THE *LET-23* GENE OF THE NEMATODE *C. ELEGANS*: GENETICS AND MOLECULAR BIOLOGY OF A MEMBER OF THE EGF RECEPTOR TYROSINE KINASE FAMILY

Thesis by

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Once upon a nematode...

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Dedications

"... and (render) to God the things that are God's."

Luke 20: 25

Pidar natawanad, Pisar tamam kunad.

(If the father cannot, the son may bring it to a conclusion.)

Get the facts straight

A guide was taking a party round the British Museum. "This sarcophagus is five thousand years old."

A bearded figure with a turban stepped forward.

"You are mistaken," said Nasrudin, "for it is five thousand and three years old."

Everyone was impressed, and the guide was not pleased.

They passed into another room.

"This vase," said the guide, "is two thousand five hundred years old."

"Two thousand five hundred and three," intoned Nasrudin.

"Now look here," said the guide, "how can you date things so precisely? I don't care if you do come from the East, people just don't know things like that."

"Simple," said Nasrudin. "I was last here three years ago. That time you said the vase was two thousand five hundred years old."

Moral: It's later than you think.

From "The Subtleties of the Inimitable Mulla Nasrudin" by Idries Shah, Octagon Press, London, 1985.

Abstract

Genetic studies indicate that the *let-23* gene affects several developmental decisions in the nematode *Caenorhabditis elegans*. *let-23* is required for the proper development of the hermaphrodite vulva, the male tail, and the posterior ectoderm. In addition, *let-23* mutations can cause lethality and hermaphrodite sterility. These five *let-23* functions can be independently mutated, suggesting that the *let-23* protein encodes tissue-specific functions. Furthermore, *let-23* controls two opposing pathways: one that stimulates and another that inhibits vulval development. These two pathways ensure that the proper level of vulval development occurs. Twenty *let-23* alleles exist: 14 eliminate function (null), three reduce function in all tissues (hypomorphic), and three reduce function in certain tissues (tissue-specific). In addition, two of these alleles are defective in the inhibitory vulval pathway.

The *let-23* primary structure resembles that of the mammalian epidermal growth factor receptor (EGFR). The *let-23* protein possesses putative ligand binding, transmembrane, and tyrosine kinase domains, as well as cysteine-rich regions, all with the characteristics of the EGFR family. Like *let-23*, mammalian EGFR is multifunctional, encodes tissue-specific functions, and functions in stimulatory and inhibitory pathways. *let-23* may be the receptor in the vulva for the anchor-cell inductive signal. Furthermore, genetic data indicate *let-23* acts upstream of the *let-60 ras* gene, supporting mammalian studies that suggest a link between EGFR and *ras*.

To investigate how EGFR primary structure relates to function, mutations in eight *let-23* alleles have been sequenced. Five null alleles alter sequences in both the kinase and the extracellular domains. These alterations suggest that *let-23* has kinase activity and that the extra cysteine domain found only in invertebrate EGFRs is important. A strong hypomorphic allele mutates one of the conserved extracellular cysteines close to the ligand binding domain. A tissue-specific allele mutates an intron/exon boundary in the C-terminus. This mutation suggests that the C-terminus can provide tissue-specific information. Finally, a hypomorphic allele that is defective in the *let-23* inhibitory vulval pathway alters a different intron/exon boundary in the C-terminus. This mutation results in numerous, unexpected transcripts. Models are suggested to account for the behavior of this allele.

Table of Contents

Summary

Chapters I, II, III and Appendix II in this thesis have been published or have been submitted for publication. This section provides an overview of each chapter in order to help unify the entire thesis. This section also provides supplementary information for the chapters and appendices.

Chapter I, submitted for publication as a book chapter, provides a comprehensive review of *Caenorhabditis elegans* (or *C. elegans*) hermaphrodite vulval development. My thesis focuses on the *let-23* gene, and a key element of this gene is its effects on vulval development. These studies of *let-23* were initiated because Paul Sternberg and Chip Ferguson, while graduate students in Bob Horvitz's laboratory, noticed that *let-23* mutations had diverse effects on the development of the hermaphrodite vulva. Most notably, the *let-23* allele *n1045* caused no vulval differentiation at 15°C and too much differentiation at 25°C. When he set up his own laboratory, Paul decided to pursue the *let-23* gene because of the pivotal role it seemed to play in the vulva. Although *let-23* studies have expanded beyond the vulva, many of the important results still relate to *let-23* role's in vulval differentiation. Chapter I includes and anticipates many of the results found in Chapters II, III, and IV. Chapter I also expands and updates some of my thinking about the models originally drawn in Chapter II. The models in Chapter I are still current (although probably not for long) with the notable addition that Russell Hill in our laboratory has cloned the *lin-3* gene. He has found that *lin-3* encodes an epidermal growth factor-like molecule, and he has accumulated convincing genetic and

molecular evidence that *lin-3* is the anchor-cell ligand for the *let-23* receptor tyrosine kinase (R. J. Hill and P.W. Sternberg, manuscript in preparation).

The publication included as Chapter II (Aroian and Sternberg, 1991) describes the first aspect of my research -- genetic characterization of the *let-23* locus. I took over from Paul two different mutagenesis screens that relied on *let-23* vulval phenotypes to isolate new *let-23* alleles. Paul isolated two and I isolated 14 new alleles. Two new *let-23* phenotypes came out of these screens. [Gregg Jongeward in the laboratory has since used two of these alleles to isolate suppressors of *let-23* and expand our understanding of vulval pathways. Helen Chamberlin in the laboratory has since characterized in detail the cellular basis of one of the new phenotypes, the male spicule defect, and has uncovered a new example of induction in *C. elegans*.] I then characterized the effects of the *let-23* gene by quantitating the severity of various phenotypes in many trans-configurations of these alleles. Briefly, the results of this analyses are: (1) complete loss of *let-23* function results in larval lethality; (2) *let-23* mutations affect at least five tissues; (3) loss of *let-23* function in the vulva leads to no vulval differentiation (*i.e.*, *let-23* is needed to stimulate vulval differentiation); (4) *let-23* appears to be required for inhibition of vulval differentiation (the opposing effects of stimulation and inhibition result in a proper level of vulval differentiation); and (5) the functioning of *let-23* in different tissues in independently mutable, suggesting that *let-23* has tissue-specific functions. I wish to acknowledge Roberta Goldstein for her help in this phase of the project. I apologize for omitting her name from the publication acknowledgements.

The publication included as Chapter III (Aroian *et al.*, 1990) describes the second aspect of my research -- the cloning of the *let-23* gene.

My contributions to this publication include: mapping of the *let-23* gene relative to the Tc5A polymorphism, YAC analysis, making and probing of Southern blots to try to find polymorphisms in our 20 alleles, design and execution of germ line transformation experiments to locate the *let-23* gene, and sequencing of the *let-23(sy5)* mutation. At the time, germ line transformation was not as commonplace as it is now, and my *let-23* transformation experiments were the first successful ones in our laboratory. Briefly, the results of this study are: (1) the *let-23* amino acid sequence resembles mammalian epidermal growth factor receptor (EGFR); (2) *let-23* provides a convincing example of a receptor tyrosine kinase used in development; (3) *let-23* is probably the receptor in the vulval precursor cells for the anchor-cell signal; and (4) the vulval pathway, which includes *let-23* upstream of *let-60 ras*, supports a link between EGFR and *ras* as inferred from studies in mammalian systems.

Chapter IV includes the final phase of my thesis and is unpublished. The aim of this final project is to unite the findings of Chapters II and III by sequencing the mutations associated with various *let-23* alleles. To date, five null alleles, one hypomorphic allele, one tissue-specific allele, and one allele defective in the vulval inhibitory pathway have been sequenced. This study makes some preliminary and novel inroads as to how *let-23* structure relates to function and demonstrates the advantages in using *let-23* to study EGFR. This study also uncovered unprecedented results in splicing.

Appendix I includes some information omitted from Aroian and Sternberg (1991). Specifically, it includes the effect of temperature on all *let-23(n1045)* phenotypes.

Appendix II includes some genetic work I performed on the *let-60 ras* project and was published as Han *et al.* (1990). My contributions

include: isolation of the first dominant negative allele of this gene; initial characterization of the mutation as dominant vulvaless, recessive lethal; initial mapping of the gene to the *dpy-20* region of LG IV; epistasis with *let-23*; gathering of other genetic data; and an active involvement in interpreting data and planning experiments.

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Chapter I

Signal transduction during *Caenorhabditis elegans* vulval determination

Raffi V. Aroian and Paul W. Sternberg

Submitted for publication in "Molecular Mechanisms of Signal Transduction in Genetically Tractable Organisms," R.P. Dottin, J. Kurjan, and B.L. Taylors, eds., Academic Press, Florida.

I. Introduction -- general aspects of signal transduction and *C. elegans* development

Development of multicellular organisms involves the proliferation of the single-celled egg into numerous cells that can subsequently differentiate into discrete cell types, *e.g.*, liver, muscle, neuronal, as the organism matures. There are thought to be two primary mechanisms responsible for this development. The identity of cells can be determined solely by factors inherited by their progenitor. This type of development is **cell autonomous** because cells take on identity without informational input from neighboring cells or the environment. Conversely, the identity of cells can be specified by their location, *i.e.*, where they are and what other cells they neighbor. This type of development is **cell non-autonomous** because the developing cells require information from the outside to determine what changes need to occur inside. This type of development requires signal transduction, the mechanism by which cells take in information from their surroundings. (For an in-depth review of the processes involved in animal development, see Davidson, 1990.)

The development of the free-living nematode *C. elegans* provides a unique opportunity to study signal transduction by means of three methods: cell biology, genetics, and molecular biology. Each adult *C. elegans* hermaphrodite contains 959 somatic nuclei, the identity and relative geometry of which are known and essentially identical. Each individual hermaphrodite can therefore be thought of as an identical "tissue-culture dish" in which a complex arrangement of known cells and tissues is faithfully reproduced, providing an excellent opportunity for *in vivo* **cell biology**. By using a laser microbeam and various mutations, the local

environment around a cell or group of cells can be perturbed and the effects on those cells assessed. This analysis can yield insights into how various cells and tissues interact. **Genetics** can be used to identify and characterize the genes involved in a given developmental pathway since small perturbations in the molecules involved in development can have major consequences on the final outcome (*e.g.*, hermaphrodites that cannot lay eggs). The interactions of the genes can also be ascertained by examining phenotypes of double mutants. Chromosomal deficiencies and duplications allow one to vary dose in a controlled way. Mosaic analysis can be used to determine in which tissues a specific gene acts. Lastly, **molecular biology** can be used to identify the proteins involved in development, and molecular genetic techniques, *e.g.*, transgenic nematodes, can be used to probe the function of these molecules. As discussed below, the study of the development of the hermaphrodite vulva makes use of all these tools to unravel what has turned out to be a complex and fascinating signal transduction pathway.

Since the power of vulval development as a system to study signal transduction relies on cell biology, genetics, and molecular biology, this review offers a comprehensive look at each. Vulval development is introduced through cell biology and cell biological experiments summarized in Figure 7. These experiments were the first to suggest that vulval induction involves signal transduction. Next, the isolation of mutations in genes responsible for vulval development will be discussed, followed by the molecular identification of some of these genes. Some are similar to signal transduction components of other systems, such as the epidermal growth factor (EGF) receptor, *ras*, and *Notch*. Genetic

experiments that order the genes in the vulval pathway and that elucidate the interactions among these genes are reviewed. This information is combined with cell biology and molecular biology data to model the signal transduction events responsible for vulval development (summarized in Figure 19) and to model how three different outcomes from those signal transduction events are produced (summarized in Figure 23).

In addition to providing a summary of data and interpretation, this review also discusses how the data were obtained, on what basis a given interpretation is made, and caveats in making that interpretation. This attention to thinking as well as data serves several purposes: 1) it provides for a deeper understanding of the field; 2) it permits many different aspects of vulval determination to be brought together in a single review; and 3) it provides a foundation for understanding future results -- not only what those results mean but how they were obtained. For a briefer review on vulval determination, see Horvitz and Sternberg, 1991.

II. Cell biology of the *C. elegans* hermaphrodite vulva – development and signal transduction

A. Development of the vulva

C. elegans comes in two sexes, hermaphrodite and male. They share many similarities, *e.g.*, pharynx, digestive system, overall musculature and nervous system, but exhibit major differences with regards to the reproductive system. Hermaphrodites can be thought of as females with a limited amount of sperm. They produce both oocytes and sperm, and self fertilization can occur in the gonad (Figure 1). A hermaphrodite's own

sperm can fertilize its own oocytes in the spermatheca, producing **self-progeny**. After a few rounds of division, the fertilized egg is ejected through an opening in the ventral side of the hermaphrodite, the **vulva**, and the zygote hatches about twelve hours later. Males, on the other hand, produce only sperm and can introduce this sperm into hermaphrodites to produce **cross-progeny**, half of which are male and half of which are hermaphrodites.

The wild-type vulva is comprised of 22 nuclei and develops the same way in each wild-type hermaphrodite (Figure 2). These 22 nuclei are in turn derived from three cells located in the ventral hypodermis called, according to *C. elegans* nomenclature, P5.p, P6.p, and P7.p (Sulston and Horvitz, 1977). From 29 hours after hatching until 34 hours after hatching, these three cells start to undergo three rounds of division (Figure 2). By comparison, the three ventral cells that flank P5.p and P7.p, namely P3.p, P4.p, and P8.p, divide only once and fuse with hyp7, a large, multinucleate hypodermal cell that extends most of the length of the body. The cells P3.p, P4.p, and P8.p therefore do not normally contribute to the hermaphrodite vulva, although they have the potential to do so (see below).

The term **lineage**, in the case of the vulva, is used to describe the set of divisions that the cells P3.p-P8.p and their progeny undergo. Thus, the cell P6.p executes the following lineage (Figure 2): it divides along the longitudinal (anterior-posterior) axis of the animal to produce two daughters, both of which divide longitudinally to produce four granddaughters, all of which then divide along the transverse (left-right) axis to form eight vulval cells. This final round of divisions from four to eight cells is abbreviated "TTTT" to indicate that all four cells divided

transversely. Since the decision of what lineage to execute is thought to occur primarily in the cells P3.p-P8.p (see below), a lineage is often abbreviated to include only a given cell and the final division in its lineage. Thus, P6.p is said to execute a "TTTT" lineage, although in reality it is the granddaughters of P6.p that divide "TTTT" to produce eight vulval nuclei.

B. Vulval development requires a signal from the gonad

Although the timing and pattern of divisions of the cells that produce the vulva are invariant from hermaphrodite to hermaphrodite, Sulston and Horvitz (1977) hypothesized that vulval development required an external influence. This hypothesis was based on the fact that even though P5.p, P6.p, and P7.p always divide in the fixed pattern described above, the cells that give rise to P5.p, P6.p, and P7.p are variably determined. For example, P5.p is derived from the cell P5 (P5.p is its posterior daughter) and P6.p is derived from P6. But the cells P5 and P6 themselves come from a pool of two cells, one of which randomly becomes P5 and the other P6 (Figure 3). Thus, even though P5.p and P6.p give rise to different division patterns (Figure 2), this difference cannot be entirely due to ancestry, suggesting that cell non-autonomous development and signal transduction are involved. Similarly, P7.p and P8.p are randomly derived from a pool of two cells.

Sulston and Horvitz (1977) also hypothesized that, based on the symmetry of the vulva relative to the somatic gonad, *i.e.*, the center of the vulva was located around the center of the gonad, the gonad was responsible for signalling the development of the vulva. The gonad is distinct from the ventral hypodermis (Figure 1) and by ancestry is unrelated to the P3.p - P8.p cells. Using a laser microbeam, Sulston and

White (1980) removed the two precursor cells that make the somatic gonad and found that no vulva was formed (Figure 4B). Rather, in the absence of a gonad, they found that P5.p, P6.p, and P7.p behaved like P3.p, P4.p, and P8.p normally do so that all six cells divided once and fused with the hypodermis.

This observation was extended by Kimble (1981) to show that one cell in the gonad, the **anchor cell**, which is normally located directly above (dorsal to) P6.p, is both necessary and sufficient for P5.p, P6.p, and P7.p progeny to form vulva (Figure 4C,D). In addition, the stimulation of these three cells by the anchor cell to form vulva occurs before the first round of division, since ablation of the anchor cell during or after the first round of division leads to generally normal production of vulval cells (Figure 4E; Kimble and White, 1981; Sternberg and Horvitz, 1986). Based on this and other observations given below, the primary determination of vulva versus hypodermis is thought to mostly occur in the P5.p, P6.p, and P7.p cells themselves, even though it is their great-granddaughters that eventually form the vulva. The action of the anchor cell on the ventral hypodermal cells P5.p-P7.p to produce vulva is called **induction** since the development of the vulva is dependent on a cell which itself does not contribute to that structure (Sulston and White, 1980).

C. Regulation of vulval versus hypodermal fate among the vulval precursor cells

Although these experiments show that the anchor cell signals the formation of vulva, they do not indicate how P6.p is instructed to take on a different fate than P5.p and how P7.p is instructed to take on a different fate

than P8.p (recall that the selection of P5/P6 is random, as is P7/P8). This question was addressed by ablation of individual cells in the ventral hypodermis (Figure 5; Sulston and White, 1980; Sternberg and Horvitz, 1986). When P6.p was ablated in an early larval stage, either P5.p or P7.p could move into the position normally occupied by P6.p and behave like P6.p normally would. Similarly, when P5.p was ablated or moved to replace P6.p, P4.p was able to replace P5.p and generate seven vulval nuclei instead of the normal two hypodermal nuclei. Also, P8.p could replace P7.p. In the most extreme case, P5.p, P6.p, and P7.p were ablated, and P3.p, P4.p, and P8.p, which normally produce hypodermis, produced vulva. However, the other P cell daughters, P1.p, P2.p, and P9.p-P11.p, were never found to take part in the formation of vulva.

The cells, P3.p, P4.p, P5.p, P6.p, P7.p, and P8.p, are therefore thought to form an equivalence group in which all are equally competent to receive the anchor cell signal and differentiate into vulva. These six cells are collectively referred to as the **vulval precursor cells** (VPCs). The presence of P6.p normally prevents the progeny of P5.p or P7.p from exhibiting the "TTTT" pattern, whereas the presence of P5.p and P7.p respectively prevent the progeny of P4.p and P8.p from forming vulva and exhibiting the "LLTN" or "NTLL" pattern (see Figure 2 for a description of division patterns). At least two cells need to be removed for P3.p to form vulva.

The lineage adopted by P6.p is referred to as **1°** because it is the "highest" in a hierarchy of lineages. That is, a VPC that normally executes a 1° lineage can be replaced by a VPC that normally executes a "LLTN" or a "S S" (hypodermal) lineage. Likewise, the "LLTN" or "NTLL" lineage is designated **2°** since it can be replaced by a VPC that normally executes a "S

S" lineage but not by a cell that executes a 1° lineage. Last, the hypodermal "S S" lineage is designated as 3°. Ablation and other experiments have shown that the VPCs are completely equipotential in that P3.p, P4.p, P5.p, P6.p, P7.p, and P8.p each can execute 1°, 2°, and 3° lineages (Sulston and Horvitz, 1977; Sulston and White, 1980; Sternberg and Horvitz, 1986; Thomas *et al.*, 1990).

The experiments above suggested that the location of a VPC is important in determining what lineage it executes. Location of a VPC could be an important factor because of its: 1) distance to the inductive anchor-cell signal, or 2) position with respect to other VPCs (*i.e.*, VPC-VPC interactions are important).

To determine if distance from the anchor cell mattered, all but one VPC were ablated in the presence of the anchor cell (Sternberg and Horvitz, 1986). Upon isolation, a VPC would often move towards the anchor cell and end up at a variable distance from it. Sternberg and Horvitz found that the closer a VPC was to the signal, the higher the fate it adopted, and that all three fates, 1°, 2°, and 3° were possible for an isolated VPC (Figure 6A). This result suggests that the anchor cell signal alone is sufficient to generate the three different cell types and that VPC-VPC interactions are not absolutely required, although such interactions cannot be ruled out by these experiments (in fact, VPC-VPC interactions probably do occur, see section IV). In the simplest interpretation, the anchor cell emits a graded signal that is received and transduced by the VPCs -- the closer a VPC is to that signal, the greater the amount of signal it receives, and the "higher" the fate that VPC executes (Figure 6B). Alternatively, the anchor cell may emit two signals, one that acts over a very short range to specify 1° fate and

another that acts over a somewhat larger range to specify 2° fate (Figure 6C).

The isolated VPC experiment suggests that the anchor cell signal can act at a distance. Data from Thomas *et al.* (1990) supports this. They examined hermaphrodites in which the anchor cell had been displaced dorsally away from the VPCs due to the *dig-1* mutation. They found that induction of the VPCs still occurred, albeit often abnormally, even though the anchor cell was presumably no longer touching the VPCs. This result suggests that the action of the anchor cell signal does not require contact between the anchor cell and the VPCs. Hence, the signal is probably diffusible.

D. Determination of VPC fate seems to occur in the VPCs

The inductive action of the anchor cell most likely acts on the VPCs and not their progeny since ablation of the anchor cell just before or during VPC division does not affect the lineages executed by the VPCs (Kimble and White, 1981; Sternberg and Horvitz, 1986). As with interpreting all ablation experiments, caution should be exercised since a cell's influence does not necessarily stop at the time of ablation. Debris from the ablated anchor cell may be affecting the VPCs, or the anchor-cell signal itself could persist if, for example, the signal is stored in the extracellular matrix. Nonetheless, the idea that the anchor cell acts on the VPCs was strengthened by studying vulval induction in *lon-1* and *dig-1* animals (Sternberg and Horvitz, 1986; Thomas *et al.*, 1990). The *lon-1* mutation results in hermaphrodites that are about 50% longer than wild type, and the anchor cell in these hermaphrodites is occasionally positioned between P6.p and P5.p, rather

than directly over P6.p. If the anchor cell signal acted on the daughters of the VPCs and not the VPCs themselves, then it should be possible to generate an intact 1° lineage from the posterior daughter of P5.p and the anterior daughter of P6.p (*i.e.*, from P3.p to P8.p, one should see the following division patterns "S S" "S LL" "TN TT" "TT NT" "LL S" "S S"; 1° lineage underlined). In fact, this pattern was never found -- either P5.p or P6.p, but never a combination of P5.p/P6.p daughters, gave rise to intact 1° lineages. Similarly, in *dig-1* hermaphrodites the anchor cell is sometimes initially located between two VPCs. As with *lon-1* mutants, in no cases did the daughters of two different VPCs combine to generate an intact 1° or 2° lineage.

These data do not exclude the possibility that other signalling occurs after the anchor cell has ceased to play its role. For example, communication among the VPC daughters could be necessary to execute the pattern established in the VPCs by the anchor cell. One way to address this possibility is to ablate VPC daughters and look for perturbations in lineage. For example, when the VPC daughter cell that gives rise to the "TN" part of a 2° lineage was ablated in several animals, the other daughter still gave rise to an "L L" lineage (Sternberg and Horvitz, 1986). Based on these negative results, a good working hypothesis is that the entire vulval lineage from P5.p, P6.p, and P7.p to their great-granddaughters is determined by inductive events that occur before P5.p, P6.p, and P7.p divide.

The 1°, 2°, and 3° lineages executed by VPCs seem to be an intrinsic property of the VPCs. The VPCs are pre-programmed to execute a fixed repertoire of lineages called **sublineages** -- either "TTTT," "LLTN," or "S S"

(Sternberg and Horvitz, 1986), the selection of which depends on the outcome of signal transduction events that occur in the VPCs. This selection is called vulval **determination**, and the sublineage selected by a VPC is often referred to as its **fate**. Thus, for example, P6.p can be said to select a 1° fate. Consistent with this notion of sublineage, no physical or genetic alteration has generated more than the three rounds of division associated with 1° and 2° lineages. With the exception of hybrid lineages (see below), genetic and physical perturbations of VPCs result in lineages generally identifiable as 1°, 2°, or 3° (Sternberg and Horvitz, 1986; Sternberg, 1988). The cell biology data are summarized in Figure 7.

E. Cell types and morphogenesis

Vulval development is more than the signal transduction events associated with induction of the VPCs. The processes that occur subsequent to vulval determination offer the opportunity to study processes downstream of signal transduction.

What are the characteristics of the differentiated vulval cells? Wild-type axes of division establish three vulval cell types, T, N, and L, which can be classified further. For example, of the twelve nuclei derived from T divisions, (*i.e.*, LLTN **TTTT** NTLL), only those derived from the central two (underlined) are capable of adherence to the anchor cell (Sternberg and Horvitz, 1989). [The components of the vulva are referred to as "nuclei" and not "cells" because some of the nuclei are syncytial (J. White, personal communication).] An important characteristic of 2° lineages is the adherence to the cuticle of the progeny of the L divisions. In some mutant backgrounds, L divisions are seen whose progeny do not adhere. These are

often interpreted as part of abnormal 1° lineages (Sternberg, 1988). Another issue is what generates the mirror image symmetry in the vulva, *e.g.*, the polarity of the 2° lineages adopted by P5.p (LLTN) and P7.p (NTLL).

Following the final set of divisions, the vulva undergoes a process of **morphogenesis** in which the 22 nuclei move in a coordinated and defined fashion (Sulston and Horvitz, 1977). This movement results in the proper positioning of the vulval cells and attachment to the uterus to form a functional vulva. Kimble (1981) demonstrated that ablation of the anchor cell just prior to VPC division results in abnormal morphogenesis, although the proper complement of cells is generated. Thus, the anchor cell is needed for both induction of the VPCs and proper movement of their great-granddaughters. Ablation of the anchor cell at or soon after the first division of the VPCs results in normal lineages and morphogenesis, but abnormal attachment of the vulva to the uterus, suggesting the anchor cell is also needed for this process. Last, during the final stages of morphogenesis, the anchor cell disappears, thereby allowing a passage between the uterus and the vulva.

An oddity of vulval cell biology involves P3.p. Fifty percent of the time, P3.p executes a 3° vulval lineage like P4.p and P8.p (lineage = "S S"). However, the other fifty percent of the time P3.p behaves like P1.p, P2.p, P9.p, P10.p, and P11.p --these cells do not divide but rather fuse directly with the hypodermis (lineage = "S", Sulston and White, 1980; Sternberg and Horvitz, 1986). In these cases, P3.p is thought to be incapable of being a part of the vulval equivalence group (Sulston and White, 1980). This decision may be pertinent to understanding the generation of the entire vulval

equivalence group (see generation mutants below). For the remainder of this review, we will ignore the nonvulval fate of P3.p.

III. Identification of mutations that affect *C. elegans* vulval development

A. Overview of genetics as a tool to study vulval development

Several characteristics make genetics a powerful approach to study *C. elegans* vulval determination, and all the genes presently known to participate in *C. elegans* vulval determination have been first identified via genetic techniques. First, the vulva is not essential for propagation of *C. elegans*, thus making it possible to select for mutations that perturb vulval development. Hermaphrodites which lack a vulva, *e.g.*, via anchor-cell ablation, cannot lay eggs but can still produce self-progeny (Horvitz and Sulston, 1980). Second, mutations that eliminate or alter the vulva are easily detectable under a dissecting microscope, making large scale morphological screens for mutants in the inductive process feasible. (For reference, a dissecting microscope is generally used at a magnification of 6X-50X and allows for visualization of up to hundreds of nematodes at a time. To visualize VPC fate (see Section II), however, a dissecting microscope is inadequate and Nomarski optics at 1000-1250X magnification is necessary. At such magnification, only one hermaphrodite at a time can be studied.) Third, *C. elegans* itself is a genetically tractable organism. The generation time is fast (an embryo can develop into a sexually mature hermaphrodite in about $3^1/_2$ days). A good genetic map exists, as do genetic tools like balancers, rearrangements, deficiencies and duplications. The hermaphroditic nature of *C. elegans* facilitates screens for random

mutations that affect the vulva and screens for suppressors of known mutations, and the availability of fertile males facilitates the ability to isolate more alleles of a given mutation (see below). Fourth, the effects of mutations can be interpreted at the cellular level, and ablations can be performed to gain greater insights into the signal transduction process.

