis indicated that *lin-3* acted early in a genetic pathway of genes required for vulval induction (Ferguson and Horvitz 1985, Ferguson et al. 1987). It was ambiguous from the available genetic evidence, however, whether *lin-3* was the vulval-inducing signal, and there was some concern whether the inductive signal had been, or could be, genetically identified. It is perhaps fitting from a genetic viewpoint, and probably especially to those who performed the initial characterization of the cell lineage mutants, that mutations in the vulval-inducing signal have been with the field essentially from the start. This thesis presents the molecular cloning of the *lin-3* locus and one step forward in the genetic analysis of *lin-3* function, the demonstration that *lin-3* is the vulval-inducing signal made by the anchor cell.

Chapter 1 presents an introduction to the role of *lin-3* in vulval development and is composed of two distinct parts: section 1 reviews the role of each of the three identified signals in determining the fates of the VPCs; and section 2 reviews the literature concerning the EGF group of growth

factors. Chapter 2 presents the molecular cloning of *lin-3* and molecular genetic experiments based upon this cloning. Together, these experiments demonstrate that *lin-3* encodes a vulval-inducing signal. Chapter 3 shows that the EGF-like domain of the Lin-3 protein is sufficient to induce both the 1° and the 2° vulval fates. These experiments support the model that *lin-3* acts in a manner similar to the EGF-like growth factors and suggest that *lin-3* is the sole vulval-inducing signal made by the gonad. Chapter 4 examines the pattern of vulval fates assumed in mutant animals with reduced *lin-3* activity and with increased *lin-3* activity. These experiments ask what information is conveyed by the *lin-3* signal. This chapter also presents possible experiments to further analyze the role of *lin-3* in establishing vulval fate pattern.

Section 1 of chapter 1 presents the current "three-signal model" (Horvitz and Sternberg 1991) of VPC fate determination. It presents a brief overview of the developmental biology of vulval development and the molecular characterization of the genetic pathway of vulval induction. Its main focus is the evidence for the three inter-cellular signals believed to control VPC fate determination, and the role of each signal in controlling the location, extent, and pattern, of vulval induction. The "three-signal model" proposes that an inductive signal made by the anchor cell is necessary to induce the VPCs to assume the 1° and 2° fates. The fate of an isolated VPC correlates with its distance from the anchor cell (Sternberg and Horvitz 1986). This result is the basis of the formal model that the inductive signal is distributed in a gradient centered on the anchor cell, and that the VPCs assume different fates in response to different levels of the inductive signal.

This interpretation suggests that the inductive signal plays an important role in establishing the pattern of VPC fates. A lateral signal (Sternberg 1988a), likely mediated by *lin-12* (Sternberg and Horvitz 1989) acts among the VPCs to prevent adjacent VPCs from assuming the 1° fate. This signal is believed to involve a feedback loop between cells that can measure and establish differences in the state of adjacent cells (Seydoux and Greenwald 1989, Sternberg 1988b). Each VPC is proposed to laterally signal its neighbors to be 2° in proportion to the level of inductive signal that it receives. An inhibitory signal that may arise from the syncytial epidermis (Herman and Hedgecock 1990) and which is mediated by a pathway of genes that includes *lin-15* (Ferguson and Horvitz 1985, Ferguson and Horvitz 1989) prevents the VPCs from assuming vulval fates in the absence of the inductive signal (Aroian and Sternberg 1991, Ferguson, et al. 1987).

The review in chapter 1 incorporates the new data on *lin-3* into the "three-signal model" including the evidence that *lin-3* encodes the vulval-inducing signal made by the anchor cell. Since *lin-3* transgenes can cause up to all six of the VPCs to assume vulval fates (Hill and Sternberg 1992), whereas reduction-of-function *lin-3* mutations prevent the VPCs from assuming vulval fates (Ferguson and Horvitz 1985), this suggests that the level of *lin-3* activity determines the extent of vulval induction. The review also notes that adjacent VPCs can assume the 1° fate in *lin-3* transgenic animals. This result suggests that high levels of *lin-3* activity specify the 1° fate. As lateral signalling among the VPCs can not prevent adjacent VPCs from assuming the 1° fate, this confirms that the dose of *lin-3* made by the anchor cell plays an important role in establishing the correct 2° 1° 2° pattern

of fates.