B. Isolation of vulvaless and multivulva mutations and associated cellular defects

Random mutagenesis screens have been fruitful in identifying important components of vulval determination (Horvitz and Sulston, 1980; Sulston and Horvitz, 1981; Ferguson and Horvitz, 1985). Without prior knowledge of what to expect, two classes of easily detectable abnormalities associated with the production of mutant vulvae were identified (Horvitz and Sulston, 1980; Sulston and Horvitz, 1981): the **egg-laying defective** or Egl phenotype, detectable by bloated worms or the bag-of-worms appearance, and the **multivulva** (or Muv) phenotype, detectable by up to five protrusions on the ventral surface (Figure 8). In a random screen, wild-type hermaphrodites are mutagenized, generally with ethyl methanesulphonate (EMS; Brenner, 1974), and allowed to produce self-progeny (Figure 9). Both the first generation progeny (F1) and the second generation progeny (F2) are screened for mutant phenotypes, allowing the isolation of dominant and recessive Egl and Muv mutations.

The phenotype associated with many Egl mutants is caused by a lack of or reduction in vulval induction -- *i.e.*, less than three and often no VPCs adopt 1° or 2° vulval fates (Figure 10C,D). In the most severe case, all VPCs execute a 3° hypodermal fate. This class of Egl mutants are specifically

called the **vulvaless** (or **Vul**) mutants, so named because no vulval tissue forms (Horvitz and Sulston, 1980). (Note that whereas all Vul hermaphrodites are Egl, not all Egl hermaphrodites are Vul; see below.) Mutations in any of the following seven genes can result in a Vul phenotype: *lin-2*, *lin-3*, *lin-7*, *lin-10*, *lin-45*, *let-23*, and *let-60* (Horvitz and Sulston, 1980; Sulston and Horvitz, 1981; Ferguson and Horvitz, 1985; Ferguson *et al.*, 1987; Sternberg and Horvitz, 1989; Han *et al.*, 1990; Beitel *et al.*, 1990; Aroian and Sternberg, 1991). The genes *lin-12* and *lin-25*, which also mutate to an Egl phenotype, are dealt with later.

Conversely, VPCs in Muv mutants lack the 3°, nonvulval fate (Figure 10E). Mutations in each of *lin-1*, *lin-13*, *lin-15*, *let-60 ras* (formerly called *lin-34*), and the synthetic multivulva genes (see below) can result in a Muv phenotype (Horvitz and Sulston, 1980; Sulston and Horvitz, 1981; Ferguson and Horvitz, 1985; Han *et al.*, 1990; Beitel *et al.*, 1990). The most severe Muv phenotype results in all six VPCs executing only vulval fates, 1° and 2° (Sulston and Horvitz, 1981; Ferguson *et al.*, 1987; Sternberg, 1988; Sternberg and Horvitz, 1989). The extra vulval tissue generated in a Muv hermaphrodite is responsible for the prominent ventral protrusions, also called pseudovulvae, discernible under the dissecting microscope (Figure 8, Sulston and Horvitz, 1981). The number of protrusions, from one to five, depends on the severity of the Muv phenotype and the exact vulval lineages executed. Although the *lin-12(d)* phenotype and the hyperinduced phenotype can outwardly resemble the Muv phenotype, the cellular basis of these other phenotypes is very different. Both *lin-12* and hyperinduction will be dealt with in their own sections below.

Besides random screens, two other types of screens have been used to isolate vulval mutations: F1 non-complementation and suppressor (Ferguson and Horvitz, 1985; Kim and Horvitz, 1990; Han *et al.*, 1990; Beitel *et al.*, 1990; Aroian and Sternberg, 1991). An F1 non-complementation screen is a powerful way to generate more mutations in a particular gene (Figure 11). For example, this scheme was used to generate 14 new *let-23 RTK* alleles (Aroian and Sternberg, 1991; *RTK* stands for Receptor Tyrosine Kinase). In a suppressor screen, mutant hermaphrodites that are either Muv or Vul are mutagenized, and suppression of that phenotype is screened for in the F1 or F2 generation (Figure 12). For instance, dominant negative *let-60 ras* Vul mutations were isolated as suppressors of the *lin-15* Muv gene (Han *et al.*, 1990; Beitel *et al.*, 1990). The hermaphroditic nature of *C. elegans* makes such a screen readily feasible, and the screen can be set up such that suppressors are easy to identify in the original mutant background. Such a screen is restrictive in that it only selects for mutations that can suppress the original mutant. Furthermore, if the original mutant results in a complete loss of gene function, the screen selects for genes which act only downstream of the original mutation.

C. Methods for cloning *C. elegans* genes

The availability of strains with active transposition, the presence of an extensive physical map, and the ability to perform germ line transformation experiments all combine to allow the facile cloning of *C. elegans* genes. To date, the molecular identities of the genes *lin-10*, *lin-11*, *lin-12*, *let-23 RTK*, and *let-60 ras* have been described (Greenwald, 1985; Yochem *et al.*, 1988; Freyd *et al.*, 1990; Kim and Horvitz, 1990; Han and

Sternberg, 1990; Aroian *et al.*, 1990). Cloning strategies have made use of transposon tagging (*lin-10*, *lin-11*, and *lin-12*) and physical mapping/germ-line transformation (*let-23 RTK*, *let-60 ras*).

Although transposons are found inserted in the genomes of all *C. elegans* strains, germ line transposition only occurs in some of them. For example, germ line transposition of the TC1 family of transposible elements does not occur in the standard laboratory *C. elegans* strain, called variety Bristol or N2, but it does occur in the *C. elegans* strain TR679 (Collins *et al.*, 1987). Therefore, a TC1 transposon in the strain TR679 can insert into a gene of interest causing a heritable, mutant phenotype. This transposition event can be identified by performing either an F2 random screen or an F1 non-complementation screen similar to those performed with EMS as a mutagen. Once a transposon mutant has been isolated in a gene of interest, genetic mapping and Southern analysis probing with cloned transposons are combined to identify the single transposition event responsible for the mutant phenotype. Inducing the transposon to excise by reverting the mutant phenotype is also useful in this regard. Once the responsible transposon has been identified, flanking DNA can be isolated, which presumably contains or is tightly linked to the gene of interest. One limitation of this technique genes is that not all genes are good targets for transposon insertion.

An alternative approach utilizes correlation of genetic and physical maps, followed by germ line rescue experiments. DNA spanning most of the *C. elegans* genome has been collected into large stretches of overlapping cosmid and yeast artificial chromosome clones called contigs (Coulson *et al.*, 1986; Coulson *et al.*, 1988), and this physical DNA map has been

correlated to the genetic map in many areas of the genome. Most genes can be readily mapped relative to genetic and/or physical (*e.g.*, restriction fragment length polymorphisms or RFLP) markers, and if the physical map is well-characterized in that area, it is possible to narrow down the region of DNA containing the gene of interest. For example, in the case of *let-23 RTK*, mapping of the gene between an RFLP and a deficiency breakpoint narrowed the region containing the gene down to about 200 kilobases (kb) on the physical map (Aroian *et al.*, 1990). The exact location of the gene in this 200 kb region was then determined by germ line transformation experiments (Figure 13).

D. Molecular identity of *lin-10*, *lin-11*, *lin-12*, *let-23 RTK*, and *let-60 ras*

In this section, the molecular identities of the vulval genes published to date are reviewed. The relevance of these identities to the vulval determination pathway is discussed in later sections.

The predicted protein sequence of *lin-10* shows no similarity to known proteins (Kim and Horvitz, 1990). Although *lin-10* mutants show only defects in the vulva, expression data indicates that the gene is transcribed in many other cells and throughout development. For example, *lin-10* RNA levels are not substantially altered in *lin-26* mutant hermaphrodites that completely lack VPCs (Kim and Horvitz, 1990).

The *lin-11* gene, which is necessary for execution of the "NT" portion of 2° lineages, encodes a protein with a homeodomain and two tandem copies of a putative metal binding domain found in two other homeodomain-containing genes (Freyd *et al.*, 1990). The *lin-11* gene is likely to encode a transcription factor.

The *lin-12* gene encodes a putative membrane spanning protein with striking similarity in overall architecture to the *C. elegans glp-1*, the *Drosophila Notch*, *Xenopus Xotch,* and human *TAN-1* proteins (Greenwald, 1985; Yochem *et al.*, 1988; Yochem and Greenwald, 1989; Austin *et al.*, 1989; Coffman *et al.*, 1990; Ellisen *et al.*, 1991). In its extracellular domain, *lin-12* contains 13 epidermal growth factor-like (EGFL) repeats and three tandem copies of a cysteine-rich motif. In its intracellular domain, *lin-12* contains six copies of a motif identified in the yeast genes *cdc10/swi6*, which may provide interaction surfaces for binding with other proteins (Thompson *et al.*, 1991). All members of this family contains these motifs, although the number of EGFL repeats varies. The *C. elegans glp-1* protein is involved in cell-cell interactions in the hermaphrodite germ line and anterior pharynx and may be a receptor for close-range interactions (Priess and Thomson, 1987; Austin and Kimble, 1987). The *Drosophila Notch* protein also appears to act as a receptor for close range signals and is involved in the determination of epidermal/neuronal fates (reviewed in Simpson, 1990).

The *let-23 RTK* protein belongs to the epidermal growth factor (EGF) receptor family of tyrosine kinases since it contains: 1) a tyrosine kinase domain with highest similarity to human EGF receptor (44% identity); 2) an appropriately positioned putative membrane spanning domain; 3) two cysteine-rich motifs in the extracellular domain with inter-cysteine spacing characteristic of the EGF receptor family; and 4) limited similarity in the ligand binding region with other members of the EGF receptor family (Aroian *et al.*, 1990). EGF receptor and its ligands can elicit diverse cellular responses including proliferation, inhibition of proliferation, and fate

determination in mammals and *Drosophila* (for reviews in mammalian systems see Ullrich and Schlessinger, 1990; Sporn and Roberts, 1988; for *Drosophila* EGF receptor see Price *et al.*, 1989; Schejter and Shilo, 1989). EGF receptor responses are elicited upon binding of ligand to the receptor extracellular domain and subsequent activation of tyrosine kinase activity in the receptor cytoplasmic domain.

The *let-60 ras* protein is highly similar to *ras* proteins of other organisms (Han and Sternberg, 1990). For example, the predicted *let-60 ras* protein is 83% identical to human N-*ras* protein in its first 164 amino acids, a region that contains the guanine nucleotide binding domains, intrinsic GTPase activity, and the so-called effector domain. Like the EGF receptor family, the *ras* family of proteins are important components of signal transduction involved in cell growth and differentiation and are thought to be downstream targets of growth factor receptors (Mulcahy *et al.*, 1985; Weissman and Aaronson, 1983). The *ras* family of proteins are small (ca. 21 kD) guanine nucleotide binding proteins that can switch from an inactive GDP-bound form to an active GTP-bound form (reviewed in Bourne *et al.*, 1991). The *let-60 ras* protein likely functions similar to other ras proteins (see below).

E. Vulval development beyond vulval induction

Although the remainder of this chapter will mostly detail the signal transduction pathway involved in vulval determination (*i.e.*, selection of 3°, 2°, or 1° fate), vulval development involves more than just vulval determination. The Vul and Muv mutants discussed above are called **determination mutants** since they alter the determination of vulval fate.

Screens for vulval mutations revealed three other groupings of mutants (Ferguson *et al.*, 1987). Like Vul mutants, **generation mutants** cannot lay eggs. However, these mutants are Egl not because of defects in vulval determination, but because the cells that make up the vulval equivalence group are never properly generated. For example, in *n300* mutant hermaphrodites, the cells P3.p-P8.p behave like P1.p, P2.p, and P9.p-P11.p normally do -- they do not divide but directly fuse with the hypodermis (Ferguson *et al.*, 1987). These cells do not become vulva precursor cells and therefore are incapable of responding to the anchor cell signal and executing 1°, 2°, or 3° fates. The **expression** or **execution genes** are good candidates for targets of the determination genes since mutations in these execution genes appear to act downstream of the determination genes. Mutations in these genes do not affect which fate the VPCs select, but rather affect the execution of specific lineages. Those known to date all affect aspects of 2° fate execution (see below). The last category consists of mutations not easily amenable to classification. For example, mutations in the genes *lin-24* and *lin-33* can result in the death of some of the cells P3.p-P8.p, and these mutations are thought to encode abnormal products that are toxic to these cells (Ferguson and Horvitz, 1985).

These different mutant categories, all of which result in abnormal vulval lineages, underscore the importance of vulval cell biology in categorizing mutants. Furthermore, not all mutations that result in an Egl phenotype or in ventral protrusions overtly affect vulval lineages. For example, mutations that affect the egg-laying musculature or neuronal circuitry can also lead to an Egl phenotype (Waterston *et al.*, 1980; Trent *et al.*, 1983; Desai and Horvitz, 1989). In these mutants, the VPCs and their

lineages are normal. The well-characterized vulval cell biology therefore allows any new mutation to be examined at the cellular level to ascertain whether or not it affects vulval lineage, and, if so, in what specific way.

Interestingly, the positioning and development of some other components of the egg-laying system noted above, such as musculature and neuronal circuitry, also depend on signal transduction events that involve both the vulva and the somatic gonad. Vulval cells from the 1° and 2° lineages are important for branching of the VC motor neurons, for positioning of the vulval muscle cells, and for the induction of an FMRFamide-like peptide in certain gonadal cells (Li and Chalfie, 1990). Furthermore, the somatic gonad, responsible for vulval induction via the anchor cell, is also important for proper migration of an important component of the egg-laying system, the sex myoblasts (Thomas *et al.*, 1990).

F. Other effects of the Vul/Muv genes

Mutations in many of the genes mentioned above and below have effects in tissues other than the vulva. The *lin-12* gene affects at least ten developmental decisions in the hermaphrodite and male, including the production of the anchor cell (Greenwald *et al.*, 1983). Mutations in the Vul genes *lin-3*, *let-23 RTK* and *let-60 ras* share in common at least three phenotypes apart from Vul including larval lethality, sterility, and male tail defects (Ferguson and Horvitz, 1985; Han *et al.*, 1990; Aroian and Sternberg, 1991; H. M. Chamberlin, M. Han, R. J. Hill, and P.W.S. unpublished observations). Muv mutants can affect the male hypodermis, the hermaphrodite head region, and hermaphrodite fertility (Sulston and

Horvitz, 1981; Ferguson and Horvitz, 1985; Ferguson and Horvitz, 1989). Mutations in *lin-11* show defects in the male tail and in movement (Ferguson and Horvitz, 1985). However, some genes, such as *lin-2*, *lin-7*, and *lin-10*, seem primarily to operate in the development of the vulva (Ferguson and Horvitz, 1985; Kim and Horvitz, 1990). The vulval determination pathway therefore utilizes genes generally involved in *C. elegans* development and also specifically tailored for the development of the vulva.

IV. Building a signal transduction pathway for determination of vulval vs. hypodermal fate

A. Genes associated with Vul mutations normally stimulate vulval fate

Genetic evidence suggests that the Vulvaless phenotype associated with mutations in either *lin-2*, *lin-3*, *lin-7*, *lin-10*, *let-23 RTK*, or *let-60 ras* is the result of loss of gene function (Table 1). That is, eliminating the activity of any one of these genes prevents the VPCs from selecting 1° and 2° vulval fate and results in the VPCs adopting 3° nonvulval fate. These genes therefore normally stimulate selection of vulval rather than hypodermal fate and could be required for producing the anchor cell signal, for reception of the signal at the VPCs, or for transduction of that signal in the VPCs.

For each of *lin-3*, *let-23 RTK*, and *let-60 ras*, the evidence suggests that complete loss of gene function results in a fully penetrant Vul phenotype -- that is, when gene activity is eliminated, all six VPCs always execute 3° fates (Ferguson and Horvitz, 1985; Sternberg and Horvitz, 1989; Han *et al.*,

1990; Aroian and Sternberg, 1991; R. Hill and P.W.S., unpublished results). [The previous statement is actually a simplification. It is not possible to directly determine the fate of the VPCs in complete loss of function *lin-3*, *let-23 RTK*, or *let-60 ras* alleles since the complete loss of function phenotype for all three is early larval lethality. However, it can be inferred that complete loss of function for any one of these results in no vulval induction based on strong reduction of function alleles, deficiency arguments, and allelic series arguments ([Han, 1990 #509]; Aroian and Sternberg, 1991; R.Hill and P.W.S., unpublished results). The lethality associated with complete loss of function *let-60 ras* alleles can be maternally rescued using activated *let-60 ras* mutations. Thus, induction in complete loss of function *let-60 ras* animals can be measured.] Given that complete loss of function in these genes fully reproduces the effect of removing the anchor cell, it is simplest to assume that these genes act directly in the pathway between the anchor cell and the selection of vulval fate (Figure 14). For *let-23 RTK*, the genetic evidence along with its molecular structure suggest that *let-23 RTK* is the receptor in the VPCs for the anchor-cell signal (Aroian *et al.*, 1990). For *let-60 ras*, the genetic and molecular evidence suggest that it is a necessary signal transduction component in the VPCs downstream of *let-23 RTK* (see below).

For *lin-2*, *lin-7*, and *lin-10*, complete loss of gene activity does not result in a fully penetrant Vul phenotype (Horvitz and Sulston, 1980; Sulston and Horvitz, 1981; Ferguson and Horvitz, 1985; Sternberg and Horvitz, 1989; Kim and Horvitz, 1990). Even in animals carrying putative complete loss of function or **null** alleles, all VPCs do not always execute 3° hypodermal fates, although the number of VPCs that execute 1° and 2° vulval fates is

markedly reduced. These data suggest that these genes do not act directly in the pathway from anchor cell to selection of vulval fate since if they did, elimination of their activities should fully reproduce elimination of the anchor cell (Figure 14). This conclusion is supported by the finding that the partial induction seen in *lin-7* and *lin-10* mutant hermaphrodites is eliminated if the anchor cell is ablated (Sternberg and Horvitz, 1989). Rather, these genes may act indirectly by modifying or stabilizing components in the direct pathway. For example, one of these genes may stabilize *let-23 RTK*. In the absence of that gene, *let-23 RTK* is unstable and is present in reduced quantity such that only a small amount of induction can occur. See legend to Figure 14 for alternative interpretations of the *lin-2*, *lin-7*, and *lin-10* data, *e.g.*, functional redundancy.

B. Genes associated with Muv mutants normally inhibit vulval fate

The Muv phenotype associated with mutations in *lin-1*, *lin-13*, and *lin-15* is caused by a decrease of gene function (Table 1). In the absence of any of these genes, all VPCs execute 1° or 2° vulval fates. Thus, these genes are required for the VPCs to inhibit 1°/2° vulval fates and to adopt 3°, nonvulval fates (Figure 15A).

In some instances, the manifestation of the Muv phenotype requires mutations in two genes (Horvitz and Sulston, 1980; Ferguson *et al.*, 1987; Ferguson and Horvitz, 1989). This is known as the synthetic Muv or syn-Muv phenotype. Syn-Muv mutations fall into two classes, A (*e.g.*, *lin-8, lin-38*) and B (*e.g.*, *lin-9, lin-35, lin-36, lin-37*). Any combination of A only mutants (*e.g.*, *lin-8* or *lin8 lin-38* double mutant) or B only mutants (*e.g.*, *lin-9* or *lin-9 lin-36* double mutant) results in a wild-type vulva, but any

combination of an A and a B mutant, *e.g.*, *lin-8; lin-9* double mutant or *lin-8; lin-35* double mutant, results in a Muv phenotype (Ferguson and Horvitz, 1989). This suggests that the A and B genes define two separate pathways that are functionally redundant -- eliminating any component of just one pathway is not sufficient to result in a phenotype but eliminating components from both pathways can result in a phenotype (Figure 15B; Ferguson and Horvitz, 1989). Some alleles of *lin-13*, and *lin-15* also exhibit syn-Muv behavior. For example, the *lin-15* allele *n767* behaves like a class A syn-Muv and the *lin-15* allele *n744* behaves like a class B syn-Muv. Nonetheless, there are alleles of each of these two genes that give rise to a Muv phenotype alone, suggesting, in the simplest case, that these genes operate in both A and B pathways (Ferguson and Horvitz, 1989). Apart from its synthetic nature, the syn-Muv phenotype is the same as the Muv phenotype.

This Muv phenotype is independent of the anchor-cell signal -- if the anchor cell is eliminated in the background of Muv mutants, the VPCs still execute 1° and 2° vulval fates (Figure 10F; Ferguson *et al.*, 1987; Sternberg, 1988; Han *et al.*, 1990; Beitel *et al.*, 1990). Thus, in the absence of Muv gene activity, a VPC automatically or **intrinsically** adopts 1° or 2° vulval fate with or without the anchor-cell signal. In other words, all that is required for a VPC to execute vulval fate is for the Muv genes to be turned off. This elimination is achieved genetically in the case of Muv mutants. In the wild-type hermaphrodite, however, the simplest model would be that the anchor-cell signal overrides the effect of the Muv genes in P5.p-P7.p (Figure 15C; Herman and Hedgecock, 1990).

Just as *lin-2*, *lin-7*, and *lin-10* appear not to act directly in the pathway from the anchor-cell signal to the selection of vulval fate, three observations suggest that at least one of the Muv genes, *lin-15*, also does not. First, although all VPCs select 1° or 2° fates in a *lin-15(lf)* (*i.e.*, *lin-15* complete loss of function) mutant irrespective of the presence of the anchor cell, the pattern of 1° and 2° fate seen is affected by the anchor cell (Sternberg, 1988; Figure 16A,B). Second, the anchor cell influences the patterns of induction seen in *lin-15(lf)* hermaphrodites in which all but two VPCs have been ablated (Figure 16C,D). Third, *lin-15(lf)* hermaphrodites which also carry a *let-60(dn)* mutant (see below) often have wild-type vulvae, and this induction is anchor-cell dependent (Han *et al.*, 1990). In all three instances, the anchor cell is able to exert an influence even though *lin-15* activity has been eliminated. It is unknown whether other Muv mutations also operate in pathways separate from the anchor-cell signalling pathway, although it is simplest to assume so.

C. Vul/Muv Interactions

The Muv and Vul genes act antagonistically on the VPCs: whereas Muv genes inhibit 1°/2° vulval fates and promote nonvulval 3° fate (loss of a Muv gene leads to no 3° fate in all VPCs), Vul genes promote vulval 1° and 2° fates (loss of a Vul gene leads to no 1° and 2° fates in all VPCs). Therefore, Muv genes must be activated to select 3° fate and turned off to select 1°/2° fate, and *vice versa* for Vul genes (Figure 17A). A gene that negatively regulates a Muv gene would be expected to mutate to a Vul phenotype, since if a Muv gene could not be turned off, 3° fate would be promoted. For example, assume *lin-2* negatively regulates *lin-15* (Figure 17B). A *lin-2(lf)*

mutant, where ***lf*** indicates a loss of function or complete loss of function allele, would therefore result in a Vul phenotype because *lin-15* cannot be turned off, thereby promoting all 3° fates. Alternatively, a gene that turns off a Vul gene would be expected to mutate to a Muv phenotype, since if a Vul gene could not be turned off, 1°/2° fate would be promoted. For example, assume *lin-15* negatively regulates *lin-2* (Figure 17B). A *lin-15(lf)* mutant would therefore result in a Muv phenotype because *lin-2* cannot be turned off, thereby promoting all 1°/2° fates. The difference between these two cases is the order in which the genes act -- the Vul gene regulates the Muv gene in the first case, whereas the Muv gene regulates the Vul gene in the second case.

For a given Muv/Vul pair, it is possible to determine their relative order by making the double Muv-Vul mutant (Figure 17). If a given Muv-Vul double mutant is Muv, then this suggests that the Vul gene normally regulates the Muv gene (Figure 17C). If the Muv gene activity is eliminated by mutation, then the presence or absence of the Vul gene is superfluous since the normal action of the Vul gene is to do just that, eliminate the Muv gene activity. Conversely, if a given Muv-Vul double mutant is Vul, then this suggests that the Muv gene normally regulates the Vul gene. It is also possible that a Muv-Vul double mutant is neither Muv nor Vul but that the two mutations are co-expressed. This would suggest that the two genes do not act in a linear order, but rather that their activities converge antagonistically on a third gene (Figure 17B). Interpretation of these experiments relies heavily on the use of mutations in each gene that eliminate activity -- *i.e.*, it is necessary that complete loss of function alleles of the Muv and Vul genes are used.

Double mutants between many of the Muv and Vul genes have been made, although for some combinations, non-complete loss of function alleles were used (Ferguson *et al.*, 1987; Han *et al.*, 1990; Aroian and Sternberg, 1991). The simplest pathway inferred from the double mutant data in which complete or near complete loss of function mutations were used is shown in Figure 18. Genes not shown in the pathway are discussed in the notes.

Of particular note is the relationship of *let-23 RTK* to *let-60 ras*. Both genetic and molecular data suggest *let-60 ras* operates downstream of *let-23 RTK*. First, hyperactive *let-60 ras* can bypass the need for *let-23 RTK* -- gain of function *let-60 ras* mutations are able to fully overcome the vulval defects in a *let-23 RTK* complete loss of function mutant (Han *et al.*, 1990). Second, the Vul phenotype of a strongly defective *let-23* allele is suppressed by overexpression of *let-60 ras* (Han and Sternberg, 1990). Thus, *let-23 RTK* is no longer needed if *let-60 ras* is activated or overexpressed, suggesting that *let-23 RTK* normally acts to activate *let-60 ras* (directly or through other genes). This data provide dramatic *in vivo* support for mammalian tissue culture data, which suggest that *ras* operates downstream of the EGF receptor, and also indicate that molecules similar to EGF receptor and *ras* can act in a linear pathway in the determination of cell fate.

The Muv gene *lin-1* is unusual. Unlike other Muv genes, the double mutant between *lin-1* and any Vul gene (except *lin-25*; see below) is Muv, suggesting that *lin-1* acts downstream of the Vul genes. Furthermore, unlike all the other Muv genes, there are no syn-Muv *lin-1* alleles. Lastly, the 1° and 2° lineages in a *lin-1* mutant are not always wild-type and can be difficult to interpret, unlike in other Muv mutants (Ferguson *et al.*, 1987;

P.W.S., unpublished observations). This gene may therefore act differently than the other Muv genes.

D. Place and time of action

The discussion so far has dealt with the role and functional relationships of various components but not where and when they act. Where a gene acts can be answered in two ways -- molecularly and genetically. Fusion of a gene's regulatory regions with *lacZ* can be used to determine where a gene is transcribed (Way and Chalfie, 1989; Fire *et al.*, 1990), and antibody staining can be used to determine in which cells protein product is present (Ruvkun and Giusto, 1989). RNA expression levels can also suggest where a gene is expressed. For example, although *lin-10* mutations are known to affect only the vulva, *lin-10* transcript levels in hermaphrodites lacking an anchor cell or lacking VPCs is not substantially altered from wild type (Kim and Horvitz, 1990). This result suggests that, whether or not *lin-10* is expressed in the VPCs, the gene is expressed in other cells.

Unlike molecular experiments, which can indicate where a gene is expressed or protein product is present, genetic mosaic experiments can suggest in which cells a gene actually performs its function (reviewed in Herman, 1989). This type of experiment makes use of a free duplication, which is essentially an unstable mini-chromosome. When the cell containing this mini-chromosome divides, the normal chromosome pairs segregate properly, but this unstable mini-chromosome has a low probability of mis-segregating. Thus, the mini-chromosome may be present in some cells and not others. If the experiment is carried out in

hermaphrodites mutant for a gene of interest (*e.g.*, *lin-15(lf)*) but containing a mini-chromosome with a wild-type copy of the gene (*e.g.*, *lin-15(+)*), then it is possible to generate mosaic hermaphrodites in which some cells are *lin-15(lf)* and some cells are *lin-15(+)*. By using other genetic markers to determine which cells are actually *lin-15(lf)* and which are *lin-15(+)* and by noting the vulval phenotype of different mosaic animals, one can determine in which cells a wild-type copy of *lin-15* is needed to generate a wild-type hermaphrodite and in which cells *lin-15* needs to be absent to generate a mutant hermaphrodite.

With respect to vulval development, such an experiment has been performed only for *lin-15* (Herman and Hedgecock, 1990). It was found that an individual hermaphrodite in which all six VPCs carried a wild-type *lin-15* gene could still sometimes express a *lin-15* Muv phenotype if other cells in the hermaphrodite were mutant for *lin-15*. Thus, wild-type *lin-15* in the VPCs is not sufficient for wild-type vulva, suggesting that *lin-15* acts outside the VPCs (although the data does not exclude that *lin-15* could also act in the vulva). Furthermore, animals in which all six VPCs were wild type for *lin-15* and that lacked an anchor cell could also express the mutant phenotype, arguing that the mutant phenotype seen in these VPCs was not caused by the anchor cell. In addition, the VPCs P3.p, P4.p, and P8.p could sometimes express a wild-type 3° phenotype when mutant for *lin-15*, indicating that induction in these VPCs was being repressed by some other cells.

The action of *lin-15* seems to be spread out among different lineages that share the commonality of contributing to **hyp7**, the large, multinucleate hypodermal cell that surrounds the VPCs. Combining all these

observations, Herman and Hedgecock (1990) hypothesized that *lin-15* exerts an inhibitory effect on vulval induction from hyp7. Thus, there appear to be three tissues involved in vulval determination -- 1) the large hypodermal syncytium, hyp7, which promotes 3° hypodermal fate in the VPCs; 2) the gonadal anchor cell, which induces some of the VPCs to overcome the effects of hyp7; and 3) the VPCs themselves. An updated molecular-genetic model for vulval determination is shown in Figure 19.

The question of when Muv and Vul genes act can be addressed using temperature sensitive alleles and temperature-shift experiments. Such experiments have been performed for the Muv genes *lin-13* and *lin-15*, the Vul genes *lin-10* and *let-23 RTK*, the 2°-determining gene *lin-12,* and *lin-25* (Greenwald *et al.*, 1983; Ferguson *et al.*, 1987; Kim and Horvitz, 1990). All of these genes except *lin-25* (dealt with below) appear to act before the VPCs undergo their first round of division, consistent with cell ablation studies which suggest that determination occurs before the VPCs divide. In addition, the Muv genes appear to act for a broad time interval that may precede the action of the Vul genes, which in turn may or may not precede the action of *lin-12*. That *lin-15* operates in the hypodermal syncytium may explain the long, gradual temperature-sensitive period seen with *lin-15* since hyp7 is accumulatively recruiting nuclei during this time. These data can be taken to support and extend the model presented above. The Muv genes establish their effects on the VPCs early, promoting 3° fate. Subsequently, the anchor-cell signal allows the VPCs to overcome these effects. The data are also consistent with the notion that these genes all act at or around the same time. Caveats for temperature shift experiments include: (1) assuming that the temperature shift affects protein activity and

not synthesis or stability; and (2) error due to the amount of time it takes to inactivate or reactivate the protein after the temperature shift.

V. VPC-VPC interactions and distinguishing between 1° and 2° fates

A. 1° fate is a VPC's default or intrinsic fate

The discussion to this point has dealt with the molecular genetics of how 1°/2° vulval fate versus 3° nonvulval fate is determined. What distinguishes the two vulval fates, 1° and 2°, from each other has not yet been addressed. As discussed below, VPC-VPC communication and the gene *lin-12* have prominent roles in this decision.

In the absence of outside influences, present data suggest that a VPC will adopt 1° fate. This is because in a *lin-15(lf)* mutant hermaphrodite (hence no hyp7 influence) with no anchor cell (hence no anchor-cell signal) and in which all but one VPC has been laser ablated (hence no VPC-VPC influences), an isolated VPC will select 1° fate (Figure 20A,B; Sternberg, 1988). Presumably, vulval fate is selected because of basal *let-23 RTK* kinase activity present in the absence of all outside influences. This basal activity then activates the *let-60 ras* pathway which results in a default of 1° fate. We call this pathway leading to selection of 1° fate the **intrinsic pathway** because it appears to be an intrinsic property of a VPC. A prediction of this model is that if a VPC were placed alone in tissue culture that VPC would select 1° fate, although we note that such an experiment is not presently possible.

B. In the absence of *lin-15*, VPC-VPC communication is responsible for selection of 2° fate

If a VPC by default selects 1° fate, what causes selection of 2° and 3° fates? As modeled above, the nonvulval 3° fate is selected when *lin-15*, and perhaps other Muv genes, acting from hyp7 inhibit *let-23 RTK* and activation of the intrinsic pathway. On the other hand, the selection of 2° fate seems to involve communication among the VPCs themselves.

Unlike in fully penetrant Vul mutants where the pattern of VPC fate is fixed, *i.e.*, all 3°, the pattern of VPC fates in fully penetrant Muv mutants is somewhat variable and differs with and without the presence of an anchor cell (Ferguson *et al.*, 1987; Sternberg, 1988; Sternberg and Horvitz, 1989). In a *lin-15(lf)* Muv mutant in which the anchor cell is present, P6.p adopts a 1° fate, P5.p and P7.p adopt 2° fate, but the fates adopted by P3.p, P4.p, and P8.p are variable and for each can be 1° or 2° (Figure 16). In the absence of an anchor cell, the fates of all six VPCs are variable, 1° or 2°. Nonetheless, despite this variability, the presence of adjacent 1° fates in the *lin-15(lf)* mutant, with or without an anchor cell, is rare, whereas the presence of adjacent 2° fates is not (Sternberg, 1988). In no case are three consecutive 2° fates seen. Most often, 1° and 2° cells alternate with one another. This suggests that some active mechanism is preventing the formation of adjacent 1° cells.

In experiments with *lin-15(lf)* animals in which all but two VPCs were ablated, both VPCs selected intrinsic, 1° fate if not in contact with one another, but one VPC selected 1° fate and the other 2° fate if the VPCs were in contact (Figure 20C,D). This result suggests that adjacent VPCs communicate with one another to prevent the formation of adjacent 1° cells. This communication has been called **lateral inhibition** or **lateral signalling** since it occurs laterally between VPCs.

Although this conclusion did not depend on the presence or absence of the anchor cell, the anchor cell did bias which cell executed the 1° fate (see Figure 16C,D). In the absence of the anchor cell, two adjacent VPCs fight it out with one another over which will adopt the 1° fate. At random, one VPC wins out, and the other VPC is forced to adopt a 2° fate. In the presence of the anchor cell, the VPC closer to the inducing signal always selects the 1° fate, forcing its adjacent VPC neighbor to adopt a 2° fate. This helps explain the patterns seen when all six VPCs are present in a *lin-15* Muv mutant (Figure 16A,B). If the anchor cell is present, the VPC closest to the anchor cell, P6.p, adopts a 1° fate, forcing P5.p and P7.p into 2° fate via lateral signalling. In the absence of the anchor cell, P6.p is no longer biased to execute the 1° fate, resulting in a more random pattern of 1° and 2° fates.

C. The gene *lin-12* is necessary and sufficient to specify 2° fate

The activation of 2° fate requires the gene *lin-12*. Mutations that eliminate *lin-12*, designated *lin-12(Ø)*, result in VPCs executing only 1° and 3° fates in an otherwise wild-type hermaphrodite (Figure 21B; Greenwald *et al.*, 1983; Sternberg and Horvitz, 1989). Thus, *lin-12* is necessary for selection of 2° fate in wild type. (Phenotypically, *lin-12(Ø)* animals often display one large protrusion at their vulva.) Furthermore, in a *lin-12(Ø); lin-15(lf)* double mutant, all six VPCs execute 1° fates (Figure 21C; Sternberg and Horvitz, 1989). This result suggests that *lin-12* is required for lateral signalling, *i.e.*, selection of 2° fate in a *lin-15(lf)* background.

In addition to *lin-12(Ø)* mutants, there are also semi-dominant, gain of function *lin-12* mutants (designated *lin-12(d)* for *lin-12 d*ominant), which

behave like they increase *lin-12* activity above wild-type levels (Greenwald *et al.*, 1983). All six VPCs in mutant *lin-12(d)* hermaphrodites that lack an anchor cell execute 2° fates (Figure 21D; Greenwald *et al.*, 1983; Sternberg and Horvitz, 1989). Thus, activation of *lin-12* is sufficient for selection of 2° fate. Consistent with this result, all six VPCs in a *lin-12(d)* hermaphrodite select 2° fates when the Vul genes are eliminated (Sternberg and Horvitz, 1989; Han *et al.*, 1990). Phenotypically, *lin-12(d)* hermaphrodites show multiple ventral protrusions due to the presence of the extra 2° lineages. In this respect these hermaphrodites share some similarity with Muv hermaphrodites. However, here we will not refer to the *lin-12(d)* phenotype as "Muv" since the cellular basis of the two phenotypes are quite distinct.

These data are summarized in Figure 22 and the conclusions from these data in Table 2 (adapted from Sternberg, 1988). In the absence of all known external influences, a VPC will intrinsically execute 1° fate via the intrinsic pathway. The selection of 2° fate ensues when such an isolated VPC is given a neighbor -- one VPC signals the other to execute 2° fate. Selection of 2° but not 1° fate requires *lin-12* since if *lin-12* activity is eliminated, VPCs do not select 2° fate. The intrinsic pathway is normally needed for both 1° and 2° fate since Vul hermaphrodites express neither 1° nor 2° fates. However, activated *lin-12* can abrogate the need for the intrinsic pathway in the selection of 2° but not 1° fate since a *lin-12(d)* mutation results in all VPCs executing 2° fate in a hermaphrodite lacking an anchor cell or the Vul genes. In addition, when *lin-12* is activated, 2° fate selection is not affected by the inhibitory signal from hyp7 since in a *lin-12(d);lin-15(+)* hermaphrodite all VPCs are 2°.

The molecular nature of *lin-12* provides a compelling link between the lateral signal inferred above and the *lin-12* phenotypes. That is, similar to what is believed to be true for the related *Drosophila* protein *Notch* (see above), *lin-12* may be a receptor for short range signal, in particular the lateral signal from a 1° VPC to its neighbor (Figure 22B; Sternberg and Horvitz, 1989). When this receptor is lacking, the lateral signal to be 2° cannot be received by a VPC. When this receptor is activated, then a VPC is biased to select a 2° fate. Consistent with the notion of *lin-12* as a receptor, mosaic analysis has shown that *lin-12* acts cell autonomously in the determination of anchor cell/ventral uterine cell fate (Seydoux and Greenwald, 1989). Alternatively, *lin-12* may not be a receptor for the lateral signal, and, in a *lin-12(Ø); lin-15(lf)* double mutant, all 1° fates are selected because VPCs are unable to select 2° fate. The lateral signal still operates but the 2° pathway is blocked due to the *lin-12(Ø)* mutation. This confusing state may explain why the lineages executed by the VPCs, interpretable as 1°, are nonetheless unusual (see Sternberg and Horvitz, 1989).

D. Modeling the selection of the three VPC fates in wild type

As summarized in Table 2, in the absence of the Vul genes 3° fate is selected unless *lin-12* is activated via *lin-12(d)*, which then leads to 2° fate. If the Vul genes are present and *lin-12* is off, then a 1° fate ensues. Thus, the combination of two binary switches, I.P. (intrinsic pathway) ON/OFF and *lin-12* ON/OFF, allows determination of the three vulval fates (Sternberg and Horvitz, 1989). Muv genes, such as *lin-15*, affect determination by influencing the first switch, I.P. ON/OFF. However, this leaves unanswered what happens when both switches are on. This

situation is important to consider because it is likely to occur in wild type since: (A) *lin-12* is required for 2° fate; and (B) the I.P. is required for 2° fate (in addition to 1° fate). Although Table 2 indicates that 2° fate ensues from *lin-12* ON - I.P. OFF, this conclusion is based on data from *lin-12(d)*, not wild type, animals.

The data suggest that the situation when both switches are on is unstable since 1° fate and 2° fates are mutually antagonistic. First, whereas VPCs in a *lin-12(d)* hermaphrodite lacking an anchor cell all select 2° fates, P6.p in a *lin-12(d)* hermaphrodite with an anchor cell selects 1° fate (the others select 2° fates). Therefore, the anchor cell signal and high activation of the intrinsic 1° pathway can override selection of 2° fate. Conversely, whereas an isolated VPC in a *lin-15(lf)* hermaphrodite always selects 1° fate, when two adjacent VPCs are present, this selection is overridden in one of them. Therefore, 2° fate can inhibit 1° fate. The mutual antagonism is perhaps best represented in a *lin-12(d); lin-15(lf)* mutant with all six VPCs present -- 1° fate is present, so some of the 2° fates from *lin-12(d)* are suppressed, but there are less 1° fates than in a *lin-15(lf)* mutant alone, so the intrinsic 1° pathway is also suppressed in some VPCs.

The above discussion has also ignored an important result from cell biology experiments in wild type -- namely that an isolated VPC at intermediate distance from the anchor cell can select 2° fate. One interpretation is that there are two ways to activate *lin-12*: (A) via the lateral signal from a neighboring VPC; and (B) via intermediate activation of the intrinsic 1° pathway by the anchor cell. Furthermore, activated *lin-12* serves two functions: (A) inhibit the intrinsic 1° pathway within the same VPC and (B) activate the subprogram required to execute 2° fate. A model

summarizing how 1° vs. 2° vs. 3° fate might be determined is shown in Figure 23.

E. Hyperinduction -- stimulatory and inhibitory pathways in the VPCs

Certain mutations in *lin-2*, *lin-7*, and *let-23 RTK* result in the **hyperinduced** or **Hin** phenotype (Ferguson and Horvitz, 1985; Aroian and Sternberg, 1991; G. Jongeward and P.W.S., unpublished results). The Hin phenotype is characterized by levels of vulval induction greater than wild type and is distinct from the Muv phenotype (Figure 10G; Aroian and Sternberg, 1991). There are more 1°/2° vulval fates in a Hin animal than in a wild-type animal, but unlike Muv induction patterns, VPCs closest to the anchor cell select higher fates than cells further from the anchor cell. In addition, induction in Hin mutants is dependent on the anchor cell. If the anchor cell is ablated, then all VPCs execute 3° fate. Therefore, unlike the VPCs in a Muv hermaphrodite which are induced independently of the anchor cell, the VPCs in a Hin hermaphrodite still require the anchor cell signal and are hypersensitive to it. Also, adjacent 1° fates can occur in Hin mutants, suggesting that lateral signalling has broken down or that higher activation of the intrinsic pathway has overridden the 2° pathway.

Surprisingly, mutations in *lin-2*, *lin-7*, and *let-23 RTK* which result in a Hin phenotype appear to reduce, but not eliminate, gene function (Aroian and Sternberg, 1991; G. Jongeward and P.W.S., unpublished results). For example, the *let-23 RTK* allele *let-23(n1045)* at 25° results in the Vul phenotype when present in one copy (*i.e.*, in *trans* to a deletion for *let-23 RTK*) but results in the Hin phenotype if present in two copies (*i.e.*, when homozygous, Aroian and Sternberg, 1991). The first result suggests that

let-23(n1045) reduces *let-23 RTK* function. The second result suggests that two copies of this reduction of function mutation somehow results in too much induction. Thus, if *let-23 RTK* is eliminated no vulval induction occurs, but if *let-23 RTK* is only partly reduced than more than wild-type induction occurs. This striking conclusion is supported by another *let-23 RTK* allele *mn224* (Aroian and Sternberg, 1991).

These data suggest that *let-23 RTK*, *lin-2*, and *lin-7* are involved in two different pathways -- one that stimulates vulval fate and another that inhibits vulval fate (Figure 24A; Aroian and Sternberg, 1991). Hin mutations such as *let-23(n1045)* might preferentially cripple the ability of these proteins to operate in the inhibitory pathway over the stimulatory pathway, resulting in too much induction because inhibition is lacking. One simple model would be that the *let-23 RTK* activated-inhibitory process is more sensitive to a decrease in *let-23 RTK* activity levels than the *let-23 RTK* activated-stimulatory process. An initial reduction in *let-23 RTK* activity would therefore result in activation of stimulation but not inhibition (Figure 24B). A further reduction in *let-23 RTK* activity, however, would affect both processes, leading to less than wild-type induction (Figure 24C). An alternative model is that the Hin mutations change quantitative aspects of these proteins. For example, the C-terminal region of EGF receptor negatively regulates receptor activity since it contains autophosphorylation and receptor downregulation sites, and sequence evidence suggest that the *let-23(n1045)* mutation results in a protein truncated for the C-terminus (R.V.A. and P.W.S., unpublished results). The *let-23(n1045)* allele might therefore be Hin because the receptor is not properly regulated. While this model could explain the *let-23* data, the *lin-2* and *lin-7* data are easier to

incorporate with the level model above. The fact that *lin-2* and *lin-7* behave similarly to *let-23 RTK* (G. Jongeward and P.W.S., unpublished results) may suggest that they exert their influence on *let-23 RTK* independent of the Muv genes, since there is no evidence that the Muv genes act in both pathways.

This inhibitory process associated with *let-23 RTK* could operate intracellularly or intercellularly (Figure 24A). In an intracellular model, the *let-23 RTK* stimulatory and the inhibitory pathways would both operate inside the same cell, consistent with, for example, the C-terminal truncation model above. On the other hand, *let-23 RTK* could be involved in intracellular stimulation and intercellular inhibition. With this notion, it is possible to combine the Hin phenotype with lateral signalling. For example, *let-23 RTK* may be involved in activating the lateral signal between cells. A Hin phenotype might then result from a weakened lateral signal. Since the proposed role of the lateral signal is to activate *lin-12* in a neighboring VPC and thereby inhibit that neighbor's intrinsic pathway (Figure 24B), a consequence of a weaker lateral signal might be to raise the overall level of induction. Alternatively, *let-23* may be involved in an intercellular signal that is unrelated to the lateral signal. (See legend to Figure 24 for more discussion.)

VI. Going deeper -- dissecting gene functions, downstream elements

A. Dissecting gene function by sequencing point mutations

The availability of cellular techniques, genetic techniques, and molecular germ line transformation techniques in the study of vulval

determination promises to verify present knowledge of *ras*- and EGF receptor-like genes and to yield new insights into their functional properties. The *let-23(n1045)* hyperinduced allele is one potential example. Mutations associated with other *let-23 RTK* alleles have also been sequenced. For example, two loss of function alleles result from missense mutations in conserved residues in the tyrosine kinase domain (R.V.A. and P.W.S., unpublished results). This suggests that *let-23 RTK* has tyrosine kinase activity and that these conserved residues are functionally important.

Mutations in *let-60 ras* provide a powerful example of dissecting gene function with point mutation studies. Three types of *let-60 ras* mutations exist. Complete loss of function and reduction of function *let-60 ras* alleles result in a recessive, Vul phenotype (as discussed above). In addition, there are dominant *let-60 ras* Muv mutations. These alleles behave like gain of function mutations in which the *let-60 ras* gene is constitutively on and are designated *let-60(gf)* (for *g*ain of *f*unction, Beitel *et al.*, 1990; Han *et al.*, 1990). These mutations result in an anchor-cell independent Muv phenotype because the requirement for *let-60 ras* activation by *let-23 RTK* is presumably bypassed, and the intrinsic pathway is constitutively on. Moreover, there are dominant Vul *let-60 ras* alleles, designated *let-60(dn)* (for *let-60 d*ominant *n*egative, Han *et al.*, 1990; Beitel *et al.*, 1990). A *let-60(dn)* allele in *trans* to a wild-type *let-60 ras* chromosome, *i.e.*, *let-60(dn)* / +, results in a Vul hermaphrodite. The data suggest that these *let-60(dn)* alleles reduce *let-60 ras* activity and can also poison wild-type *let-60 ras* activity, resulting in a dominant Vul phenotype (Han *et al.*, 1990; Han and Sternberg, 1992). Thus, the *let-60 ras* gene can be mutated three ways: loss

of function alleles display a recessive Vul phenotype, dominant gain of function alleles display a dominant Muv phenotype, and dominant negative alleles display a dominant Vul phenotype.

The sequences associated with these alleles therefore indicate residues that, when altered,: 1) eliminate or reduce *ras* function; 2) activate *ras* function independent of upstream genes such as *let-23 RTK*; and 3) poison *ras* function in *trans*. Sequence of all these types of mutations have been analyzed (Beitel *et al.*, 1990;Han and Sternberg, 1992). Many of the recessive *let-60 ras* mutations affect codons highly conserved in the *ras* family, and these residues might be important for activation of, but not for activity of, *ras* (Beitel *et al.*, 1990). One recessive reduction of function allele alters a residue in the effector domain (Beitel *et al.*, 1990). The dominant gain of function alleles all alter the conserved glycine at codon 13 to glutamic acid (Beitel *et al.*, 1990). Alterations of this glycine in other systems have been associated with oncogenic activation of *ras*. Thus, the effects of *let-60 ras* mutations on vulval development highly parallel what is known of *ras* mutations in other systems and, with the *let-23 RTK* data above, suggest that *C. elegans* vulval development provides a good system for studying important processes of more complex organisms.

The residues altered by many *let-60(dn)* mutations and the properties of these mutations have also been studied (Han and Sternberg, 1992). These data suggest that the *let-60(dn)* mutations likely influence guanine nucleotide binding and that the *let-60(dn)* protein interacts non-productively with some positive factor required by the wild-type *let-60* protein.

B. Mutations that affect execution, and not determination, of vulval fate.

The phenotype of Muv-Vul double mutants suggest that the Vul genes discussed above are defective in determination, but not execution, of 1°/2° vulval fate (Sternberg and Horvitz, 1989). For example, in a *let-23(lf)* background, *let-60 ras* gain of function mutations still result in selection of all 1° and 2° fates by the VPCs (Han *et al.*, 1990). Thus, even if *let-23 RTK* activity is eliminated, the VPCs are still competent to execute 1° and 2° fate subprograms.

Three genes are known to affect execution and not determination of vulval fate: *lin-11*, *lin-17* and *lin-18* (Figure 25; Ferguson and Horvitz, 1985; Ferguson *et al.*, 1987; Sternberg and Horvitz, 1988). Mutations in *lin-11* result in abnormal "LLLL" 2° lineages (Figure 25; Ferguson *et al.*, 1987). Given its molecular structure, the *lin-11* gene may be a transcription factor that is activated by *lin-12* and is required in VPC daughter cells for execution of the "NT" portion of 2° lineages. The genes *lin-17* and *lin-18* mainly affect P7.p. Although mutations in these genes cause variable effects, one important effect is to sometimes reverse the polarity of P7.p's 2° lineage from "NTLL" to "LLTN" (Figure 26; Ferguson *et al.*, 1987; W. Katz and P.W.S., unpublished observation). P5.p's polarity is unaffected, suggesting that some mechanism, operating via *lin-17* and *lin-18*, is actively required to make P7.p's polarity the mirror image of P5.p's. Mutant *lin-11* hermaphrodites display a single ventral protrusion and are Egl whereas mutant *lin-17* and *lin-18* hermaphrodites sometimes show two small ventral protrusions, one at and one posterior to the vulva (Ferguson and Horvitz, 1985; W. Katz and P.W.S., unpublished observation).

C. *lin-25*

Mutations in the gene *lin-25* do not fit nicely into any one category because they seem to fit into many categories. Most *lin-25* mutant hermaphrodites are Egl, though some are sterile (Ferguson and Horvitz, 1985). The *lin-25* mutation often results in extra VPCs and the precocious division of VPCs, suggesting that *lin-25* is involved in proper generation of the VPCs (Ferguson *et al.*, 1987). Furthermore, in those mutant animals in which the VPCs divide at their normal time, there are no 2° lineages, only 3° lineages and an abnormal 1° lineage at P6.p (Ferguson *et al.*, 1987). Therefore, in conjunction with *lin-12*, *lin-25* is needed for proper determination of 2° fate. Also, the temperature sensitive period of *lin-25* extends beyond the first division of the VPCs, past the time when determination is thought to occur (Ferguson *et al.*, 1987). Thus *lin-25* is also likely to affect execution of vulval lineages. Consistent with this, *lin-25* mutations result in abnormal 1° lineages and are epistatic to *lin-1* (*i.e.*, the *lin-25; lin-1* double mutant is Egl not Muv, E. Ferguson, P.W.S., and H.R. Horvitz, unpublished observations), suggesting that *lin-25* performs some downstream function.

D. Non-standard vulval lineages -- a possible link between determination and execution

Hybrid lineages associated with mutations in the Vul determination genes and *lin-12* may provide evidence for a link between determination and execution. Hybrid lineages are not uncommon and are characterized by selection of half nonvulval and half vulval fate (Figure 26A). Although they are associated with reduction of function mutations in the Vul determination genes, they are most simply explained as occurring due to

defects in execution of vulval fate. In the case of *let-23 RTK* mutants, hybrid lineages show a polarity bias centered toward the anchor cell such that the vulval half of the lineage is always closest to the anchor cell (Aroian and Sternberg, 1991). This suggests that the anchor cell is polarizing these lineages. Furthermore, there is no obvious correlation between selection of hybrid fate and the fate executed by neighboring VPCs, suggesting that perhaps hybrids are determined cell autonomously (Aroian and Sternberg, 1991)

It has been proposed that such lineages reflect a defect in execution of vulval fate in one of the daughters of a VPC (Sternberg and Horvitz, 1986; Aroian and Sternberg, 1991). In one simple model, determination of 1° and 2° vulval fate by the Vul determination genes *lin-2*, *lin-3*, *lin-7*, etc.. results in the production of a Factor X that is segregated to the daughters of the VPCs and is required for those daughters to execute a vulval lineage (Aroian and Sternberg, 1991). Furthermore, the anchor-cell signal biases the distribution of Factor X such that more Factor X is localized in the part of a VPC closest to the anchor cell (Figure 26B). VPCs in which the vulval determination pathway is reduced (but not eliminated) may undergo proper determination but improper execution because not enough Factor X is produced and the amounts that are produced are preferentially localized to the anchor-cell proximal part of a VPC (Figure 26B). Such a model is consistent with the hybrid data, but is not the only one possible. For example, hybrid lineages may represent truly novel lineages that result from defective determination and not execution.

VII. Prospects

The development of the *C. elegans* vulva provides a powerful system to study signal transduction via cell biology, genetics, and molecular biology. The ability to analyze the vulva at the cellular level and to perturb the surroundings makes it possible to understand in some detail what processes are occurring, and how various mutations affect those processes. Furthermore, the ease of genetic manipulation makes it possible to isolate many mutations in the pathways involved and to probe the relationships among these genes. Lastly, molecular analysis is now showing that these signal transduction processes share many elements in common with other important signal transduction processes in other organisms. The combination of this molecular information with the other tools of studying the vulval determination system are verifying and extending what we know of these elements. In addition, as more genes in the pathway are characterized and cloned, novel relationships and processes in these pathways might well be uncovered that will shed new light on related pathways and processes in other systems.

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IX. Acknowledgements

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Table 1. See text for discussion of complete/incomplete Vul phenotypes. Note that mutations in some of these genes can affect more than just vulval determination (*e.g.*, *lin-3*, *let-23 RTK*, and *let-60 ras* can be mutated to larval lethality).

Criteria: (1) Recessive phenotype. Consistent with being loss of function, mutations in these genes are recessive. (*let-60* also has dominant alleles. See text.)

(2) Number of alleles. Isolation of many alleles with the same phenotype suggests such alleles are complete loss of function alleles since these alleles are apt to be the most common.