The second section of chapter 1 reviews the literature on the EGF-like growth factors. The EGF-like growth factors are inter-cellular signalling molecules that can act as secreted factors, but which are made as membrane bound precursors. As a group, these precursors contain essentially no sequence in common outside of the sequence of a structural domain called the EGF repeat (reviewed in (Carpenter and Wahl 1990)). EGF repeats are a structural motif that consist of six cysteine residues with semi-conserved spacing. The Lin-3 protein is a membrane-spanning protein with essentially no sequence similarity to any other protein. It does, however contain a single EGF repeat and thus its lack of sequence similarity makes it a respectable citizen of the EGF-like growth factor family. Many aspects of the molecular biology of the EGF-like growth factors are likely relevant for the function of *lin-3*. First, the EGF repeat of the growth factors is the ligand domain and is sufficient by itself to activate the EGF-Receptor. It is confirmed in chapter 3 that the EGF repeat of the Lin-3 protein is sufficient to induce vulval development. Second, processing of the EGF repeat from the membrane bound precursor does not appear to be necessary to activate the growth factor (Brachmann et al. 1989, Mroczkowski et al. 1989, Wong et al. 1989). Thus, it is possible that Lin-3 could act either as a membrane-bound ligand or as a secreted factor in the development of different tissues of *C. elegans*. Third, the processing of the EGF-like growth factors appears to be a regulated event. The regulation of where and when Lin-3 is processed could control the developmental specificity of *lin-3*. Fourth, in mammals there are multiple EGF-like growth factors that can act as ligands of the EGF-R. This raises the

possibility that there are multiple *lin-3*-like proteins that act in the development of *C. elegans*. It should be noted that *lin-3* and the gene *let-23* that encodes the putative receptor for Lin-3 have a similar range of phenotypes. Thus, there is currently no genetic evidence that the receptor encoded by *let-23* has multiple ligands.

On the other hand, the analysis of *lin-3* function should have relevance for the study of the EGF-like growth factors of mammals. First, the function of the EGF-like growth factors in development is largely unknown and the role of *lin-3* in development and be studied genetically. Second, the proteins involved in the processing of the EGF-like growth factors have not been identified. It might be possible to identify these proteins, or other proteins important in EGF-like growth factor function, by isolating genetic suppressors of reduction-of-function and gain-of-function *lin-3* genotypes. Third, *lin-3* shows alternative RNA-splicing that should result in proteins that differ in the region between the EGF domain and the transmembrane domain. This is where the membrane-bound precuror would be processed to release the EGF repeat. NDF/heregulin/GGF also show variable splicing in this region (Holmes et al. 1992, Marcchionni et al. 1993, Wen et al. 1992). It will be interesting to see if this alternative splicing is used to control growth factor activity in *C. elegans* and mammals.

Chapter 2 presents the molecular cloning of the *lin-3* locus. A transposon-induced allele of *lin-3* was obtained in a non-complementation screen for new alleles of *lin-3*. The transposon insertion *syP1* was shown to be genetically inseparable from the vulvaless phenotype of the transposon-induced allele. The DNA sequence flanking *syP1* was directly amplified from

genomic DNA by inverse PCR and was used to isolate genomic DNA clones. A 3.3-kb genomic region was shown to be sufficient for DNA-mediated transformation rescue of *lin-3* mutations. The nucleotide sequence of this 3.3-kb region and of homologous cDNAs was determined. This region could encode a membrane-spanning protein of over 400 amino acids that contains a single EGF repeat in the amino terminal domain. The identification of this transcription unit as *lin-3* was confirmed by two facts. First, a second DNA polymorphism associated with the mutation *lin-3(n1058)* is present in this genomic region. Second, the introduction of site-directed mutants that would disrupt the disulfide-bonding pattern of the EGF repeat abolished the ability of the DNA clones to rescue *lin-3* mutations.

The molecular cloning of *lin-3* generated multicopy *lin-3* transgenes that confer a strong multivulva phenotype. The multivulva phenotype of the transgenes was a significant breakthrough and was important for demonstrating that *lin-3* could act as a vulval-inducing signal. The evidence that *lin-3* is a vulval inducing signal is as follows. First, the vulvaless reduction-of-function and multivulva gain-of-function *lin-3* phenotypes together suggest that the extent of vulval development is sensitive to the dose of *lin-3* activity. Second, the Muv phenotype of the transgenes enables a direct test of genetic epistasis between *lin-3* and *let-23*. The vulvaless phenotype of a strong reduction-of-function mutation in *let-23* is epistatic to the multivulva phenotype of the *lin-3* transgenes. This suggests that *lin-3* is an upstream activator of *let-23* and is consistent with the model that *lin-3* encodes a ligand for the EGF-Receptor-like protein encoded by *let-23*. Third, ablation of the gonadal precursor cells reduces the ability of the *lin-3*

transgenes to stimulate vulval development suggesting that *lin-3* acts in the gonad and not in the VPCs to stimulate vulval development. Fourth, a *lin-3::lacZ* fusion transgene that contains the EGF repeat of Lin-3 and that retains the ability to induce vulval fates is expressed specifically in the anchor cell at the time of vulval development.