(3) Allelic series. If alleles with more severe phenotypes (*e.g.*, lethality) enhance the penetrance of the vulval phenotype of less severe alleles, this suggests the vulval phenotype is due to loss of function. For example, the reduction of function *let-23* allele *let-23(sy1)* shows little vulval induction but does not show larval lethality. The *let-23* allele *let-23(sy15)* has a more severe phenotype (*i.e.*, larval lethality) and presumably has less *let-23* activity than *sy1*. The trans heterozygote *let-23(sy1)/let-23(sy15)* shows no vulval induction, suggesting that as *let-23* activity is lowered from *let-23(sy1)/let-23(sy1)* to *let-23(sy1)/let-23(sy15)*, vulval induction is completely eliminated and that loss of *let-23* function results in no vulval induction.

(4) Amber alleles. Amber alleles often, but not always, result in decreasing or eliminating gene activity since they often result in a truncated protein.

(5) Deficiency enhances phenotype. If alleles in *trans* to a deficiency (*i.e.*, a deletion) show a more penetrant phenotype, this suggests that the phenotype is loss of function. In the case of *lin-10*, a deficiency did not

enhance the phenotype, suggesting that the phenotype was already the null phenotype.

(6) Other. For *lin-10*, alleles generated by transposon insertion are Vul. For *let-60*, see reference E.

References: (A) Ferguson and Horvitz (1985). (B) Ferguson *et al.* (1987). (C) Sternberg and Horvitz (1989). (D) R. Hill and P.W.S., unpublished observations. (E) Kim and Horvitz (1990). (F) Aroian and Sternberg (1991). (G) Han *et al.* (1990). (H) Han and Sternberg (1992). (I) L. Huang and P.W.S., unpublished observations.

Table 1 Vulval phenotypes resulting from loss of function in various genes.

gene	complete loss of function vulval phenotype	criteria	references
lin-2	Vul - incomplete	1,2	A,B,C
lin-3	Vul - complete	1,3	A,B,C,D
lin-7	Vul - incomplete	1,2,4	A,B,C
lin-10	Vul - incomplete	1,2,4,5,6	A,B,C,E
let-23	Vul - complete	1,3,5	A,B,C,F
let-60	Vul - complete	1,6	G,H
lin-1	Muv	1,2,4	A,B
lin-13	Muv	1,5	A,B
lin-15	Muv	1,5	A,B,I

Table 2. If the intrinsic pathway (I.P.) is on and *lin-12* pathway is off, then a VPC selects 1° fate. If the I.P. is off and *lin-12* is on, then 2° fate ensues. If both pathways are off, then 3° fate ensues. Since the pathways are mutually antagonistic, then what happens when both pathways are on depends on how strongly each is activated (see text). Adapted from Sternberg (1988).

Table 2. Two pathways determine selection of three vulval fates.

lin-12	I.P. ON	I.P. OFF
ON	?	2°
OFF	1°	3°

Figure 1. Schematic of reproduction in an adult *C. elegans* hermaphrodite. Syncytial germ line nuclei are located at the distal end of each gonad arm. As they move proximal towards the vulva, they mature into oocytes, which are then fertilized by sperm as they pass through the spermatheca. The hermaphrodite stores its own sperm in the spermatheca, but it can also store sperm from a male. The fertilized eggs end up in the uterus where, after several rounds of division, they are ejected through the vulva. The adult hermaphrodite length is about 1 mm.

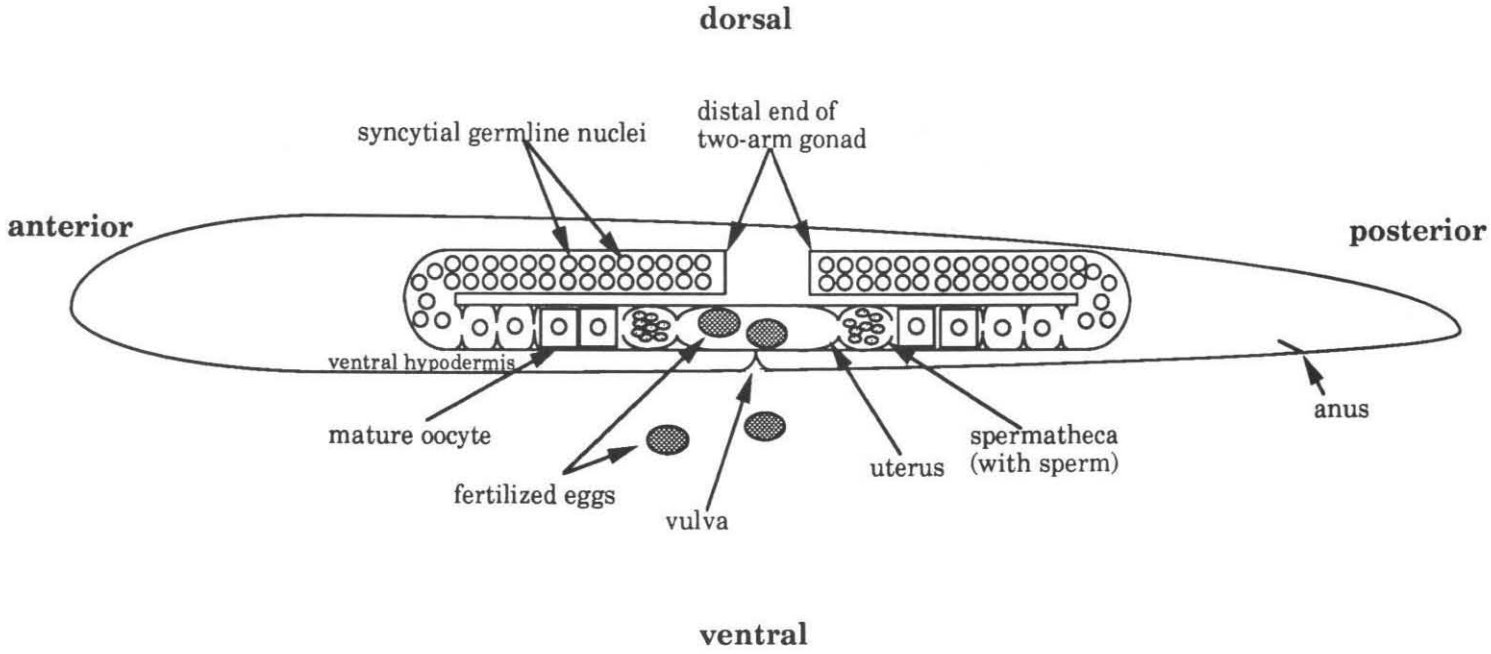
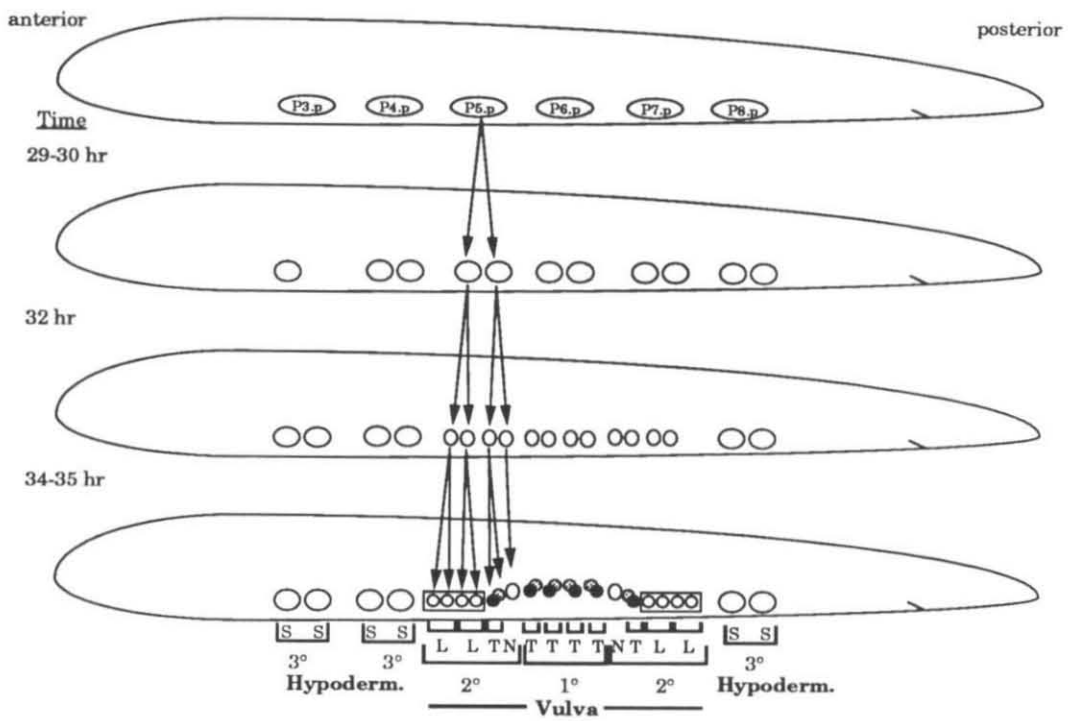

dorsal
syncytial germline nuclei
distal end of
two-arm gonad
anterior
posterior
ventral hypodermis
mature oocyte
fertilized eggs
vulva
uterus
spermatheca
(with sperm)
anus
ventral

Figure 2. Cell divisions responsible for producing the vulva. (A) A *C. elegans* hermaphrodite is schematically shown in the same orientation as in Figure 1. The divisions of P5.p, P6.p, and P7.p to generate the 22 vulval nuclei are shown. The divisions of P5.p and its progeny are connected by arrows to show the relationship between the cells of each generation. Numbers to the left show the approximate time in hours from hatching that a given round of division occurs. The first two rounds of division for P5.p, P6.p, and P7.p and their progeny are similar. They and their daughters divide longitudinally along the ventral hypodermis of the hermaphrodite. The division patterns diverge at the third and final round. All four granddaughters of the P6.p cell divide transversely (T) – perpendicular to the long axis of the nematode along the left-right axis (shading indicates that cells are not located in the plane of the page). We refer to this pattern of division as "TTTT." The four granddaughters of P7.p execute the mirror image division patterns of the four P5.p granddaughters, which include longitudinal (L), transverse (T), and no (N) divisions. From anterior to posterior, the pattern of division for P5.p is "LLTN" and for P7.p "NTLL." The "LL" portion of P5.p and P7.p are boxed and the other vulval nuclei are shown pulled away from the ventral cuticle to emphasize that the vulval nuclei resulting from the "LL" divisions adhere to the cuticle. The final round of division is shown schematically – although the orientation of the divisions (L,T,N) is correct, the final relative placement of the cells is not exact. The surrounding cells, P3.p, P4.p, and P8.p, divide once at about the same time and in the same orientation as P5.p, P6.p, and P7.p, but their daughters do not divide. These daughters do not contribute to the vulva but rather fuse with the hypodermal syncytium and become hypodermis (lineage abbreviated "SS" for "Syncytial Syncytial"). The designations 1°, 2°, and 3° are discussed later in the text. (B). This schematic is often simplified as a lineage tree. The "L" divisions of P5.p and P7.p are bold-faced to emphasize adherence to the cuticle. Hypoderm. = hypodermis. Data of Sulston and Horvitz (1977).

A.


anterior
posterior
P3.p
P4.p
P5.p
P6.p
P7.p
P8.p
Time
29-30 hr
32 hr
34-35 hr
S S
S S
L L T N
T T T T
N T L L
S S
3°
3°
3°
Hypoderm.
2°
1°
2°
Hypoderm.
Vulva

B.

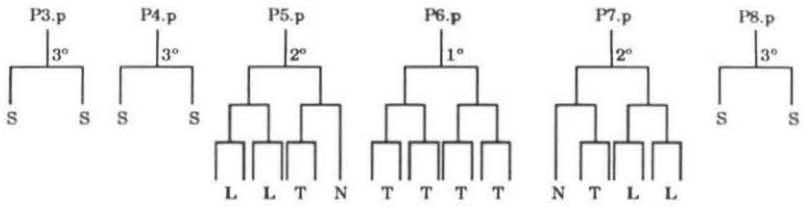
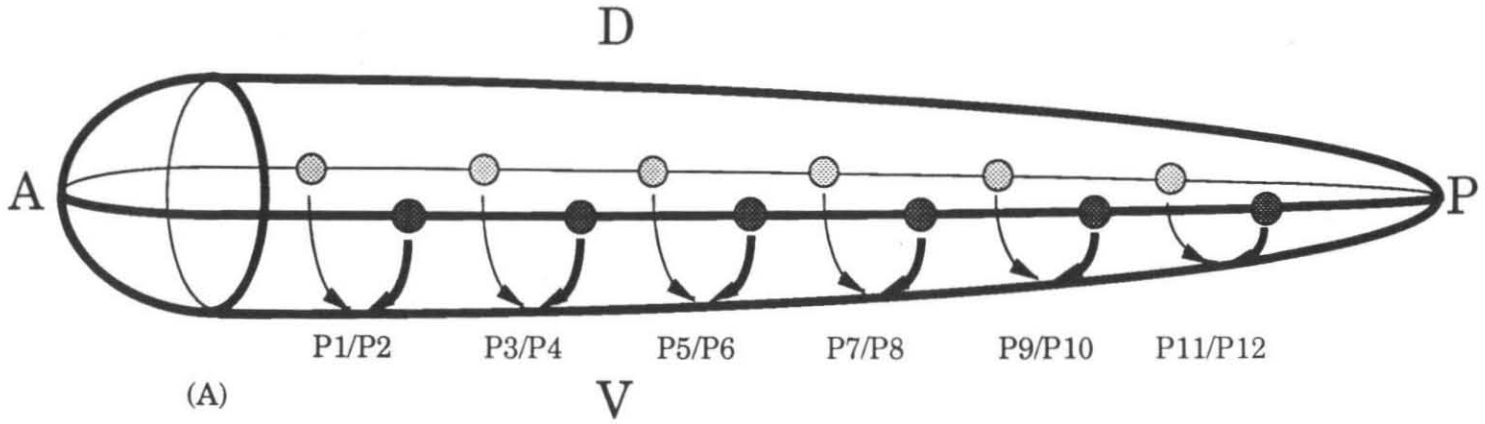
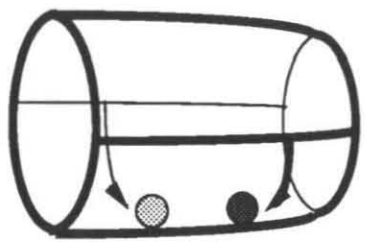

P3.p
P4.p
P5.p
P6.p
P7.p
P8.p
3°
3°
2°
1°
2°
3°
S S
S S
L L T N
T T T T
N T L L
S S

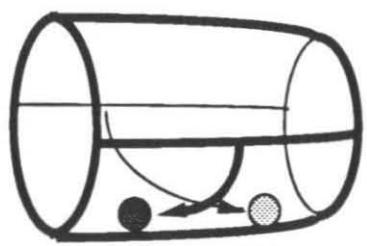
Figure 3. Random intercalation of the cells that produce the vulva. A = anterior; P = posterior; D= dorsal; V = ventral. Cells on the right side are lightly shaded; cells on the left side, heavily shaded. (A) At hatching, there are twelve cells located in six left-right pairs along the length of a hermaphrodite. Soon after hatching, these cells migrate circumferentially down into the ventral cord. (B) As shown for the third left-right pair, half the time the cell located on the right side will intercalate anterior of the cell on the left. (C) The other half the time the cell located on the right will intercalate posterior of the cell on the left . Since the cell that ends up anterior becomes P5, the cell that becomes P5 is not fixed but can be either the left or the right cell of the third left-right pair. The other cell becomes P6. P5 will divide soon after migration and give rise to P5.p (the posterior daughter of P5), and P6 will give rise to P6.p. The granddaughters of P5.p always divide in the "LLTN" pattern whereas the granddaughters of P6.p divide in the "TTTT" pattern (Figure 2). Thus, the determination of which cell's progeny will execute the "TTTT" pattern (P6.p granddaughters) and which cell's progeny will execute the "LLTN" pattern (P5.p granddaughters) is not fixed entirely by ancestry (*i.e.*, cell autonomous development) but must be determined interactively (*i.e.*, cell non-autonomous development). This determination must occur after P5 and P6 migrate but before the granddaughters of P5.p and P6.p divide. Since P7 and P8 also form a variable left/right pair, the decision to produce vulval progeny (P7.p) versus hypodermal progeny (P8.p) must also be cell non-autonomous. Data of Sulston and Horvitz (1977).



(A)



(B)



(C)

Figure 4. The anchor cell of the somatic gonad is necessary and sufficient to induce a vulva. (A) Abbreviated vulval development is schematically shown from the cells P3.p-P8.p to the final set of cells derived from these cells. The gonad and the anchor cell (which is in the gonad) are also shown. The orientation is the same as in Figure 1. (B) If the entire gonad is eliminated before the cells P3.p-P8.p divide (designated by "X's"), then P3.p - P8.p all divide once and fuse with the hypodermis. No vulva forms. (C) The elimination of one of the somatic gonad cells, the anchor cell, produces the same result, indicating that the anchor cell is necessary for differentiation of the vulva. (D) Elimination of all the gonad cells but the anchor cell results in normal production of the vulva, indicating that the anchor cell is also sufficient for vulval differentiation. (E) However, if the anchor cell is ablated after the cells P3.p-P8.p have first divided, then subsequent rounds of division occur normally. Data of Sulston and White (1980); Kimble (1981); Sternberg and Horvitz (1986).

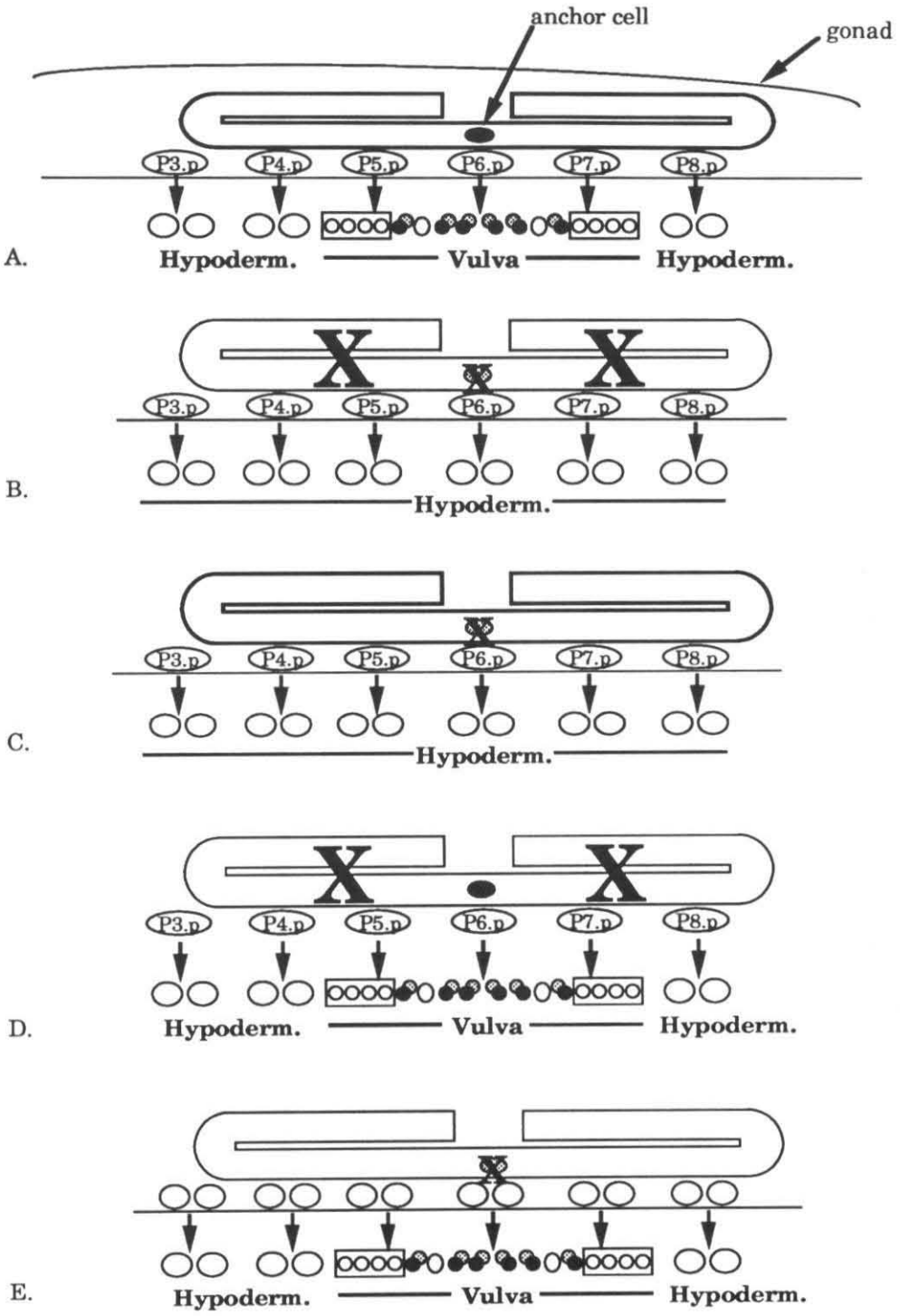

anchor cell
gonad
P3.p
P4.p
P5.p
P6.p
P7.p
P8.p
A.
Hypoderm.
Vulva
Hypoderm.
B.
Hypoderm.
C.
Hypoderm.
D.
Hypoderm.
Vulva
Hypoderm.
E.
Hypoderm.
Vulva
Hypoderm.

Figure 5. Regulation among the cells P3.p-P8.p. (A) Schematic of wild-type induction. The cells P5.p through P7.p are induced to form vulva. (B) If P6.p is ablated, either genetically or by laser, then P5.p can replace P6.p and produce a "TTTT" lineage, instead of the usual "LLTN". P4.p can then fill the space vacated by P5.p and generate the vulval "LLTN" lineage instead of the usual hypodermal "S S" lineage. (C) Sometimes P7.p and not P5.p will replace P6.p. In this case, P8.p replaces P7.p. (D) If all three of the cells that normally give rise to vulva, *i.e.*, P5.p-P7.p, are ablated, then the three cells that normally produce hypodermis, P3.p, P4.p, and P8.p, can replace them. The other "Pn.p" cells, P1.p, P2.p, P9.p, P10.p, and P11.p were never found to take part in vulval replacement. Although these five cells produce hypodermis like P3.p, P4.p, and P8.p, they differ from these latter three cells in that they do not divide before fusing (lineage = "S"). The "S S" hypodermal lineage is therefore thought to be indicative of a cell in the vulva equivalence group, where the "S" lineage is indicative of a cell that is not. See Figure 2 for a description of lineage designations. Data of Sulston and White (1980); Sternberg and Horvitz (1986).

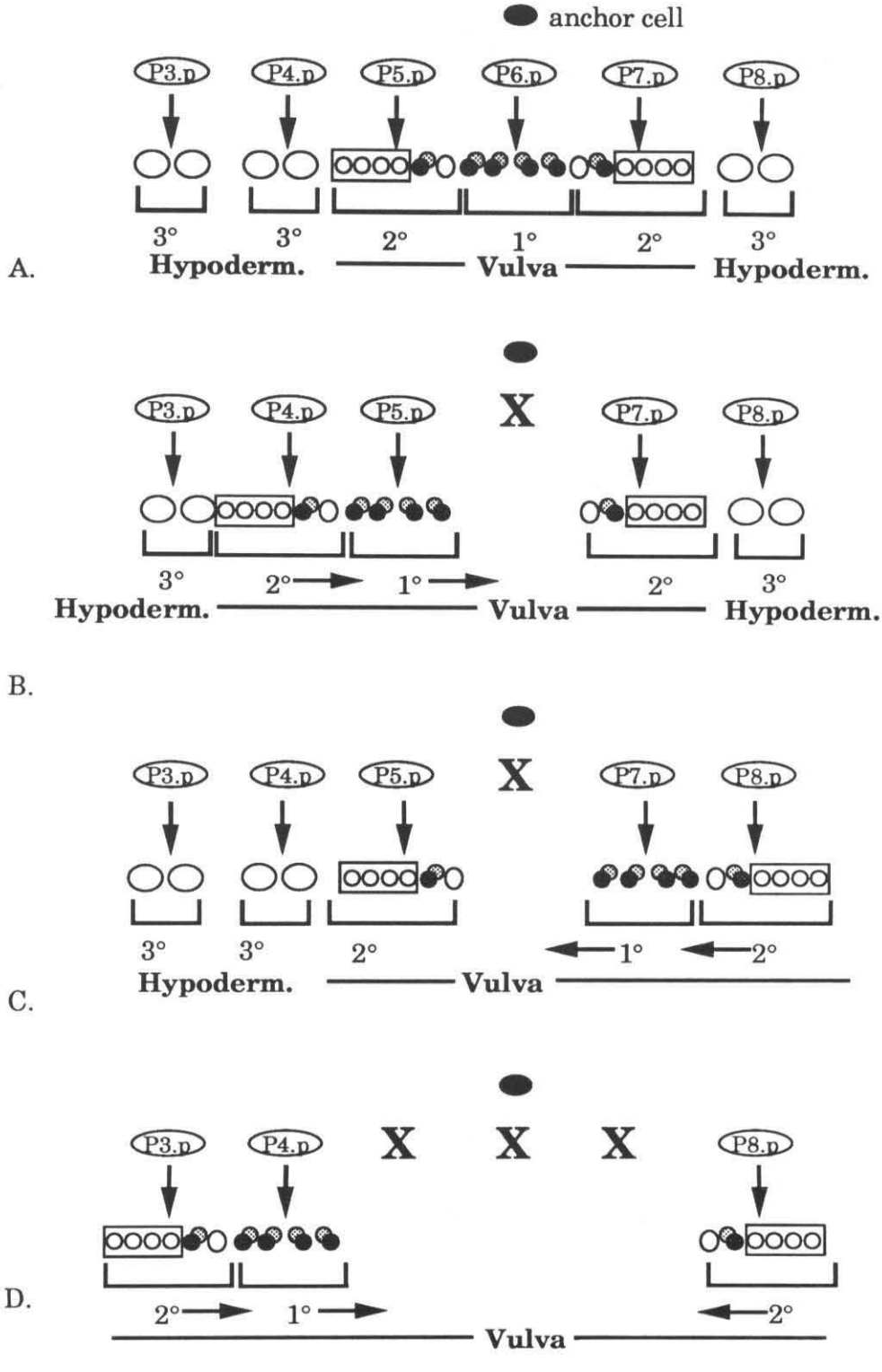
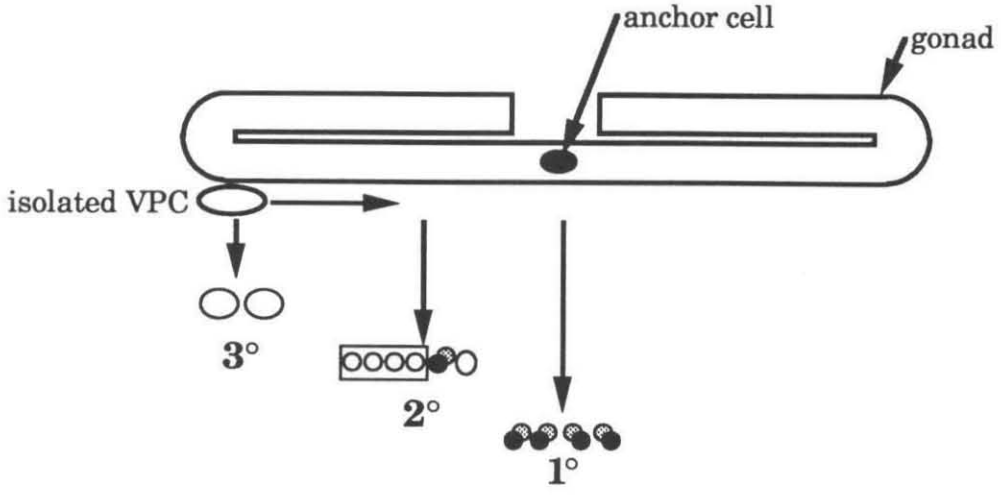
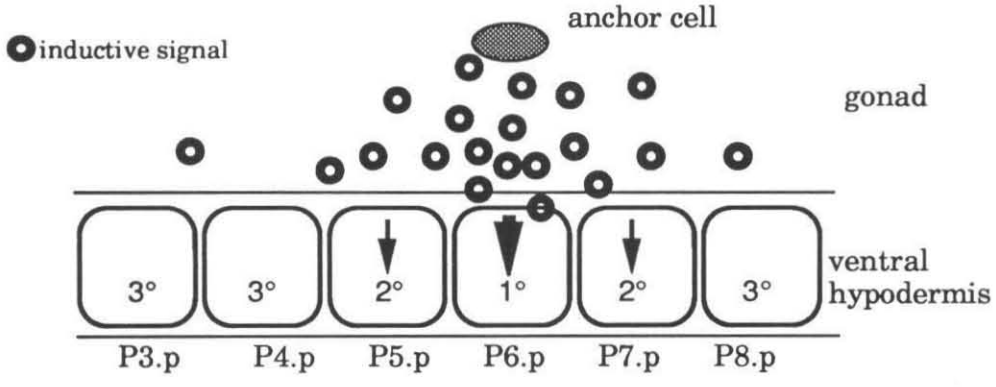

anchor cell
P3.p
P4.p
P5.p
P6.p
P7.p
P8.p
3°
3°
2°
1°
2°
3°
A.
Hypoderm.
Vulva
Hypoderm.
X
B.
C.
D.

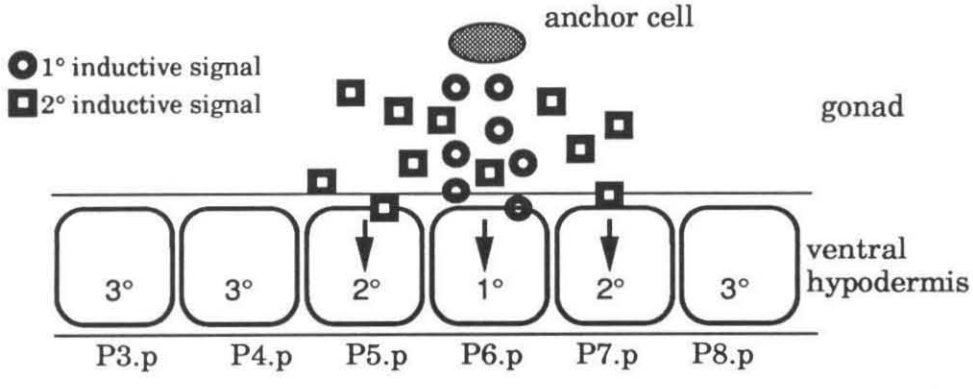
Figure 6. Fates of an isolated VPC suggest that the anchor-cell signal is graded. Five of the six VPC cells were ablated. (A) The remaining cell sometimes migrated toward the anchor cell, and the lineage executed by that cell correlated with its final distance from the anchor cell. If its final position was close to the anchor cell, it executed a 1° fate. If it ended up further away, it executed a 2° fate. And if its ultimate location was yet further, it executed a 3°° fate. (B) One simple model is that the strength of the anchor cell signal is interpreted by each cell, resulting in 1°, 2°, or 3° fate depending upon how strongly or at what time the signal transduction apparatus is activated in a VPC. (C) Alternatively, there may be two signals, one that acts over a very short range to promote 1° fate, and the other that acts over a somewhat larger range to promote 2° fate. Data of Sternberg and Horvitz (1986); P.W.S., unpublished results; M. Herman and R. Horvitz, unpublished results.


anchor cell
gonad
isolated VPC
3°
2°
1°

A.

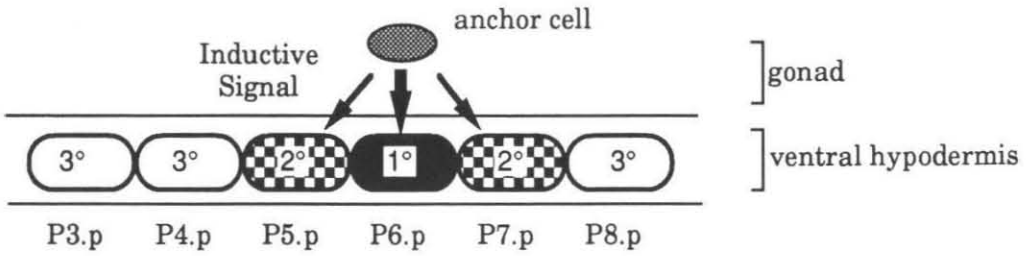

inductive signal
anchor cell
gonad
3°
3°
2°
1°
2°
3°
ventral hypodermis
P3.p
P4.p
P5.p
P6.p
P7.p
P8.p

B.

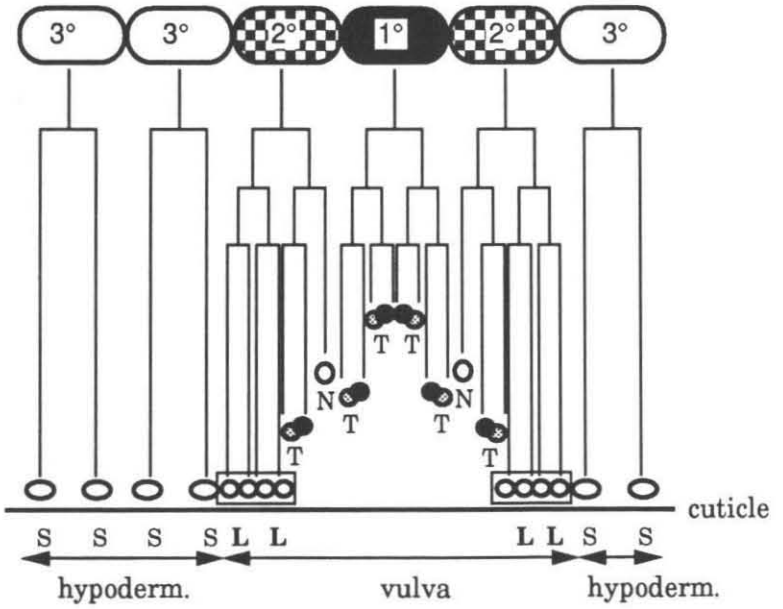

1° inductive signal
2° inductive signal
anchor cell
gonad
3°
3°
2°
1°
2°
3°
ventral hypodermis
P3.p
P4.p
P5.p
P6.p
P7.p
P8.p

C.

Figure 7. Model of vulval development. The following model is suggested by cell biological data. See Section II for details. (A) The anchor cell in the gonad releases a diffusible, graded signal that is transduced by the six vulval precursor cells or VPCs. These cells all have the potential to select any one of three possible fates; the anchor-cell signal plays an important role in determining which fate each VPC selects. The cell closest to the anchor cell, P6.p, receives the most signal and selects 1° vulval fate, the next two cells, P5.p and P7.p, receive less signal and select 2° vulval fate, and the outer three cells select 3° nonvulval fate. In the absence of the anchor cell, all six cells select 3° fate. (B) Once the VPCs have selected their fate, no further inputs appear to be required to execute vulval fate; development proceeds cell autonomously. The outer three cells divide once and fuse with the hypodermis. P5.p and P7.p each give rise through three rounds of division to seven vulval nuclei, four of which adhere to the cuticle (2° lineage). P6.p gives rise to eight vulval nuclei, none of which adhere to the cuticle but the central four of which adhere to the anchor cell (1° lineage). The 22 nuclei from P5.p, P6.p, and P7.p make up the full complement of cells required to make a wild-type vulva. The relative geometry of the 22 nuclei shown in this figure approximates the *in vivo* geometry just after the nuclei have all formed. See Figure 2 for orientation and definition of "T", "N", "L", and "S".


anchor cell
Inductive Signal
gonad
ventral hypodermis
3°
3°
2°
1°
2°
3°
P3.p
P4.p
P5.p
P6.p
P7.p
P8.p

A.


3°
3°
2°
1°
2°
3°
T
T
N
T
N
T
T
T
cuticle
S S S S L L L L S S
hypoderm.
vulva
hypoderm.

B.

Figure 8. Schematics illustrating two broad phenotypes of vulval mutants as seen with a dissecting microscope (see Figure 1 for orientation and structures). (A) Wild-type hermaphrodites generally have zero or one small protrusion at the vulva, and they are able to lay eggs. (B) Mutations that prevent vulval formation have an egg-laying defective or Egl phenotype. In the absence of a vulval opening, oocytes are fertilized as normal but they subsequently cannot be ejected. Fertilized eggs hatch internally, eventually resulting in a bag-of-worms appearance. The hatched larvae feed off the mother and eventually eat their way out, killing the mother. (C) Mutations that increase the amount of vulval tissue show a Muv phenotype. The extra vulval tissue generated results in ventral protrusions easily discernible under a dissecting microscope.

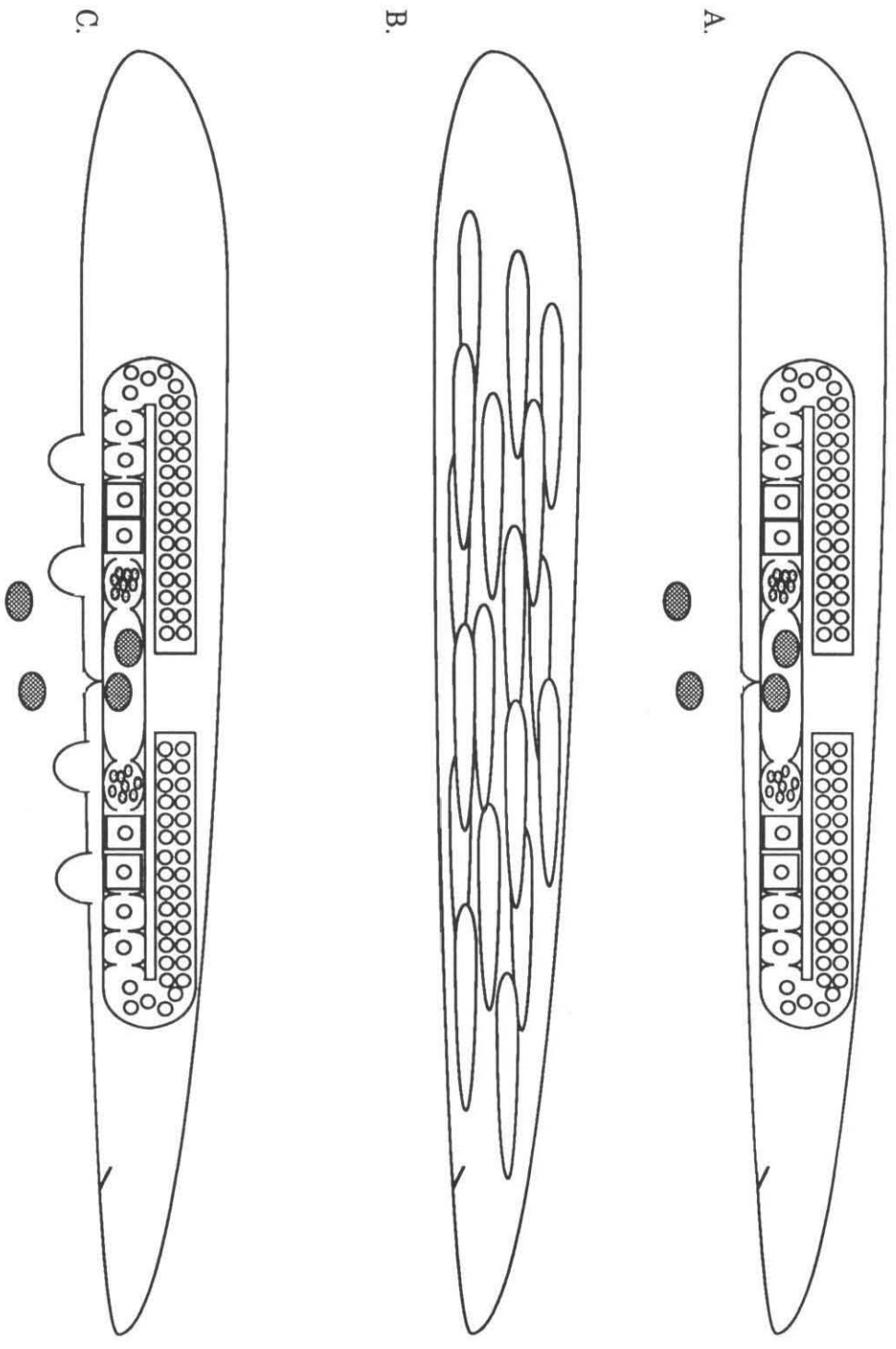

A.
B.
C.

Figure 9. Random screen for vulval mutants. Fourth stage wild-type (+/+) larval hermaphrodites are incubated with EMS to mutagenize their germ line. First generation (F1) self-progeny are often wild-type (+/+) like their mother, but occasionally a mutation is introduced in a gene involved in vulval development. If this mutation is dominant (Mutant or **M**), then Egl or Muv worms may be detected in the self-progeny of the original mother, *i.e.*, the F1 generation. If the mutation is recessive (mutant or **m**), then Egl or Muv worms may be detected in the self-progeny of the F1, *i.e.*, the F2 generation. Random screens for vulval mutations are technically easy and can be completed in about a week -- hermaphrodites are mutagenized, allowed to produce self-progeny for two generations, and each generation is screened for Egl or Muv worms.

mutagenize
self progeny
F1
+ +/−
+ m/m
+ M/m
F2
+ +/−
+ +/−
+ +/−
+ +/−
+ m/m
m m/m
+ +/−
+ M/m
M M/m
Egl
OR
Muv
=

Figure 10. The effects of vulvaless and multivulva mutations on vulval determination. For reference, (A) wild-type induction and (B) induction in a hermaphrodite lacking an anchor cell. (C) The anchor-cell minus phenotype is mimicked in a fully penetrant Vul mutant -- all VPCs divide and fuse with the hypodermis (3° fate), although the anchor cell is now intact. No vulval tissue forms. The "?s" signify that the anchor-cell signal may or may not be normal in a given Vul mutant since the Vul phenotype mimics the anchor-cell ablation. (D) Although this fully penetrant phenotype occurs often with Vul mutations, most alleles of Vul mutations will occasionally show partial induction. This may reflect the fact that the mutations are not nulls or that the genes involved can be bypassed to some extent (see text). (E) Conversely, in a Muv hermaphrodite no 3° fates are seen, and all VPCs execute 1° or 2° vulval fate. (F) This induction of vulval fate occurs even if the anchor cell is ablated so presumably the Muv mutations are not affecting the anchor-cell signal. Weaker Muv mutations, such as *lin-13* or weak alleles of *lin-15*, result in an intermediate phenotype in which more than three but less than six of the VPCs execute vulval fates (Ferguson *et al.*, 1987; P.W.S., unpublished observations). (G) The Hin phenotype is discussed later in the chapter but is shown here for comparison. Like the Muv phenotype, there are extra 1° and 2° fates. Unlike the Muv phenotype, the VPCs in a Hin mutant still behave as if they are responding to the anchor cell signal: (1) there is a gradation of VPC fate around the anchor cell; and (2) VPCs in a Hin hermaphrodite will select 3° fate if the anchor cell is ablated. See text.

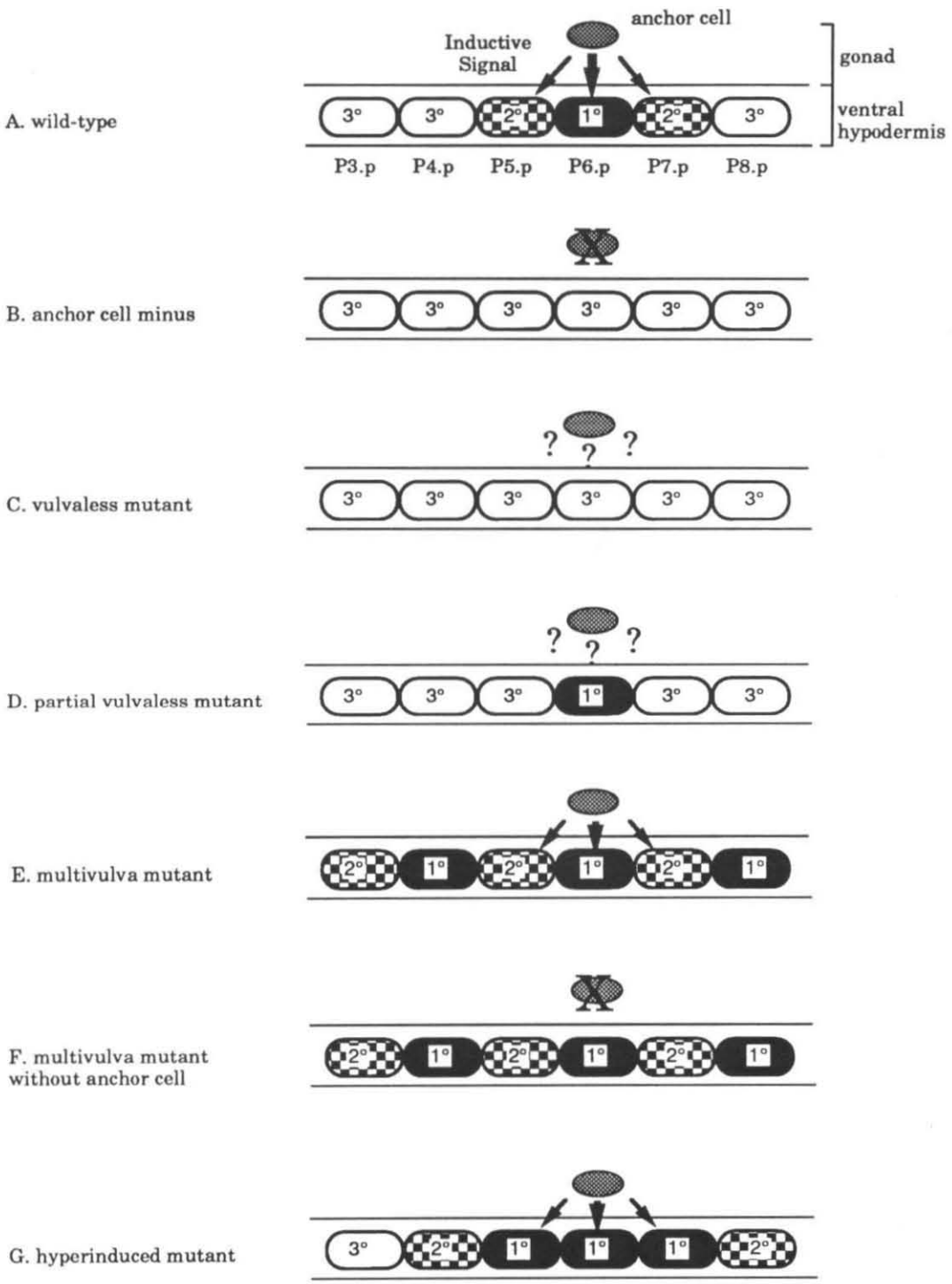

A. wild-type
anchor cell
Inductive Signal
gonad
ventral hypodermis
3° 3° 2° 1° 2° 3°
P3.p P4.p P5.p P6.p P7.p P8.p
B. anchor cell minus
3° 3° 3° 3° 3° 3°
C. vulvaless mutant
? ? ?
3° 3° 3° 3° 3° 3°
D. partial vulvaless mutant
? ? ?
3° 3° 3° 1° 3° 3°
E. multivulva mutant
2° 1° 2° 1° 2° 1°
F. multivulva mutant without anchor cell
2° 1° 2° 1° 2° 1°
G. hyperinduced mutant
3° 2° 1° 1° 1° 2°

Figure 11. F1 non-complementation mutagenesis screen. The germ line of a wild-type hermaphrodite (+/+) is mutagenized and mated with homozygous mutant males (**m**/**m**) (the male in the figure is lightly shaded as are the mutant **m** copies contributed by the male). Self progeny from the hermaphrodite will be phenotypically wild type, unless a new, dominant mutation has been introduced (this can be sorted out later). Most cross progeny from the mating between the male and the hermaphrodite will be **m**/+. Since **m** is recessive, these will show a wild-type phenotype in the F1. Occasionally, however, a new mutation in the same gene (**m***) will be generated in an oocyte of the original mutagenized hermaphrodite. When this gamete is fertilized by the mutant male, the resulting F1 progeny (**m**/**m***) will show a mutant vulval phenotype (dark shading) since the new allele fails to complement the allele introduced by the mutant male. The screen can also be performed in reverse such that wild-type males are mutagenized and the original mutant chromosome **m** is introduced via hermaphrodites. A recessive, unrelated marker often is included with either screen to allow cross-progeny from self-progeny to be distinguished.

F1
m*
m
+
cross progeny
m
m
X
+
+
self progeny
mutagenize

Figure 12. Suppressor screening. New mutations in the vulval determination pathway can be isolated by mutagenizing mutant Muv or Vul hermaphrodites and screening for suppression of that phenotype. In the example shown, Muv hermaphrodites with multiple ventral protrusions are mutagenized and allowed to produce self-progeny. A dominant suppressor (**S**) will result in an F1 progeny with no ventral protrusions (muv/muv ; S/+) (it may or may not be Egl). A recessive suppressor (**s**) will result in some F2 progeny with no ventral protrusions (muv/muv ; s/s). The suppressor gene can be isolated away from the Muv mutation and analyzed. In the case of suppressing a Vul mutation, suppressors can be identified by the presence of eggs on a plate or by hermaphrodites that no longer form a bag of worms.

muv
mutagenize
F1
muv ; ± +
muv ; + ±
muv ; + +
F2
muv ; S +
or
muv ; ± ±
or
muv ; ± S
muv ; S ±

Figure 13. Germ line transformation in *C. elegans*. The distal gonad syncytium of a hermaphrodite mutant for a Vul or Muv gene (either heterozygous m/+ or homozygous m/m) is injected simultaneously with two DNAs: (1) candidate DNA that may encode the wild-type Vul or Muv gene; and (2) marker DNA that tests for expression of injected DNAs. Here, the marker DNA is a plasmid containing a dominant cuticle mutation abbreviated *rol-6(d)*. The injected DNAs are sometimes expressed in the F1 progeny of the injected mother. Such progeny will have a twisted cuticle and will gyrate on the plate due to expression of the dominant cuticle mutation. These progeny are also likely to express the candidate Vul/Muv DNA since the *rol-6(d)* and candidate DNAs concatamerize into long, linear arrays. Progeny that roll are therefore tested for rescue of the mutant Vul or Muv phenotype. Rescue of the mutant phenotype indicates that the candidate DNA contains a functional, wild-type Vul or Muv gene. Non-rescue indicates that the candidate DNA does not contain a functional gene. Since the candidate DNA can be cosmid or plasmid, one can test ≥40kb of genomic sequence at a time and quickly narrow in on the region containing the Vul or Muv gene. Germ line transformation is also useful for assessing the *in vivo* effects of mutations made *in vitro*. For transformation protocols see Fire (1986) and Mello *et al.* (1991). For actual instances of germ line rescue of vulval mutations see Kim and Horvitz (1990), Freyd *et al.* (1990), Han and Sternberg (1990), and Aroian *et al.* (1990). For using germ line transformation to study mutations made *in vitro*, see Han and Sternberg (1992).

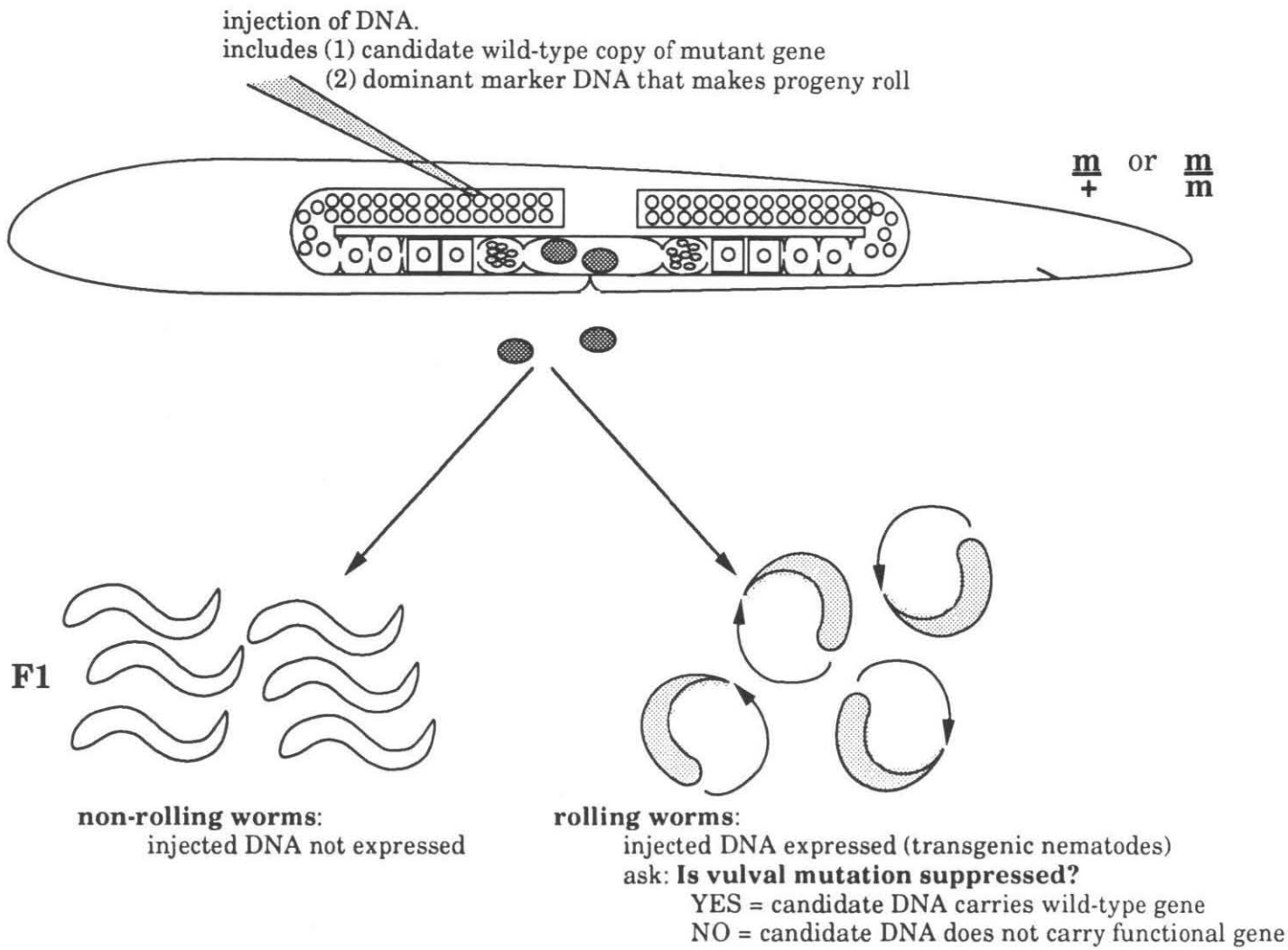

injection of DNA.
includes (1) candidate wild-type copy of mutant gene
(2) dominant marker DNA that makes progeny roll
m/+ or m/m
F1
non-rolling worms:
injected DNA not expressed
rolling worms:
injected DNA expressed (transgenic nematodes)
ask: Is vulval mutation suppressed?
YES = candidate DNA carries wild-type gene
NO = candidate DNA does not carry functional gene

Figure 14. Assigning Vul genes in a simple pathway. The genes *lin-2*, *lin-3*, *lin-7*, *lin-10*, *let-23 RTK*, and *let-60 ras* are all required for stimulation (indicated by —>) of vulval fate since eliminating any of these genes results in no or little selection of vulval fate. The anchor cell's influence on the selection of vulval fate is hypothesized to go through *lin-3*, *let-23 RTK*, and *let-60 ras* since elimination of any one of these genes mimics ablation of the anchor cell. The genes *lin-2*, *lin-7*, and *lin-10*, however, are shown to act in a pathway not in line with the anchor cell since elimination of any one of these genes does not fully mimic ablation of the anchor cell. For simplicity, these genes are assumed to act through *lin-3*, *let-23 RTK*, and/or *let-60 ras*, but they could act through other genes. An alternative explanation is that these three genes are indeed in line with the anchor cell signal but that this involves a different anchor cell signal or that there is another slightly redundant component that acts with these three genes that can weakly compensate. A more complete model is given later in Figure 18.

anchor cell
lin-3
let-23 RTK
let-60 ras
lin-2
lin-7
lin-10
select vulval (1°/2°) fate

Figure 15. Action of the Muv genes. (A) The Muv genes are necessary to turn off (indicated by —|) vulval fate, since in their absence vulval fate proceeds. (B) The Muv genes also appear to act in two redundant pathways. Elimination of one or more components in either the "A" or "B" pathway still results in normal vulval differentiation. However, simultaneously eliminating components from both pathways does result in a lack of vulval fate inhibition (*i.e.*, a Muv phenotype). Some genes, such as *lin-15*, can be mutated to an A-, B-, and A-B- phenotype, suggesting that this gene operates in both pathways. (C) Muv gene activity can be eliminated in two ways. A wild-type Muv gene produces a protein that inhibits vulval fate selection (left panel). If this gene is eliminated by mutation, then this inhibitory activity is eliminated (center panel). In a wild-type hermaphrodite, the activity of the Muv gene is presumably eliminated in P5.p-P7.p by the anchor cell signal, which overrides the Muv gene (right panel). As shown in the final panel, the anchor cell signal probably does not go through the Muv genes (see text and Figure 16). See Ferguson and Horvitz (1989) and Herman and Hedgecock (1990).