The third chapter describes the construction of transgenes that express a secretable version of the EGF domain of Lin-3 under the control of a tissue general heat shock promoter. These transgenes can induce vulval development when the entire gonad has been destroyed at hatching with a laser microbeam. This surgery prevents the development of the anchor cell and removes the normal source of the vulval-inducing signal. This result indicates that Lin-3 is sufficient, at least when overexpressed, to replace the function of the anchor cell in inducing vulval fates. This result also indicates that the EGF domain of Lin-3 can induce vulval development without the rest of the Lin-3 protein. This indicates that Lin-3 can function in a manner analogous to the EGF-like growth factors. Single VPCs isolated by the ablation of the gonad and the five other VPCs can assume either the 1° or the 2° vulval fates in response to these Lin-3 transgenes. This result confirms that Lin-3 can specify either vulval fate without the action of a lateral signal from one VPC to another VPC. The results presented in this chapter suggest that *lin-3* is the sole vulval-inducing signal made by the anchor cell.

The fourth chapter returns to the issues raised in the introduction and examines the question of what information is carried by the inductive signal. In particular, are there distinct doses of *lin-3* activity that specify a VPC to assume the 1° or the 2° fate? It is noted that in *lin-3* transgenic animals that

adjacent VPCs can assume the 1° fate and thus that the *lin-3* transgenes override, or interfere with, the lateral signal among the VPCs that normally inhibit adjacent 1° fates. This result suggests that a high dose of *lin-3* specifies the 1° fate. Specification of the 2° fate appears to require activity of the gene *lin-12* (Greenwald et al. 1983) and Lin-12 protein is probably activated in wild-type development by lateral signalling among the VPCs. This could indicate that the 2° fate is specified not by a particular dose range of *lin-3* activity but rather by the combinatorial action of both the lateral and the inductive signal. However, isolated VPCs have been observed to assume the 2° fate (Hill et al. in preparation, Sternberg and Horvitz 1986) which indicates that lateral signalling between VPCs is not required to get the 2° fate. This result indicates that there are situations in which the inductive signal can specify the 2° fate, either independently of *lin-12,* or perhaps by activating the Lin-12 protein within that cell. It has not been demonstrated if there is a dose of *lin-3* activity that consistently specifies the 2° fate. Moreover, it appears that in certain dose ranges of the inductive signal that the lateral signal can specify a cell to assume either the 1° or the 2° fate. Experiments that would test if there are different doses of *lin-3* activity that specify different fates are described in chapter 4.

Chapter 4 also presents the data from the analysis of the fate patterns assumed by the VPCs in hypomorphic *lin-3* mutant animals. This analysis tests two possible predictions of the "three-signal model." First, if there is a distinct dose of *lin-3* activity that specifies the 2° fate, then P6.p should be consistently exposed to this dose range in some *lin-3* genotypes. This has not been observed, although it is possible that this is because an insufficient

number of animals has been examined. Second, if the inductive signal is distributed in a gradient, then the VPCs should still respond in a graded fashion as the activity of the inductive signal is genetically lowered. In some reduction-of-function genotypes the VPCs usually respond in a graded manner. However, the VPCs behave in ways that suggest that VPC fate determination is not as simple as stated in the "three-signal model." These behaviors seem to be particularly pronounced in *lin-3* genotypes that have average levels of vulval induction that are 35% of the wild-type level. These behaviors include non-graded patterns of vulval induction in which VPCs further from the anchor cell assume vulval fates while VPC closer to the anchor cell assume epidermal fates; hybrid vulval/non-vulval fates; and apparent cooperativity in the extent of induction. Explanations for these effects and experiments to test these models are given in chapter 4. A "boundary signal" that regulates the decision of a VPC receiving low levels of the inductive signal to assume a vulval or non-vulval fate could explain the hybrid fates and the non-graded patterns of induction. The apparent cooperativity in the extent of vulval induction could result from positive feedback loops in the production of the inductive signal, or from lateral signals that act among the VPCs.

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