A.

Muv genes ⊣ select 1°/2° vulval fate

Muv X genes select 1°/2° vulval fate →

B.

C.

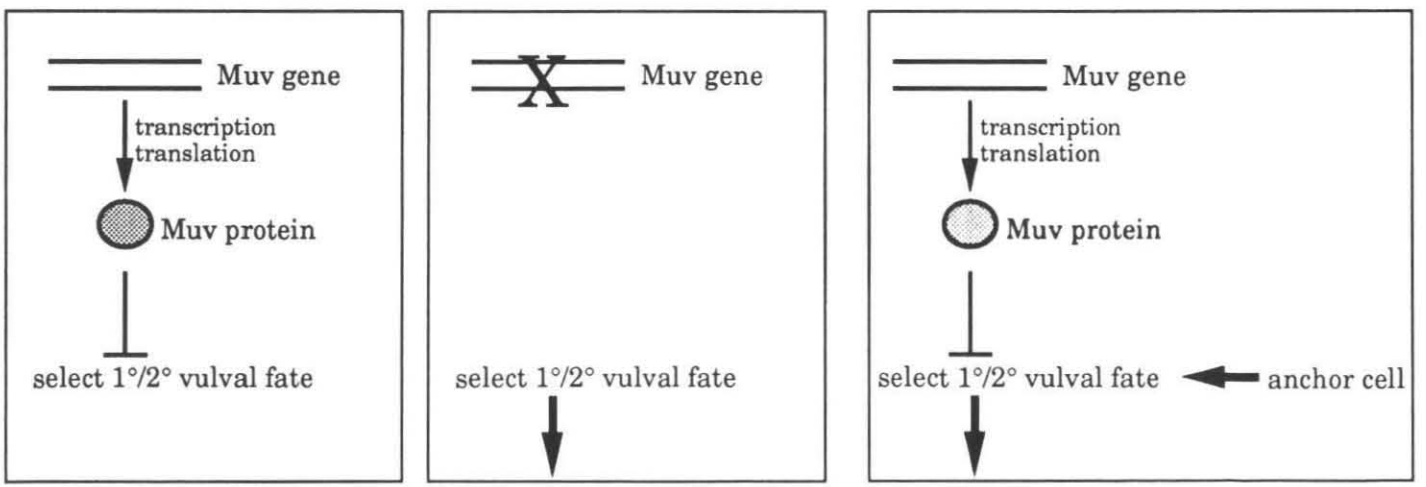
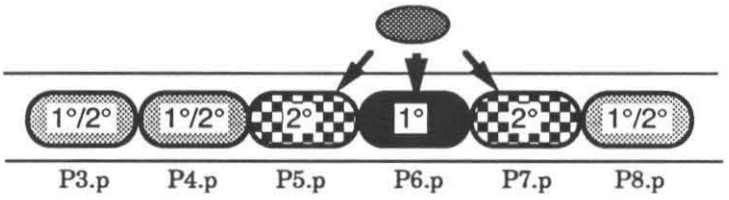
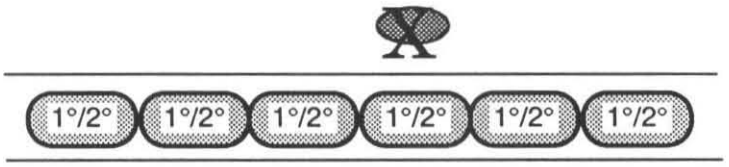


Figure 16. The Muv gene *lin-15* likely acts in a pathway independent of the anchor-cell signal. In a hermaphrodite completely lacking *lin-15* activity (*i.e.*, *lin-15(lf)*), the VPCs select only 1° or 2° vulval fates. (A) If the anchor cell is present in these mutant animals, then P6.p always selects 1° fate, P5.p/P7.p select 2° fate, and the other VPCs select either 1° or 2°. (B) However, if the anchor cell is ablated in these mutant animals, then the fates of all VPCs are variable. For example, P6.p can be 1° or 2° with equal probability. Thus, the anchor cell can affect the VPCs even though *lin-15* is absent, suggesting that the anchor cell signal does not go through *lin-15*. (C) Similarly, if, in a *lin-15(lf)* mutant hermaphrodite, all but two VPCs are ablated, then the VPC closest to the anchor cell always adopts a 1° fate. (D) However, if the anchor cell is ablated, then the fate of both VPCs is randomized. Data taken from Sternberg (1988).



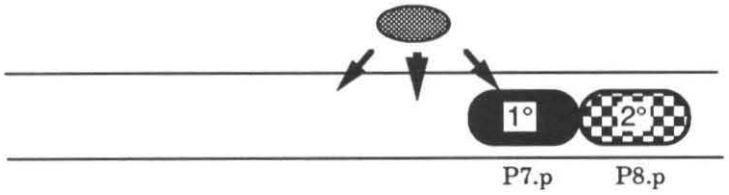
lin-15(lf)

A.



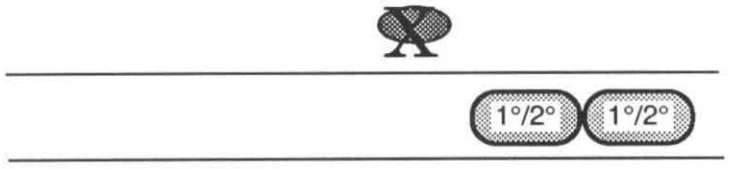
lin-15(lf)

B.



lin-15(lf)

C.



lin-15(lf)

D.

Figure 17. Ordering genes in a regulative pathway. (A) The gene *lin-2* is required for selection of vulval fate (1) since if *lin-2* is eliminated, vulval fate is not selected (2). Similarly, *lin-15* is required for inhibition of vulval fate (3) since in its absence vulval fate is selected (4). (B) There are three simple ways in which these two genes can act: (1) *lin-2* negatively regulates *lin-15* which negatively regulates vulval fate; (2) *lin-15* negatively regulates *lin-2* which stimulates vulval fate; and (3) *lin-2* and *lin-15* exert their influences independently of the other. (C) If the first combination were true, then elimination of *lin-2* would result in inhibition of vulval fate (*i.e.*, Vul), because *lin-15* could not be inactivated (1). Elimination of *lin-15* would result in automatic selection of vulval fate (*i.e.*, a Muv phenotype) since *lin-15* is required for inhibition of vulval fate (2). Since *lin-2* requires the presence of *lin-15* to affect vulval fate, the **double mutant**, *lin-2(lf) lin-15(lf)*, and the single mutant *lin-15(lf)* would exhibit the same phenotype – **Muv** (3). Similarly, if the order of the genes is reversed (*i.e.*, *lin-15* inhibits *lin-2*), the **double mutant** *lin-2(lf) lin-15(lf)* would exhibit the same phenotype as *lin-2(lf)* alone – **Vul**. Thus, if the two genes act together in the same pathway, the relative order of the two genes can be determined by examining the phenotype of the double mutant.

The third combination, *i.e.*, *lin-2* and *lin-15* affect vulval fate independently, is more difficult to prove. The double mutant *lin-2(lf) lin-15(lf)* could be Muv or Vul depending on the default fate selection in the absence of both genes. However, an unambiguous result would be obtained if the double mutant were neither Muv nor Vul but rather showed an intermediate phenotype (*e.g.*, wild-type). This result is referred to as **co-expression** of phenotypes, and indicates either that the genes are acting in independent pathways and/or that the mutations used do not completely eliminate of function.

A.

(1) *lin-2* → vulval fate on →

(2) *lin-2* X (vulval fate)

(3) *lin-15* ⊣ (vulval fate)

(4) *lin-15* X vulval fate on →

B.

(1) *lin-2* ⊣ *lin-15* ⊣ vulval fate

(2) *lin-15* ⊣ *lin-2* → vulval fate

(3) *lin-2* → vulval fate ⊢ *lin-15*

C.

(1) *lin-2* X *lin-15* ⊣ (vulval fate)

(2) *lin-2* ⊣ *lin-15* X vulval fate on →

(3) *lin-2* X *lin-15* X vulval fate on →

Figure 18. Pathway of vulval determination (1°/2° vs. 3° fate) as determined by double mutant phenotypes. The anchor cell signal is received by *let-23 RTK* which allows *let-23 RTK* to overcome inhibition by *lin-15*. This then leads to activation of *let-60 ras*. The effect of activated *let-60 ras* is to inhibit *lin-1*, which then allows vulval induction to occur. There are likely to be other genes between *let-60 ras* and *lin-1* and possibly between *let-23 RTK* and *let-60 ras*.

This pathway was put together using the logic detailed in Figure 17 and incorporates published data in which complete loss of function alleles were used to make the double mutants or in which the epistatic relationship was clear (Ferguson *et al.*, 1987; Han *et al.*, 1990; Aroian and Sternberg, 1991). The following additional assumptions were used. First, *lin-7*, *lin-10*, and *lin-15* are assumed to operate in a pathway independent of the anchor cell. Second, if no co-expression of phenotype occurred, the genes were arranged in dependent pathways, although independent pathways are also possible (see notes to *lin-1* and *lin-7* & *lin-10*).

Notes: *lin-1* is shown as acting in line with the anchor cell signal since it is fully epistatic to all Vul genes shown. However, analogous to *lin-15*, *lin-1* may act in a pathway independent of the anchor cell (see box A).

lin-2 probably acts at the same step as *lin-7* and *lin-10*.

lin-3 may act upstream in the pathway, but a non-null allele was used in making double mutants.

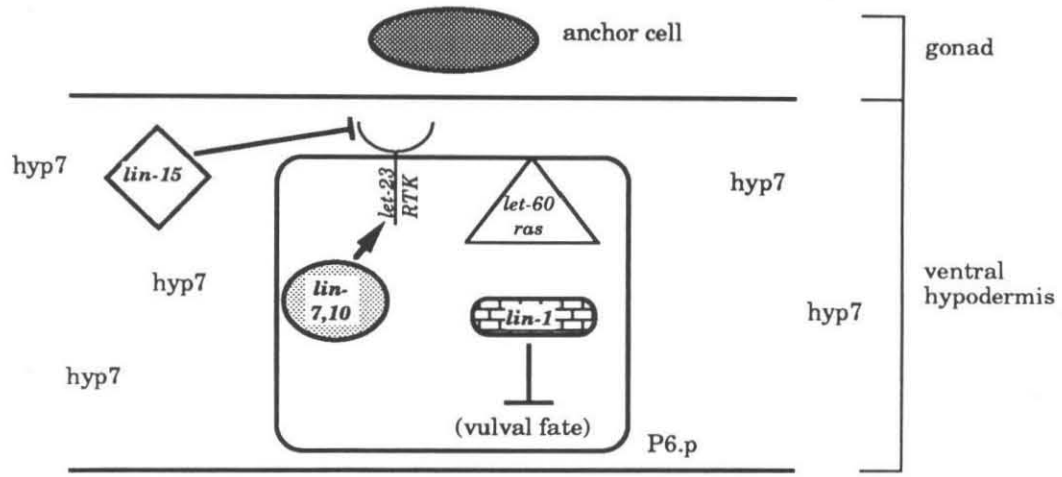
lin-7 and *lin-10* are shown in the same pathway as and upstream of *lin-15* because the double mutant phenotypes were Muv. However, other considerations (*e.g.*, hyperinduced phenotypes; see text) suggest that *lin-7* and *lin-10* may act with *let-23 RTK* in a *lin-15*-independent pathway (see box B).

The *lin-8; lin-9* synthetic Muv phenotype appears to be co-expressed with *lin-7* and *lin-10*. If so, then these syn-Muv genes would act at the same step as *lin-7* and *lin-10*. However, non-null alleles of both *lin-8* and *lin-9* were used so their relationship to *lin-7* and *lin-10* are unclear.

The *lin-13* Muv phenotype was suppressed by all Vul mutations but *lin-3*. However, only weak, non-null alleles exist of this gene.

anchor cell
let-23 RTK
let-60 ras
lin-1
vulval fate
lin-15
lin-7, 10
lin-7, 10
let-23 RTK
lin-15
B
let-60 ras
vulval fate
lin-1
A

Figure 19. Molecular genetic model for determination of vulval versus hypodermal fates. For simplicity, only P6.p is shown. The pathway is taken from Figure 18 except the alternative given in Box B has been chosen (due to hyperinduction data discussed later). (A) In a wild-type hermaphrodite without an anchor cell signal, *lin-15* acting from hyp7 (shown surrounding P6.p) inhibits *let-23 RTK* kinase activity in the VPCs. As a result, *let-60 ras* is not activated and vulval fate is not turned on because *lin-1* is preventing selection of vulval fate. A *lin-15(lf)* mutant displays an anchor-cell independent Muv phenotype because we hypothesize that *let-23 RTK* has a basal kinase activity that can activate *let-60 ras* even in the absence of ligand. (B) In a *lin-15(+)* animal, the anchor cell ligand reaches P6.p, is bound by *let-23 RTK*, and allows *let-23 RTK* to overcome inhibition by hyp7. Kinase activity from *let-23 RTK* activates *let-60 ras* which in turn activates vulval fate, either by inhibiting *lin-1* or by overcoming *lin-1* inhibition. The specific mode of action of *lin-15* shown, *i.e.*, to inhibit the extracellular domain of *let-23 RTK*, is arbitrary. The genes *lin-7* and *lin-10* are shown to act in the VPCs to stabilize *let-23 RTK*. Alternatively, *lin-7* and *lin-10* may act in hyp7 or in the VPCs to inhibit *lin-15* (see Figure 18).


anchor cell
gonad
hyp7
lin-15
let-23 RTK
let-60 ras
hyp7
hyp7
lin-7,10
lin-1
ventral hypodermis
hyp7
hyp7
(vulval fate)
P6.p

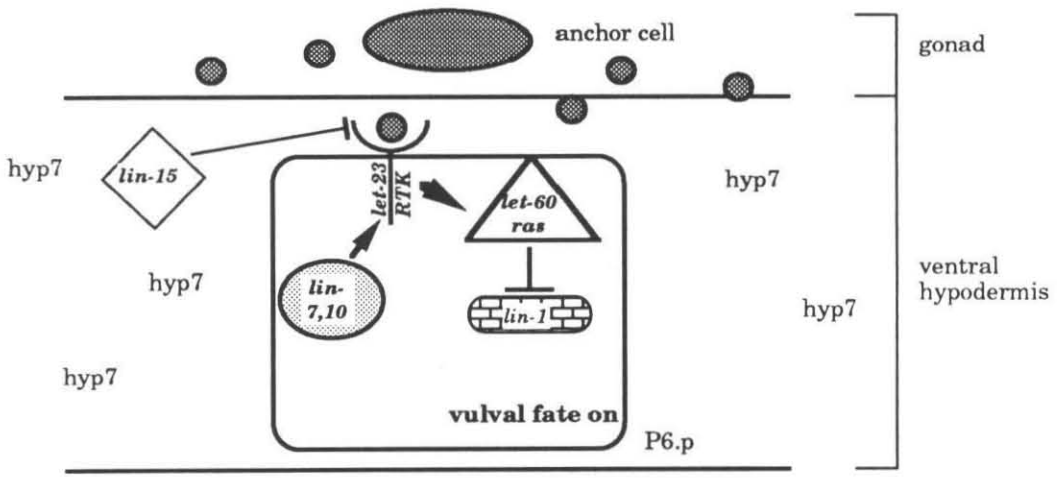
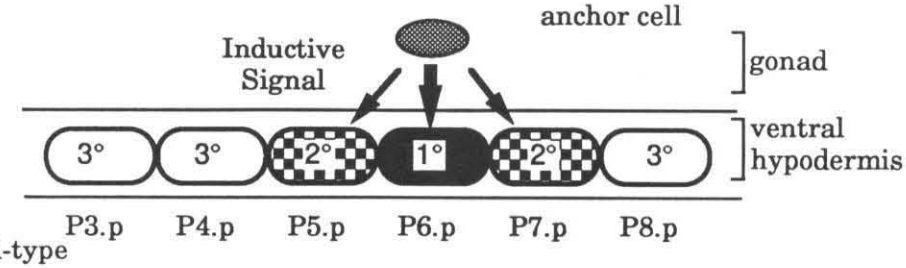

anchor cell
gonad
hyp7
lin-15
let-23 RTK
let-60 ras
hyp7
hyp7
lin-7,10
lin-1
ventral hypodermis
hyp7
hyp7
vulval fate on
P6.p

Figure 20. The intrinsic pathway and VPC-VPC interactions. (A) In the absence of the anchor-cell signal, *lin-15*, and neighboring VPCs, a VPC will select 1° fate. (B) One possible interpretation is that *let-23 RTK* has basal kinase activity in the absence of ligand, provided *lin-15* has been turned off. Furthermore, this "default" or "intrinsic" mode results in selection of 1° fate. (C) In an anchor-cell minus *lin-15(lf)* hermaphrodite, two non-adjacent VPCs will both select 1° fates. (D) However, if the two VPCs are adjacent, then one will select 1° fate and the other 2° fate, suggesting the VPC-VPC communication results in lateral inhibition of the intrinsic pathway in one of the VPCs and the selection of 2° fate. Data from Sternberg (1988).

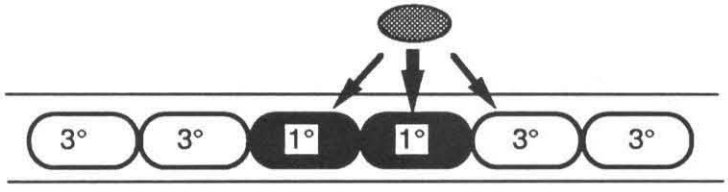
anchor cell
1°
A.
lin-15
1°
1°
C.
lin-15
2°
1°
D.
lin-15

anchor cell
hyp7
lin-15
hyp7
let-23
RTK
let-60
ras
lin-1
hyp7
vulval fate on
P6.p
B.

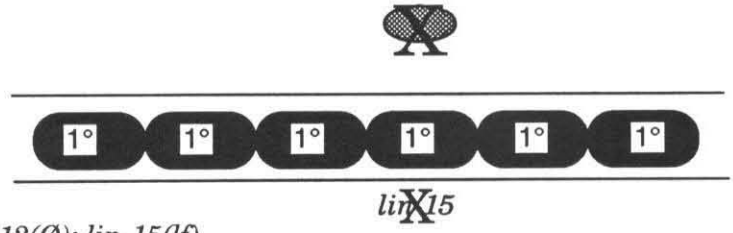
Figure 21. *lin-12* is necessary and sufficient for 2° fate selection. (A) Wild-type vulval induction. (B) When *lin-12* is eliminated, only 1° and 3° fates are selected. Note, (1) although two VPCs are shown selecting 1° fate, the actual number can vary; and (2) although only one anchor cell is shown, *lin-12(Ø)* hermaphrodites can have multiple anchor cells. (C) If, in a *lin-12(Ø)* hermaphrodite, the intrinsic pathway is activated by the elimination of *lin-15*, then all VPCs select 1° fate. Thus, the selection of 2° fate in a *lin-15(lf)* animal requires *lin-12*. See text for interpretation. (D) Gain of function *lin-12(d*ominant*)* mutations result in all VPCs executing 2° fate. The anchor cell is missing because *lin-12(d)* mutations also result in no anchor cell being produced. Data from Greenwald *et al.* (1983); Sternberg and Horvitz (1989); W. Katz and P.W.S., unpublished results.



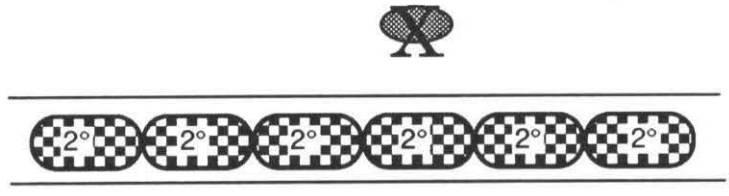
A. wild-type



B. *lin-12(Ø)*

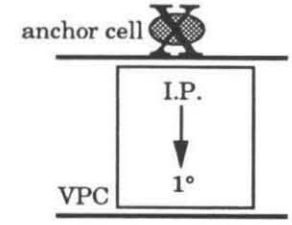


C. *lin-12(Ø); lin-15(lf)*

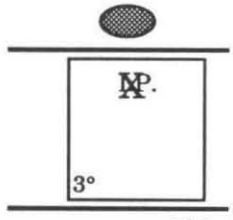


D. *lin-12(d)*

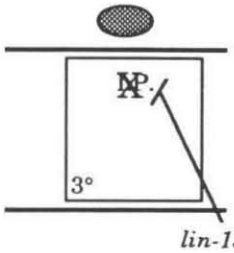
Figure 22. Summary of data on how VPCs select the different fates. (A) A VPC isolated from the influence of neighboring VPCs, the anchor cell signal, and presumably hyp7 (via *lin-15(lf)*) will select 1° fate via an intrinsic pathway (= I.P. which includes *let-23 RTK* and *let-60 ras*; see Figure 20B). (B,C) This I.P. is necessary because if *let-23 RTK* or *let-60 ras* is severely reduced in a *lin-15(lf)* or *lin-15(+)* background, then all 3° fates are selected. (D) However *lin-15*, when present, is sufficient to inhibit the I.P. as long as the anchor cell signal is off. (E,F) If the anchor-cell signal is on, then the inhibitory effects of *lin-15* can be overcome. (E) If the anchor cell signal is close, then an isolated VPC will select 1° fate via the I.P. (F) If the anchor cell signal is further away, then an isolated VPC will select 2° fate. Presumably, this selection of 2° fate also requires the I.P. because if the I.P. is eliminated then neither 2° nor 1° fate can be selected (see C). (G) However, this requirement for the I.P. can be by-passed if *lin-12* is activated via a *lin-12(d)* mutation. This result suggests that the role of the I.P. in selection of 2° fate may be to initially activate *lin-12*. Once activated, *lin-12* no longer needs the I.P. (H) Another way to activate the 2° pathway is via lateral signalling. Although the default fate of an isolated VPC is 1° (see A), this default can be overridden if a VPC has a neighbor. Thus, *lin-12* can be activated intracellularly and intercellularly. (I) If *lin-12* is eliminated, then 1° fate is selected by all VPCs in a *lin-15(lf)* background. Perhaps, *lin-12* is a receptor for the lateral signal. Data for A, D, E, and F are based on isolated VPC experiments. Data for B, C, G, H, and I are based on intact VPC experiments. Data for H and I also include experiments with two isolated VPCs.


anchor cell
I.P.
1°
VPC
lin-15

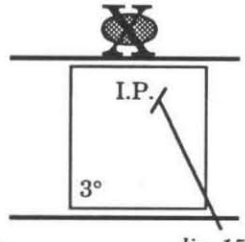
A.


I.P.
3°
lin-15

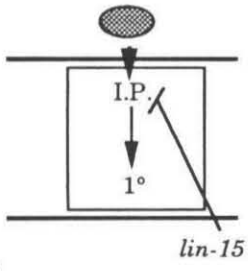
B.


I.P.
3°
lin-15

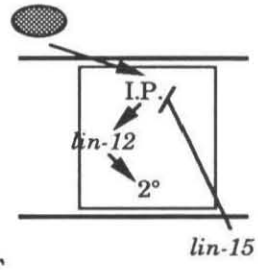
C.


I.P.
3°
lin-15

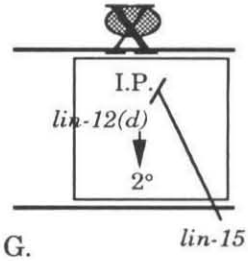
D.


I.P.
1°
lin-15

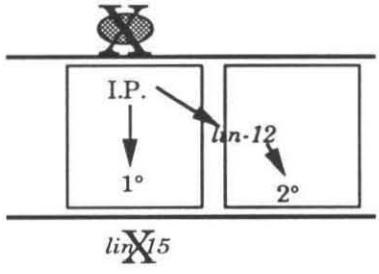
E.


I.P.
lin-12
2°
lin-15

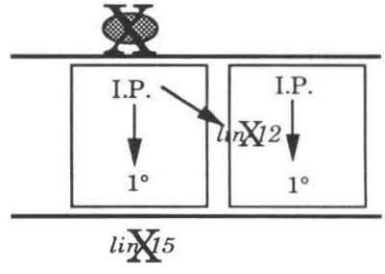
F.


I.P.
lin-12(d)
2°
lin-15

G.


I.P.
1°
lin-12
2°
lin-15

H.


I.P.
1°
lin-12
I.P.
1°
lin-15

I.

Figure 23. Model for determination of 1° vs. 2° vs. 3° fate.

In P6.p, the proximity of the anchor cell signal strongly activates the 1° intrinsic pathway (I.P.). This has three consequences. First, *lin-12* is activated in the same cell, although not enough to inhibit the strongly activated intrinsic pathway. Second, *lin-12* is activated in the neighboring cell P7.p via lateral signalling. Third, the 1° sublineage is selected. Net state: I.P. ON - *lin-12* ON.

In P7.p, an intermediate level of anchor cell signal is received, moderately activating the intrinsic pathway which then activates *lin-12*. In addition, *lin-12* is activated by lateral signalling from P6.p. As a result, *lin-12* is activated to a high enough level and the intrinsic pathway to a low enough level that 1° fate is successfully inhibited by *lin-12* and 2° fate ensues. It is also possible that P7.p emits a lateral signal, but this is apt to be weak since the intrinsic pathway is turned off. Net state: I.P. OFF - *lin-12* ON.

In P8.p, only a small amount of anchor cell signal is received, weakly activating the intrinsic pathway and *lin-12*. Furthermore, *lin-12* is weakly activated by P7.p lateral signal. As a result, the intrinsic pathway is inhibited and *lin-12* is never on to a high enough level to promote 2° fate. As a result, 3° fate ensues. Net state: I.P. OFF - *lin-12* OFF.

The essential features of this model are:

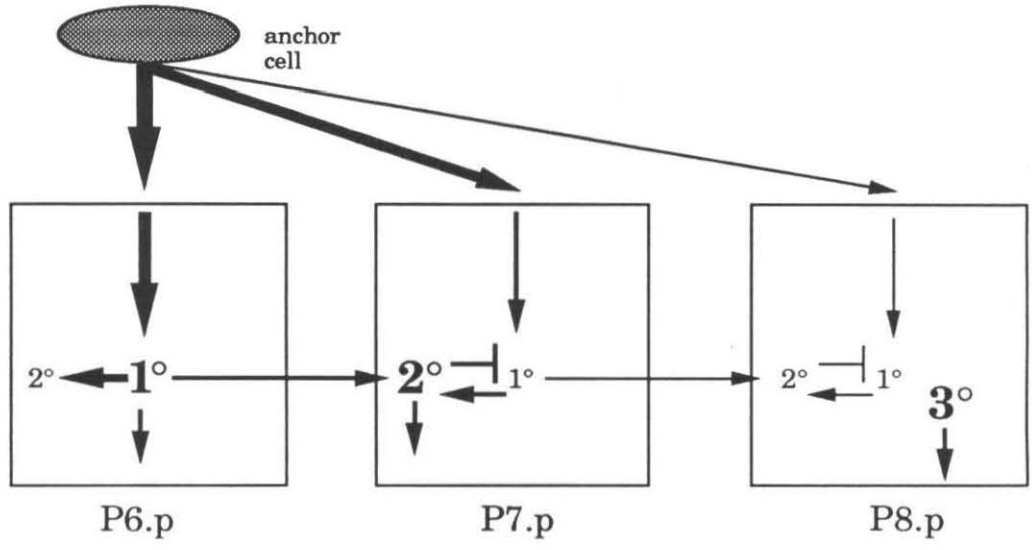
(1) The level of anchor-cell signal is important for how strongly the I.P. is turned on.

(2) The intrinsic pathway turns on 2° fate and the lateral signal.

(3) 2° fate is activated in two ways – by the intrinsic pathway and by the lateral signal coming from a neighboring cell.

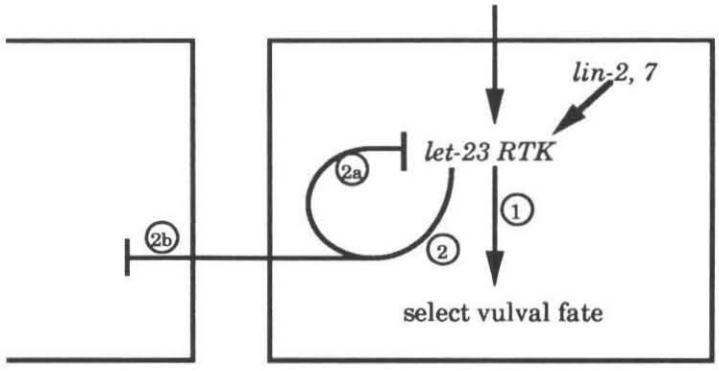
(4) The effect of 2° fate is to inhibit the intrinsic pathway. In a 2° VPC, this ensures that 1° fate is turned off and that 2° fate ensues (because *lin-12* is on). In a 3° cell this reinforces the low level of anchor cell signal. Presumably, the *lin-12* pathway is never highly activated in these 3° cells.

Adapted from Sternberg and Horvitz (1989).

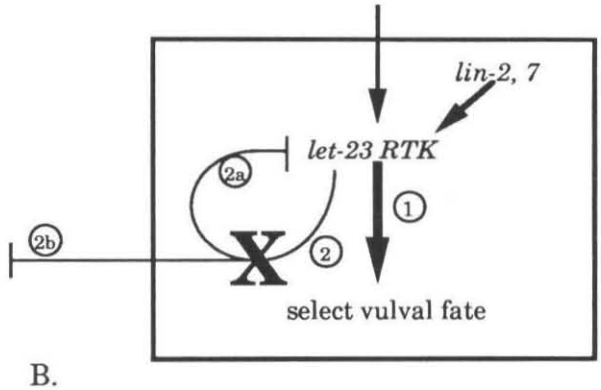


I.P.:	ON	OFF	OFF
lin-12:	ON	ON	OFF

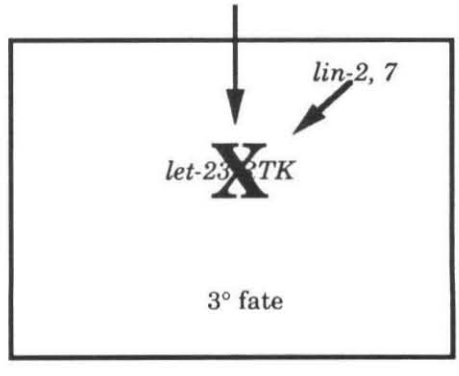
Figure 24. Modeling hyperinduction. (A) To explain the hyperinduction data, *let-23 RTK*, *lin-2*, and *lin-7* are likely to direct two pathways in the VPCs (1) a stimulatory pathway required for selection of vulval fate; and (2) an inhibitory pathway that normally acts to inhibit the stimulatory pathway either in the same cell (2a) or in a neighboring cell (2b). For example, along the lines of intracellular inhibition (2a), EGF receptor is thought to activate phospholipase C-γ, which, among other things, results in activation of protein kinase C, which in turn inactivates receptor (reviewed in Ullrich and Schlessinger, 1990). Thus, one pathway of *let-23 RTK* stimulates vulval induction via *let-60 ras* but another pathway of *let-23 RTK* might inhibit vulval induction via a negative feedback loop. An alternative intracellular inhibition model is given in the text. Along the lines of intercellular inhibition (2b), *let-23 RTK* might result in an inhibitory signal sent to a neighboring VPC. In fact, such a signal is consistent with the lateral signal discussed previously since one aspect of the lateral signal might be to inhibit the I.P in a neighboring cell. (B) Hyperinduction, associated with reduction of function *let-23 RTK* alleles, might result from a loss of the inhibitory pathway, but not the stimulatory pathway, such that inhibition (intracellular or intercellular) is lacking. As a result, there is an overall increase in the levels of induction. This loss of only the inhibitory pathway could occur because activation of the inhibitory pathway (*e.g.*, via PLC-γ or lateral signal) is more sensitive to an initial reduction in *let-23 RTK* levels than activation of the stimulatory pathway (via *let-60 ras*). Alternatively, loss of only the inhibitory pathway could occur because the hyperinduced mutations perturb the interactions of *let-23 RTK* with a substrate necessary for inhibition (*e.g.*, PLC-γ) and not another (see also text for another model along these lines). (C) Finally, complete loss of *let-23 RTK* function eliminates both pathways. The end result is no induction. Models adapted from Aroian and Sternberg (1991).


lin-2, 7
let-23 RTK
2a
1
2b
2
select vulval fate

A.


lin-2, 7
let-23 RTK
2a
1
2b
X
2
select vulval fate

B.


lin-2, 7
let-23 RTK
X
3° fate

C.

Figure 25. Effects of execution mutants. (A) Wild-type induction. (B) In a *lin-11* loss of function mutant (designated *lin-11(lf)*), the "TN" portion of 2° lineages is lacking and the "LL" portion is reiterated. (C) One of the effects of *lin-17(lf)* and *lin-18(lf)* mutants is to randomize the polarity of P7.p. Sometimes the polarity is wild-type (NTLL) and sometimes the polarity is reversed (LLTN). Data from Ferguson *et al.* (1987) and W. Katz and P.W.S., unpublished results.

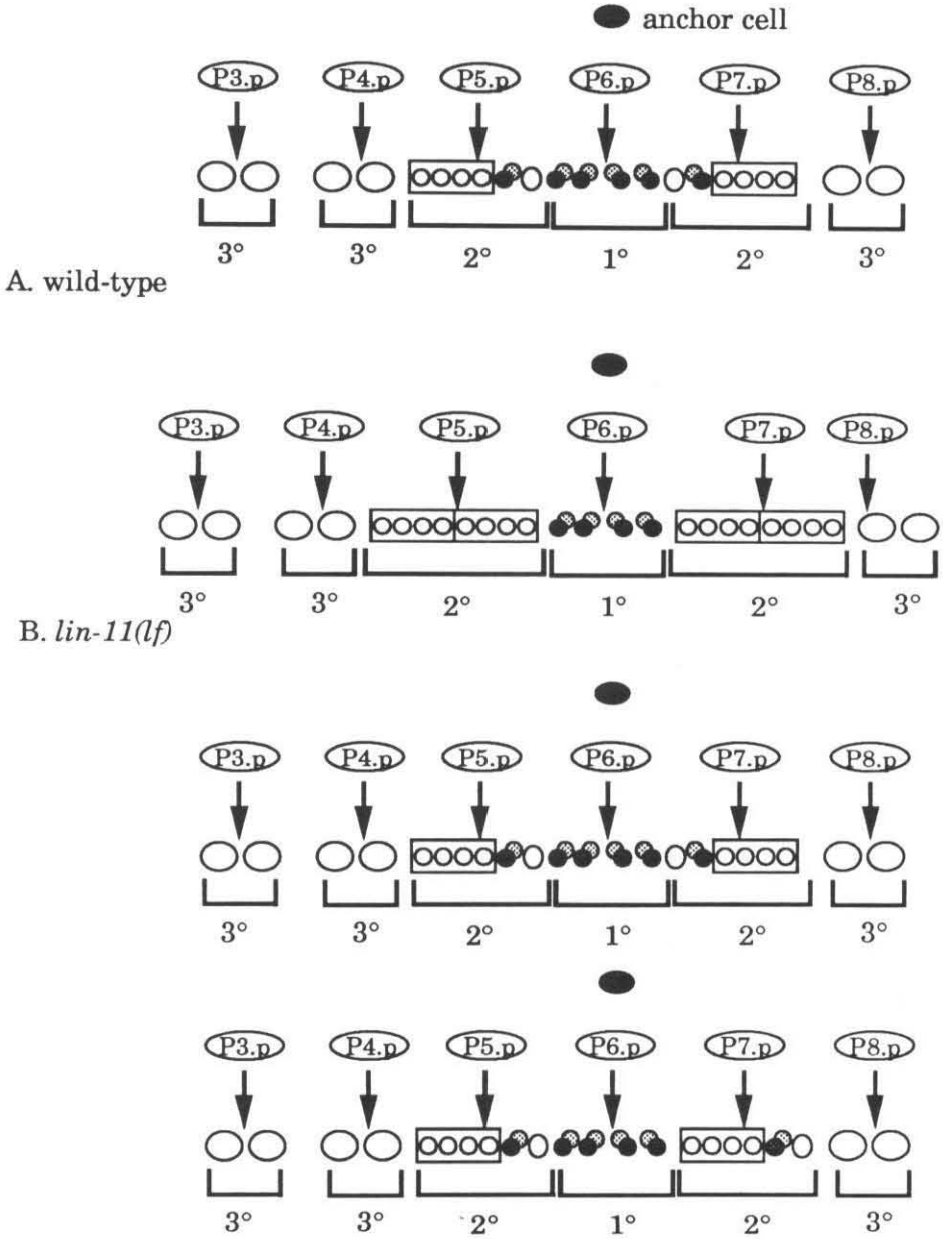
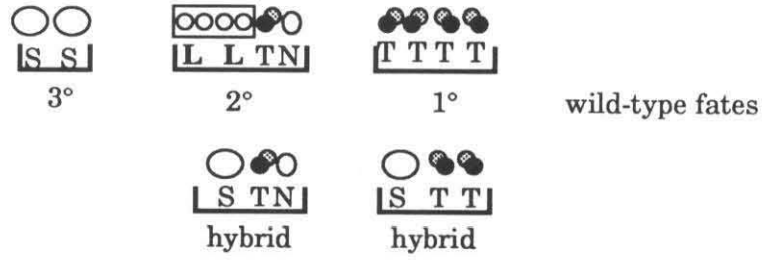
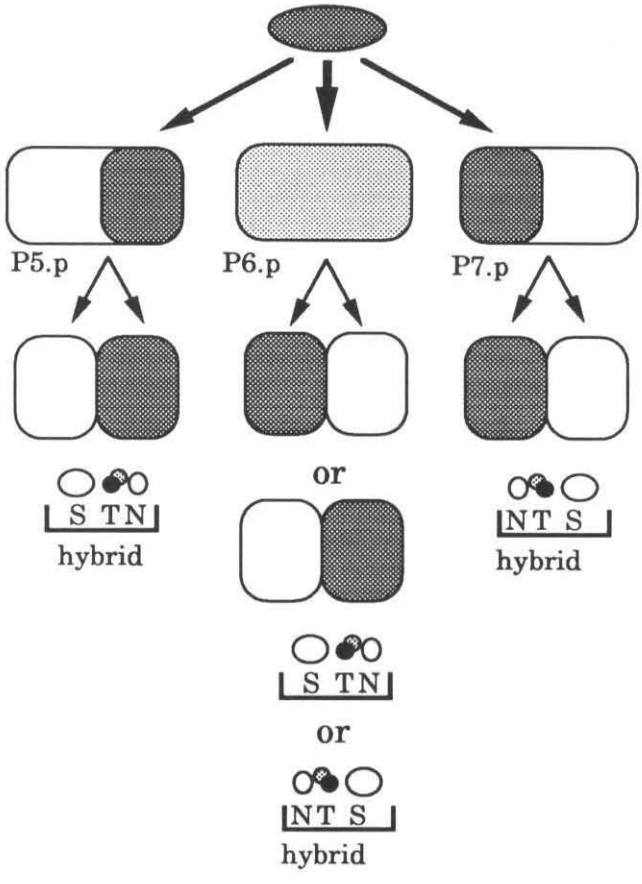

anchor cell
P3.p
P4.p
P5.p
P6.p
P7.p
P8.p
3°
3°
2°
1°
2°
3°
A. wild-type
B. lin-11(lf)
C. lin-17(lf) or lin-18(lf)

Figure 26. Hybrid lineages. (A) Hybrid lineages result from a partial reduction of function in any of the Vul genes (*e.g.*, *let-23 RTK* and *let-60 ras*). They are most often half 3°-half 2° or half 3°-half 1° as shown. The half-vulval portion of the lineage coincides with the location of the anchor cell relative to the hybrid lineage such that for P5.p, the vulval portion faces posterior, for P7.p the vulval portion faces anterior, and for P6.p the vulval portion faces either anterior or posterior. (B) This data is interpreted to mean that some factor necessary for execution of vulval lineages is being asymmetrically distributed in each VPC (shaded part of the cells) based on the direction of the anchor cell signal. In a VPC with lower signal transduction levels, this factor is limiting and results in only the anchor-cell proximal half of a VPC receiving enough of it to divide past one round. See Aroian and Sternberg (1991) for data and further discussion.


S S
3°
L L TN
2°
T T T T
1°
wild-type fates
S TN
hybrid
S T T
hybrid

A.


P5.p
P6.p
P7.p
S TN
hybrid
or
NT S
hybrid
S TN
or
NT S
hybrid

B.

Chapter II

Multiple functions of *let-23*, a *Caenorhabditis elegans* receptor tyrosine kinase gene required for vulval induction

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Multiple Functions of *let-23*, a *Caenorhabditis elegans* Receptor Tyrosine Kinase Gene Required for Vulval Induction

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ABSTRACT

The *let-23* gene, which encodes a putative tyrosine kinase of the epidermal growth factor (EGF) receptor subfamily, has multiple functions during *Caenorhabditis elegans* development. We show that *let-23* function is required for vulval precursor cells (VPCs) to respond to the signal that induces vulval differentiation: a complete loss of *let-23* function results in no induction. However, some *let-23* mutations that genetically reduce but do not eliminate *let-23* function result in VPCs apparently hypersensitive to inductive signal: as many as five of six VPCs can adopt vulval fates, in contrast to the three that normally do. These results suggest that the *let-23* receptor tyrosine kinase controls two opposing pathways, one that stimulates vulval differentiation and another that negatively regulates vulval differentiation. Furthermore, analysis of 16 new *let-23* mutations indicates that the *let-23* kinase functions in at least five tissues. Since various *let-23* mutant phenotypes can be obtained independently, the *let-23* gene is likely to have tissue-specific functions.

INDUCTION of the six vulval precursor cells (VPCs) in *Caenorhabditis elegans* to differentiate into mature vulval tissue provides an opportunity to study mechanisms of signal transduction and cell-type determination. The six VPCs in wild type adopt the following fates (from anterior to posterior): 3° 3° 2° 1° 2° 3°, where 3° fate is an unspecialized, hypodermal fate and 2° and 1° are specialized, vulval fates (SULSTON and HORVITZ 1977; SULSTON and WHITE 1980; STERNBERG and HORVITZ 1986). This pattern of cell fate is established primarily by an inductive signal generated by the anchor cell in the gonad (SULSTON and WHITE 1980; KIMBLE 1981; STERNBERG and HORVITZ 1986; THOMAS, STERN and HORVITZ 1990). In addition, lateral signalling between VPCs prevents the adoption of adjacent 1° fates (STERNBERG 1988).

Genetic and molecular analyses are being combined to study the different aspects of vulval induction. Mutations have been identified that prevent induction of the VPCs (all 3° fate; vulvaless or Vul phenotype), that cause signal-independent induction (all 1° and 2° fates; multivulva or Muv phenotype), that are necessary for determination of 2° vulval fate, and that perturb the execution of vulval fates (HORVITZ and SULSTON 1980; SULSTON and HORVITZ 1981; GREENWALD, STERNBERG and HORVITZ 1983; FERGUSON and HORVITZ 1985 1989; FERGUSON, STERNBERG and HORVITZ 1987; STERNBERG and HORVITZ 1989; KIM and HORVITZ 1990; HAN, AROIAN and STERNBERG 1990; BEITEL, CLARK and HORVITZ 1990). The *lin-12* gene, necessary for the 2° fate (GREENWALD, STERNBERG and HORVITZ 1983), encodes a putative transmembrane receptor similar to the *Drosophila Notch* product and may be a receptor for the lateral signal between VPCs (YOCHEM, WESTON and GREENWALD 1988; SEYDOUX and GREENWALD 1989; STERNBERG and HORVITZ 1989). The gene *let-60*, required for vulval induction (HAN, AROIAN and STERNBERG 1990; BEITEL, CLARK and HORVITZ 1990), encodes a member of the *ras* family (HAN and STERNBERG 1990), and the gene *lin-11*, which is required for vulval fate execution (FERGUSON, STERNBERG and HORVITZ 1987), encodes a putative transcription factor (FREYD, KIM and HORVITZ 1990). The *let-23* gene encodes a tyrosine kinase of the EGF receptor subfamily (AROIAN *et al.* 1990) and functions via *let-60 ras* (HAN, AROIAN and STERNBERG 1990; HAN and STERNBERG 1990). The structure of the *let-23* gene suggests it might be the receptor for the inductive signal (AROIAN *et al.* 1990).

The *let-23* gene was originally defined by a larval lethal mutation (HERMAN 1978), but initial observations of the subviable allele *n1045* indicated that *let-23* plays a key role in vulval induction (FERGUSON and HORVITZ 1985; FERGUSON, STERNBERG and HORVITZ 1987; STERNBERG and HORVITZ 1989). At 15° *n1045* hermaphrodites are Vul (*i.e.*, result in no vulval induction and cannot lay eggs), but at 25° they are egg-laying proficient (Egl^+) and sometimes display one or two ectopic vulva-like structures reminiscent of Muv mutations (FERGUSON and HORVITZ 1985). Here we characterize the properties of *let-23* mutations in the vulva and other tissues. We demonstrate that complete loss of *let-23* function results in no vulval induction. Furthermore, we show that the "weak Muv"

appearance of *n1045* hermaphrodites grown at 25° results from VPCs that still require, but seem hypersensitive to, inductive signal. It is therefore distinct from previously described multivulva mutations, which are signal-independent. This *n1045* phenotype apparently results from a partial reduction in *let-23* function. These data suggest that the *let-23* gene is involved in two opposing pathways: stimulation of vulval fates and inhibition of vulval fates. The multiplicity of roles assumed by the *let-23* gene again surfaces during the development of other *C. elegans* tissues. The *let-23* gene displays mutant phenotypes in multiple tissues, and we find that these phenotypes are, to a large extent, independently mutable.

MATERIALS AND METHODS

General methods: Methods for culturing, handling, mutagenesis and genetic manipulation of *C. elegans* were performed as described by BRENNER (1974). Unless otherwise noted, we performed all experiments at 20°. The standard *C. elegans* cellular and genetic nomenclature, defined by SULSTON and HORVITZ (1977) and HORVITZ *et al.* (1979), respectively, is followed in this paper. "VPCs" are the six cells (P3.p, P4.p, P5.p, P6.p, P7.p and P8.p) that have the potential to participate in vulval development. Measurements of dead larvae were obtained using a calibrated ocular micrometer.

Strains: The standard wild-type strain N2 and marker strains were from BRENNER (1974) and the Caenorhabditis Genetics Center. Below is a list of mutants and alleles used throughout the paper; references are given where appropriate. If only one allele of a gene is used (*e.g.*, *dpy-10*), the allele is given only in this section. Unless otherwise stated, *him-5* is *him-5(e1490)*.

LGII single mutations: dpy-10(e128), vab-9(e1744), rol-6(e187), unc-4(e120), the balancer *mnC1[dpy-10(e128) unc-52(e444)]* (II) (HERMAN 1978), *mnDf61, mnDf67 unc-4(e120), mnDf68 unc-4(e120)* (all SIGURDSON, SPANIER and HERMAN 1984).

LGII linked double mutations: dpy-10(e128) rol-6(e187), dpy-10(e128) unc-4(e120), rol-6(e187) unc-4(e120).

let-23 mutations: let-23(mn23) unc-4(e120), let-23(mn216) unc-4(e120), let-23(mn224) unc-4(e120) (HERMAN 1978; SIGURDSON, SPANIER and HERMAN 1984), *let-23(sy97); him-5(e1490) V* (from H. CHAMBERLIN). Ten other lethal alleles not specifically mentioned and linked to *rol-6(e187): sy5, sy6, sy7, sy9, sy11, sy13, sy14, sy16, sy17* and *sy18* (all this paper).

LGV: him-5(e1467), him-5(e1490): (HODGKIN, HORVITZ and BRENNER 1979).

LGX: lin-15(n309): (FERGUSON and HORVITZ 1985).

Unlinked triple mutant: let-23(sy97) II; him-5(e1490) V; lin-15(n309) X (from H. CHAMBERLIN).

Isolation of *let-23* alleles as suppressors of *lin-15*: At 15°, *n1045* hermaphrodites display a Vul phenotype (all six VPCs often execute the 3° nonvulval fate) and are egg-laying incompetent or Egl due to the lack of a vulva (FERGUSON and HORVITZ 1985). Conversely, Muv *lin-15* hermaphrodites all have multiple ventral protrusions and are egg-laying competent. When grown at 15°, the double mutant, *let-23(n1045); lin-15*, is often Vul and Egl with no ventral protrusions. We therefore sought to isolate new *let-23* mutations as suppressors of *lin-15*. Since this was an F2 screen, only viable, fertile *let-23* alleles could be isolated. Homozygous *lin-15* hermaphrodites were mutagenized with ethyl methanesulfonate (EMS) and allowed to self for two generations. We then picked nonmultivulva hermaphrodites that were retaining eggs or had formed "bags of worms." (The larvae are trapped within the cuticle of the Egl mother, subsequently eating their way out.) The phenotypes viewed under the dissecting microscope for Egl and Muv hermaphrodites were described by HORVITZ and SULSTON (1980). If these Egl, non-Muv hermaphrodites bred true, we mated them with *rol-6/mnC1* males, tested for linkage by examining segregation, and performed complementation tests. For *let-23(sy1); lin-15* this proved easy. While *let-23(sy97)* hermaphrodites have never been observed to copulate with males, the presence of *lin-15* in the original isolate allowed copulation. Although the *let-23(sy97); lin-15* double mutant is most often Vul (36% average induction; $n = 27$; 13/27 had no induction; see Figure 2 for description of induction), it still displays greater vulval differentiation than that of *sy97* hermaphrodites. Since *lin-15* mutants are 100% Muv (200% induction, all six VPCs are induced), these data suggest that *let-23* is epistatic to *lin-15*. In over 100,000 mutagenized gametes screened (see HAN, AROIAN and STERNBERG 1990), we have found only these two *let-23* alleles.

We linked these alleles to *dpy-10* and *unc-4* by placing each of them in *trans* to the double mutant *dpy-10 unc-4*, picking Dpy non-Unc and Unc non-Dpy recombinant progeny, and then selecting for progeny carrying the *let-23* mutation. We similarly constructed a *rol-6 cis* double mutant using the double *rol-6 unc-4*.

Noncomplementation screen against *sy1*: In the second screen for new alleles, we made use of the fact that *sy1* hermaphrodites are 100% viable but completely Vul, even in *trans* to the deficiency *mnDf68*. Thus, any hypomorphic *let-23* allele should be completely viable in *trans* to *sy1*, even if that allele eliminates function of the locus. We screened for new mutations that failed to complement *sy1* for the Vul phenotype and expected that these would include homozygous inviable *let-23* alleles. We mutagenized *rol-6* hermaphrodites and set up crosses of 3–4 hermaphrodites with 4–5 *sy1; him-5* males. The parents were transferred onto new plates after 36 hours and both sets of F_1 progeny were scored for non-Rol Egl hermaphrodites that retained eggs or hermaphrodites that had formed bags (it is often not possible to score Rol in bags of worms). These worms were picked and allowed to self. The absence of Rol progeny but presence of Egl progeny in their F_2 brood indicated the presence of a lethal *let-23* allele. All alleles were selected from independent matings except the pairs *sy13* and *sy10*, and *sy14* and *sy12*. Since in each case the pair behave differently (*sy13* and *sy14* are 100% lethal but *sy10* and *sy12* are not), we believe they are independent mutations. These alleles were subsequently recovered by mating F_3 non-Rol males, which can be *rol-6 let-23(new)/sy1; him-5* or *sy1/sy1; him-5* at roughly a 2:1 ratio, with *rol-6 unc-4* hermaphrodites, and selecting for Rol non-Uncs. We balanced these alleles over *mnC1* and screened for both *him-5* and non-*him-5* strains.

The 12 lethal alleles recovered in this screen were isolated because they fail to complement *sy1* for the vulval defect. We further tested these alleles by: (1) mating *rol-6 let-23(n1045)/mnC1* males with each of the strains and scoring hermaphrodites under the dissecting microscope and spicules of several of the males under Nomarski optics, (2) mating *rol-6 let-23(sy8)/mnC1; him-5* males with each lethal and looking for F_1 Rols, and (3) mating males from each lethal (*e.g.*, *rol-6 let-23(sy8)/mnC1; him-5*) with the deficiency *mnDf67* and looking for F_1 Rols (*mnDf67* deletes *let-23* and *rol-6 /mnDf67* is Rol).

We linked *dpy-10* to *sy10* and *sy12* by picking Dpy non-Unc recombinants from the heterozygote *rol-6 let-23(sy10 or sy12)/ dpy-10 unc-4* and selected recombinants that segregated the *let-23* mutation but not the *rol-6* mutation. Since *dpy-10* is epistatic to *rol-6*, we determined the absence of *rol-6* by complementation.

The original *rol-6 let-23(sy12)/mnC1* strain (PS227) is 100% sterile. During maintenance of the strain *dpy-10 let-23(sy12)/ rol-6 unc-4* (derived from PS227; see above), we fortuitously picked a recombinant which resulted in the strain *let-23(sy12)/ rol-6 unc-4*. We then linked *rol-6* and *unc-4* to this *sy12* by picking Rol non-Unc and Unc non-Rol recombinants, which were then balanced in *trans* to *mnC1*. The new *sy12 unc-4/mnC1* strain is 100% sterile like its parent. The new *rol-6 sy12/mnC1* strain (PS716) was, however, approximately 30% fertile. This partially fertile *rol-6 let-23(sy12)/mnC1* strain, PS716, was used for additional experiments since it has undergone the most back-crossing. We cannot detect any differences between PS227 and PS716 for the other phenotypes. We were also able to pick a similar recombinant for *sy10* and linked it to *rol-6* and *unc-4* as for *sy12*. This *rol-6 sy10* strain is still, as the parent, 100% sterile.

Complementation matrix: Since homozygous *let-23* hermaphrodites have difficulty mating with males, and since most homozygous *let-23* alleles produce males that cannot mate, all allele combinations were made using analogous sets of hermaphrodites and male strains balanced in *trans* to a *let-23*(+) chromosome. This protocol also maintains internal consistency for the comparison of results among different allele combinations. We mated N2 males with *dpy-10 rol-6* hermaphrodites and picked wild-type males (*dpy-10 rol-6*/++). These males were mated with *dpy-10 let-23(X)/ rol-6 unc-4* hermaphrodites where *let-23(X)* is any *let-23* allele. In the next generation we picked Dpy L4 hermaphrodites, many of which are cross progeny *dpy-10 let-23(X)/ dpy-10 rol-6*. Self progeny, homozygous *dpy-10 let-23(X)*, are also Dpy but can be eliminated in the next cross since they do not segregate Rol. Individual Dpy hermaphrodites were mated with 4 to 5 *(rol-6 let-23(Y))/mnC1* males where *let-23(Y)* is any *let-23* allele (*rol-6 let-23/mnC1* male strains are well balanced and are easily propagated). These parents were transferred to new plates first after 2 days, then every day for a total of four plates per cross. This procedure allows for synchrony among the progeny on a plate. Self progeny are all Dpy hermaphrodites. Cross progeny are hermaphrodites and males, either Dpy, (*dpy-10 let-23(X)/mnC1*[*dpy-10 unc-52*] and *dpy-10 rol-6/mnC1*), Rol (*rol-6 let-23(Y)/dpy-10 rol-6*), or non-Dpy non-Rol (*dpy-10 let-23(X)/rol-6 let-23(Y)*). A wild-type chromosome is introduced for *let-23(X)*, by substituting *dpy-10* hermaphrodites for *dpy-10 let-23(X)/ rol-6 unc-4*. To substitute a wild-type chromosome for *let-23(Y)*, we replace *rol-6 let-23(Y)/mnC1* males with *rol-6/mnC1* males. Since *mnDf68* deletes *rol-6* and *let-23*, *mnDf68 unc-4/ mnC1* males were directly used in place of *rol-6 let-23(Y)/ mnC1* males.

We controlled for recombinants as follows. Loss of *rol-6* in *dpy-10 rol-6*/++ males are detected by the lack of Rol animals segregating in the final set of crosses (this is why we used individual hermaphrodites in each cross). Since *dpy-10 let-23(X)/rol-6 unc-4* hermaphrodites are maintained clonally, we verified the mother's genotype by segregation. The only troublesome recombinant would be loss of *let-23(X)* from *dpy-10*. This recombinant picks up *unc-4* and thus segregates Dpy Uncs in the final crosses.

Two other recombinants could affect our data. Class I recombinants result from recombination between *rol-6*(+) and *let-23(X)* in the mother [*dpy-10 let-23(X)/dpy-10 rol-6*]. This *dpy-10 rol-6*(+) *let-23*(+) gamete can give rise to a non-Dpy non-Rol animal that lacks *let-23(X)*, namely *dpy-10/rol-6 let-23(Y)*. Class II recombinants result from breakdown of the *mnC1* balancer which removes *let-23(Y)* from *rol-6*, potentially giving rise to a non-Dpy non-Rol progeny that lacks *let-23(Y)*, namely *rol-6 /dpy-10 let-23(X)*. The other recombinant, which removes *rol-6* from *let-23(Y)*, would pick up *dpy-10* from the *mnC1* chromosome and thus not give a false non-Dpy non-Rol.

Recombinant classes I and II both effectively separate *rol-6* and *let-23*. To ascertain the frequency of such recombinants, we mapped *rol-6* relative to *let-23* in the strain *rol-6 let-23(sy15)/vab-9(e1744)*. We found 11 Rol nonlethal recombinants out of a total of 7232 Vab and wild-type animals. The calculated *rol-6* to *let-23* distance is 0.23 ± 0.08 map units. Therefore, if *let-23(X)/let-23(Y)* is 100% viable, we would expect about 1/400 of non-Dpy non-Rol animals to be class I recombinants. In the cases where *let-23(X)/let-23(Y)* is 10% viable, this ratio becomes 1/40, which is about what we found. We therefore do not expect these recombinants to seriously affect our results. We expect class II recombinants to be even more rare since in these cases a recombination event between *rol-6* and *let-23* needs to occur in addition to the breakdown of the balancer *mnC1*. We have indeed found this class of recombinants to be rare except in the presence of homozygous *him-5*, which increases recombination in this region in the presence of *mnC1*. (We do not know if recombination is increased in the absence of *mnC1*.) For this reason, we only used non-*him-5* male strains in our crosses. In many cases, we recovered hermaphrodites that had wild-type vulvae and verified that they were nonrecombinant by segregation. It is not practical to do this with the males that have wild-type spicules, since individual matings and scoring of subsequent segregation is involved. It is thus possible there is a slight underestimation of the penetrance of the spicule defect shown in Table 1C. Assuming a viability of 10%, recombinants would introduce a 3% (1/40) error. Since the viability of most allele combinations is greater than 10%, the error introduced for most combinations is less than 3%.

Temperatures of 15°, 20° and 25° were kept within 0.5°. Crosses with *mn224* were performed differently since *mn224* is linked to *unc-4* and not *rol-6*. In the first cross we mated N2 males with *dpy-10 unc-4* and carried out all other crosses as above, in which *let-23(Y)* is introduced via *let-23(mn224) unc-4/mnC1*, and the Unc-4 phenotype replaces Rol-6 in the scoring.

Since *dpy-10* and *rol-6* both affect the cuticle and such genes can have unusual interactions (Cox *et al.* 1980), we mated *dpy-10 rol-6* hermaphrodites with *rol-6 unc-4/mnC1* males. We found 373 Dpy males, 384 Rol males, and 381 Rol hermaphrodites, the 1:1:1 ratio as expected. Therefore, ignoring *unc-4*, *dpy-10 rol-6/dpy-10* + animals are Dpy and *dpy-10 rol-6/+ rol-6* animals are Rol. For the *dpy-10* +/+ *rol-6* control, see +/+ results in Table 1A–E.

Data points for all phenotypes were often taken over more than one day to avoid age-related biases. We have not seen any age-effects for either vulval induction, male spicules or fertility.

Since all crosses were performed with balanced *let-23(mutant)/let-23*(+) hermaphrodites, we tested for maternal rescue of any of the phenotypes. Larval lethality is not maternally rescued in any of our lethal alleles. Homozygous *sy1*, *sy97* and *n1045* mutations result in no quantitative differences in vulval, lethal, male tail, or sterile phenotypes whether coming from homozygous or heterozygous mothers (see below). There is no maternal rescue of the fully pene-

trant *sy10* sterility. The P12 transformation is, however, partly rescued maternally (see RESULTS).

We chose *sy15* as our standard lethal allele in these crosses at random from fourteen possible null lethal alleles. All lethals were tested in *trans* to *sy1* and *n1045(20°)* and fail to complement vulval and male tail defects (except *mn224*). Furthermore, all lethals isolated in this paper fail to complement the lethal allele *let-23(sy8)* and are lethal in *trans* to *mnDf67*, a deficiency which deletes *let-23* (SIGURDSON, SPANIER and HERMAN 1984; AROIAN *et al.* 1990).

Measuring vulval induction/hybrid lineages: Non-Dpy non-Rol L4 hermaphrodites were placed live on pads of 5% Noble agar (as described by SULSTON and HORVITZ 1977; STERNBERG and HORVITZ 1981) and examined for their extent of vulval induction. The final induction pattern was checked for internal consistency in both the number of syncytial nuclei and the number of nonsyncytial VPC progeny. To check the genotype of hermaphrodites, we pulled individual animals off slides in S Basal with a capillary and suction apparatus and transferred them to a Petri plate, and examined their genotype by segregation. To eliminate the anchor cell in *n1045* hermaphrodites, we laser ablated somatic gonad precursor cells during the L1 larval stage (SULSTON and WHITE 1980; STERNBERG and HORVITZ 1981).

Hybrid vulval lineages in *let-23* mutant hermaphrodites appear to arise from VPCs that have a reduced levels of signal transduction since they are associated with reduction of function *let-23* alleles which lower induction below wild type but do not eliminate it. This conclusion is supported by induction patterns seen in eighty homozygous *sy97* hermaphrodites (Table 1A and other data not shown). Only four of these hermaphrodites had any VPC induction and in all cases a single VPC executed a hybrid lineage (all other VPCs executed 3° fate). Hybrid lineages also correlate with the lower induction levels seen in Table 1A. Collating our data from the genotypes with the lowest induction (<5%; 108 animals), we see that 8/11 instances of VPC induction were hybrid (compare this to 72/156 instances from non-hyperinduced genotypes with average induction >30%; 106 animals).

Hybrid lineages also show a strong polarity bias in *let-23* mutants, which correlates with the position of the anchor cell relative to the VPC daughters. When P5.p executed a hybrid fate, the anchor cell-proximal daughter, P5.pp, executed the vulval fate in 49/53 instances. When P7.p executed a hybrid fate, the anchor cell-proximal daughter, P7.pa, executed the vulval fate in 41/46 instances. For P6.p, the anterior daughter executed the vulval fate 20/54 times, and the posterior daughter 34/54 times. Although these data are collected from all *let-23* allele combinations, these trends do not significantly differ among the various alleles.

Last, the decision for a given VPC to execute a hybrid lineage does not show any obvious correlation with fates of neighboring VPCs. P5.p can execute a hybrid lineage whether P6.p executes a hypodermal fate (24/53), a hybrid fate (11/53), or a vulval fate (18/53). Similarly, P7.p can execute a hybrid lineage whether P6.p executes a hypodermal fate (14/46), a hybrid fate (18/46), or a vulval fate (18/46). P6.p can execute a hybrid lineage when both neighbors execute hypodermal fates (19/54), when one neighbor executes a nonhypodermal (*i.e.*, hybrid or vulval) fate (21/54), and when both neighbors execute a non-hypodermal fate (14/54). These data do not rule out a possible role for neighbor-neighbor interactions in establishing hybrid fate, but they do suggest that neighboring VPCs alone do not cause a given VPC to adopt a hybrid fate.

Measuring survival: We expect the ratio of Dpy:Rol:non-Dpy non-Rol males to be 2:1:1 if there is no lethality associated with *let-23(X)/let-23(Y)*. An approximate sample size of the total number of non-Dpy non-Rol males, both viable and inviable, is therefore the number of Dpy males plus the number of Rol males divided by three. We counted all males on all the plates in a set and calculated the percent survival (s) as

$$s = 3 \cdot (\text{No. non-Dpy non-Rol males})/(\text{No. Dpy males} + \text{No. Rol males}).$$

The 95% confidence limits are $3 \cdot 1.96 \cdot \sqrt{(s \cdot (1 - s)/N)}$, where N is the number of Dpy plus Rol males divided by three. For example, *n1045/n1045* at 20° is 42% ± 13% viable, *n1045/sy10* is 56% ± 17% viable, *n1045/mn224* is 15% ± 12% viable, and *sy97/sy15* is 0.4% ± 1.7% viable. Additionally, in all but one case (*n1045/mnDf68* at 20°), we found that the ratio of Dpy:Rol males was, within 95% confidence limits, consistent with the expected 2:1 ratio (assuming lethality is recessive).

Measuring wild-type spicules: We examined both spicules of live non-Dpy non-Rol adult males usually within 1–2 days of adulthood under Nomarski optics. We have also noted defects in the male gubernaculum, but these may be a consequence of spicule disorganization.

Measuring fertility: Due to the demands of scoring all the phenotypes, most fertility data points were collected in a separate set of crosses identical to those used for the other phenotypes. We picked L4 and young adult hermaphrodites over several days. These hermaphrodites were checked every 12 hours for four days. Any worm that was Egl (turned into a "bag of worms") was removed from the plate; if an Egl$^+$ worm was found, all the other hermaphrodites were transferred to a new plate. This allowed unambiguous confirmation of whether or not a given hermaphrodite was fertile. Hermaphrodites left after the four days were counted as sterile. They were also identifiable by mottled uteri.

We found two classes of fertile hermaphrodites. Class A fertiles had healthy brood sizes (including dead larvae) and healthy looking uteri; class B fertiles had small brood sizes (<6 including dead larvae) and mottled uteri typical of *let-23* sterile hermaphrodites (see RESULTS). Class B fertiles are counted as fertile in Table 1D, but the results are not substantially altered if these are classified as sterile: *sy1* and *sy97* hermaphrodites in *trans* to any allele give class A fertiles, and the same allelic series holds true (see Table 4). However, the fertile hermaphrodites counted for both *sy15* and *mn224* in *trans* to *n1045* at 20° are mostly class B (10/15 and 1/1, respectively). If these class B fertiles are classified as sterile, then the percent fertility for *sy15* and *mn224* in *trans* to *n1045* changes to 12% and 0% respectively. Thus, these alleles are not as different from *mnDf68* as they appear.

Studies on the oocyte basis of sterility were carried out as follows. We took eleven individual *dpy-10 n1045/mn224 unc-4* hermaphrodites from the matrix cross and put them each on a plate with five N2 males. No progeny were produced on any of the plates. We also took seven individual *dpy-10 n1045/mn224 unc-4* males and mated them with several *dpy-10 unc-4* (2 plates) and several *unc-4* (5 plates) hermaphrodites. We found cross-progeny on four of the seven plates.

Measuring wild-type P12: We assessed the fate of P12 in the same hermaphrodites in which we measured vulval induction. Maternal rescue of the P12 phenotype was checked as follows. Penetrance of the P12 transformation was measured in the Unc progeny of *sy97 unc-4* and *sy97 unc-4/mnC1* mothers. Likewise, the penetrance was meas-

ured in the Unc progeny of *n1045 unc-4* mothers grown at 15° and *n1045 unc-4/mnC1* mothers grown at 15°. In the case of *sy12*, the penetrance was measured in F_1 progeny of the complementation crosses (mother = *dpy-10 sy12/dpy-10 rol-6*) and in their F_2 progeny (mother = *dpy-10 sy12/rol-6 sy12*).

Since in *let-23(n1045)* males grown at 15° there is a transformation both of P12.p to P11.p and of P12.aap to P11.aap (P. W. STERNBERG and R. HORVITZ, unpublished results), and since P11 and P12 form an equivalence group in males (SULSTON and HORVITZ 1980), we believe that the lack of P12.pa and presence of an extra P11.p-like cell (presumably P12.p) represents a transformation of P12 to P11. This inference assumes that the male and hermaphrodite behavior of these cells are the same.

Maternal rescue and marker controls: We have examined vulval induction in the following strains: *sy1*, *sy1 unc-4* from homozygous and heterozygous mothers, *sy97*, *sy97 unc-4* from homozygous and heterozygous mothers, *n1045 unc-4* 20° from homozygous and heterozygous mothers, and *sy10 unc-4* from heterozygous mothers. We have seen no substantial differences in vulval induction from homozygous and heterozygous mothers in any of these strains. For example, vulvae in *n1045 unc-4* hermaphrodites at 20° (this strain behaves like the original *n1045* strain; see legend to Table 3) were hyperinduced in 6/19 animals, wild-type in 11/19 animals, and Vul in 2/19 animals when coming from a homozygous mother (see RESULTS and Figure 2 for a description of the hyperinduced phenotype). Similarly, when coming from a heterozygous *n1045 e120/mnC1* mother, vulvae in *n1045 unc-4* hermaphrodites at 20° were hyperinduced in 7/19 animals, wild type in 9/19 animals, and Vul in 3/19 animals. With the exception of *n1045* (see legend to Table 3), the vulval induction seen in *let-23* mutants is roughly the same as that seen in the complementation matrix crosses. That similar vulval induction occurs in both our controls and complementation scheme also suggests that our sample sizes for each data point in the complementation scheme are sufficient to show general trends in induction.

We observed that *sy1/sy1; him-5* males roughly mate as well as *him-5* males, *n1045/n1045him-5(e1467) 20°* males mate poorly, and *sy97/sy97; him-5* males do not mate at all (>40 males attempted). These data are consistent with our complementation results. Qualitatively, the lethality associated with *sy1/sy1*, *n1045/n1045* and *sy97/sy97* is as observed in the complementation matrix. The percent *let-23* survival in the strains *rol-6 sy10/mnC1* (20%), *rol-6 sy12/mnC1* (11%), and *sy97 unc-4/mnC1* (15%) (in all cases the number of animals scored is less than in the matrix) is consistent with complementation data. Mating *rol-6 let-23(Z)/mnC1; him-5* males (where *let-23(Z)* = *sy10*, *sy12* and the lethal *sy14*) into *dpy-10 n1045* hermaphrodites at 20° and measuring percent survival yields similar results as found in the matrix (56, 53 and 20%, respectively), further arguing against maternal rescue of larval lethality.

RESULTS

Isolation of new *let-23* alleles and characterization of *let-23* phenotypes: To understand the role of *let-23* in the VPCs and other cells, we isolated and genetically characterized new *let-23* alleles. Prior to this study, there were four known alleles: *n1045* (FERGUSON and HORVITZ 1985) and the larval lethal alleles *mn23*, *mn216* and *mn224* (HERMAN 1978; SIGURDSON, SPANIER and HERMAN 1984). We isolated two viable Vul *let-23* alleles, *sy1* and *sy97*, as *lin-15(n309)* suppressors (see MATERIALS AND METHODS). We also isolated fourteen *let-23* alleles in an F_1 noncomplementation screen against *sy1*. Twelve of these 14 alleles are 100% penetrant larval lethals; the other two alleles, *sy10* and *sy12*, have a partially penetrant larval lethality.

Two of the new alleles, *sy97* and *sy10*, revealed novel defects in the male tail and hermaphrodite fertility. Previously, *let-23* mutations have been associated with vulval defects (Figure 1, A–D; FERGUSON and HORVITZ 1985), larval lethality (HERMAN 1978), and a loss of the cell P12.pa with concomitant apparent duplication of P11.p in the hermaphrodite tail (Figure 1, E and F; FIXSEN *et al.* 1985), which may actually represent a transformation of P12 to P11 (see MATERIALS AND METHODS). During attempted crosses with hermaphrodites, we found that *sy97/sy97* males could not produce cross progeny. Comparison of wild-type (Figure 1G) with mutant males (Figure 1H) revealed abnormal spicules in the tail that vary from slightly shortened and broken to severely crumpled and disorganized. We also found that, although 15% of *sy10/sy10* hermaphrodites survive past the first larval stage, these survivors are sterile. These sterile adults appear not to fertilize their oocytes, which subsequently degenerate in the uterus. As these adults age, the degenerate oocytes accumulate, giving the uterus a mottled appearance. Sterility is likely caused by oocyte and not sperm defects: *n1045/mn224* hermaphrodites (which often have wild-type vulvae) are sterile whereas *n1045/mn224* males are fertile, and sperm from wild-type males cannot rescue *n1045/mn224* hermaphrodite sterility.

To characterize the larval lethal phenotype, we examined the cellular anatomy of *let-23* mutant dead larvae. These larvae appear to arrest at the mid-late L1 stage: they have the "lateral alae" characteristic of wild-type L1 larvae, and their gonads contain between 8 and 16 cells (KIMBLE and HIRSH 1979; KIMBLE and WHITE 1981). The dead larvae homozygous for the lethal alleles isolated in this study arrest at approximately the same length (average length 320 μm), indicating growth arrest at a similar time of development.

The *let-23* vulva and P12 defects involve a transformation of cell fate, as does the male spicule defect (H. CHAMBERLIN, personal communication). We do not know the cellular basis for either *let-23* lethality or sterility. However, since neither the vulva nor P12 nor male spicules are required for viability or hermaphrodite fertility (SULSTON and HORVITZ 1977; SULSTON and WHITE 1980) and since the lethal and sterile phenotypes appear distinct and are somewhat separa-

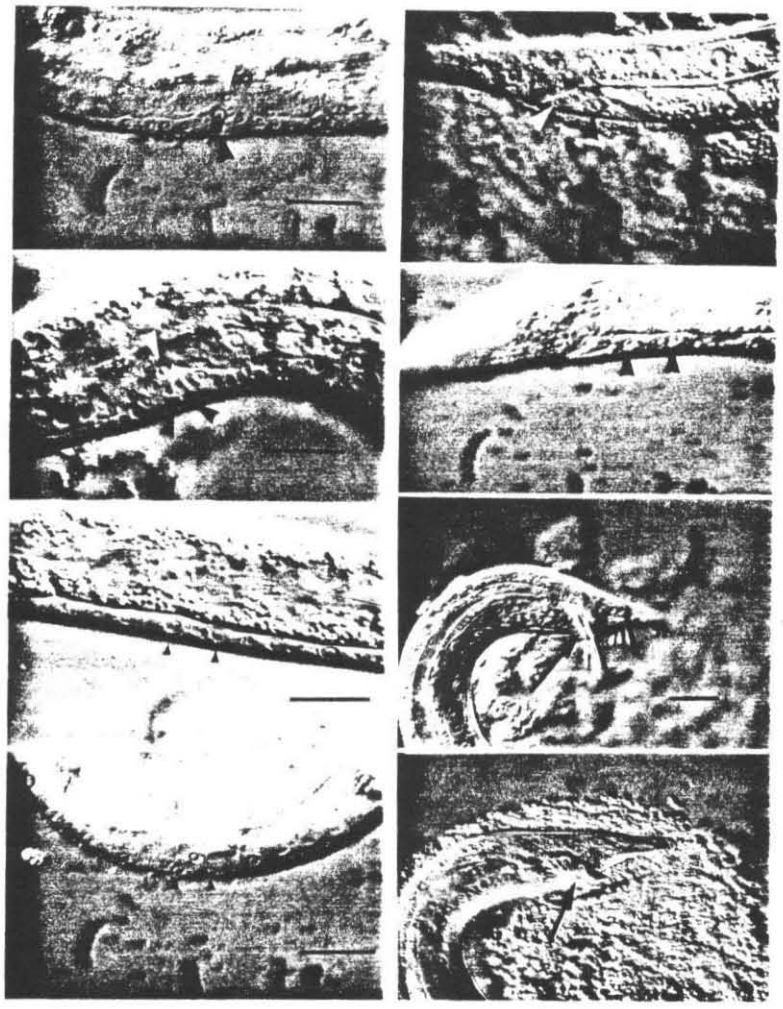

FIGURE 1.—Gallery of Nomarski photomicrographs illustrating *let-23* phenotypes. **A**, Induction in a wild-type L3 lethargus hermaphrodite. Black arrow points to the single vulval invagination; white arrow points to the anchor cell nucleus. **B**, L3 molt *n1045* animal grown at 25° displaying more vulval induction than wild type (phenotype = hyperinduced; see text). The lineages of the VPCs in this animal were observed to execute (anterior to posterior) 3° 3° 2° 1° 1° 2° fates. Black arrows point to the double vulval invagination caused by the extra primary cell; white arrow points to the anchor cell nucleus. **C**, Induction in an L3 molt *n1045* animal grown at 25° with gonad ablated. Note the lack of an anchor cell. All cells executed the 3° fate, demonstrating that hyperinduction is anchor-cell dependent. The two P6.p daughters are indicated with black arrows. **D**, Induction in a Vul *let-23(sy1)* animal at the L3 molt. Despite the presence of an anchor cell (white arrow), all VPCs executed 3° fate. The two P6.p daughters are indicated with black arrows. **E**, Wild-type P11.p (large nucleus; black arrow) and P12.pa (small nucleus; white arrow). **F**, Absence of P12.pa in a *let-23(sy97)* hermaphrodite and appearance of two P11.p-like nuclei (two large nuclei; black arrows). **G**, Wild-type spicules in a *him-5(e1490)* male. The spicule on the right side of this animal (black arrow) is in focus in this photomicrograph and partly projects out from the tail of the animal. It is long and straight. **H**, Crumpled spicules in a *let-23(sy97); him-5(e1490)* male (black arrow). Scale bar = 20 μm. Scale in E and F is same as in A; scale in H is same as in G. For A, C and D, anterior is right and posterior left. For B, anterior is left, posterior right. E and G printed in reverse orientation for ease of comparison with F and H.

ble (see below), we infer that all phenotypes arise from defects in different cells.

***let-23* null phenotype:** Complete loss of *let-23* function results in larval lethality based on the following criteria. First, larval lethals arose at a frequency typical for null mutations (1/1600) in our F_1 noncomplemen-

tation screen against *sy1* (BRENNER 1974; GREENWALD and HORVITZ 1980). This screen allows recovery of mutations that completely eliminate *let-23* activity, and larval lethals were the most common allele obtained. Second, larval lethality is the most severe phenotype associated with *let-23* mutations. Third, an allelic series can be made with larval lethals retaining least *let-23* function. Fourth, larval lethals behave like deletions in *trans* to other alleles. Lastly, one larval lethal is due to a premature stop codon located in the tyrosine kinase domain (AROIAN *et al.* 1990). As discussed below, the larval lethal *mn224* is an exception to the third and fourth criteria. The phenotype associated with complete loss of *let-23* function is probably not more severe than larval lethality since a *mnDf61/mnDf67* heterozygote, which deletes the *let-23* gene and a small region around it (SIGURDSON, SPANIER and HERMAN 1984), results in larval lethality. Thus no zygotic embryonic lethal gene resides in the overlap of these two deficiencies.

Loss of *let-23* in the vulva leads to the vulvaless phenotype: To study the role of the *let-23* gene in vulval induction, we made all possible *trans* heterozygotes using our five viable and subviable alleles, *n1045* (20°), *sy1*, *sy10*, *sy12* and *sy97*, and determined the percent vulval induction (a measure of vulval differentiation; see Figure 2) for each allele combination (Table 1A). We included in this analysis a representative null allele, *sy15*, the deficiency *mnDf68*, and the lethal allele *mn224*, which is phenotypically distinct from other lethal alleles. We also determined vulval induction in *n1045* hermaphrodites grown at 25° (see Table 3 and below) as homozygotes, as heterozygotes in *trans* to a *let-23*(+) chromosome, and in *trans* to *mnDf68*, a deficiency that deletes the *let-23* gene and the nearest genes on either side (SIGURDSON, SPANIER and HERMAN 1984).

Wild-type vulval induction is invariant (Figure 2A). The three VPCs closest to the anchor cell, P5.p, P6.p and P7.p, execute 2°, 1° and 2° fates, respectively. The other three VPCs, P3.p, P4.p and P8.p, divide once and fuse with the hypodermal syncytium, executing a "lower," 3° fate (see Figure 2 for definition of the 1°, 2° and 3° fate hierarchy). For comparison, induction is shown for *let-23* vulvaless animals (Figure 2, B and C) and for *let-23* hyperinduced animals (Figure 2D; see below).

Loss of *let-23* function in the vulva leads to a completely Vul phenotype (all VPCs 3°) based on the data in Table 1A and the following arguments. First, vulval induction of either *sy1* or *n1045* is lowered in *trans* to either a deletion or the *sy15* null, and in some cases decreases to 0% (*e.g.*, *sy1/mnDf68*). Second, we can infer an allelic series of decreasing *let-23* vulval activity, and lack of induction is consistent with least *let-23* function. Thus, in *trans* to any given allele, *n1045* at

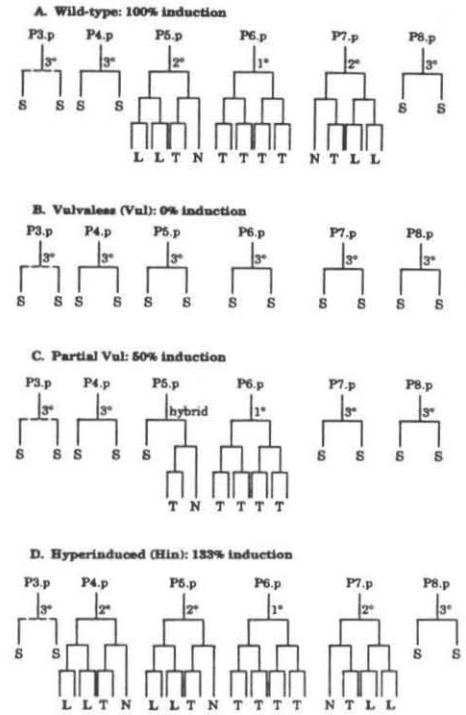

FIGURE 2.—Vulval induction in wild-type and *let-23* mutants. **A**, In wild-type animals, three VPCs, P5.p, P6.p and P7.p, divide more than once and execute vulval fates 2°, 1°, 2°, respectively. The other three VPCs, P3.p, P4.p and P8.p, do not form vulval tissue but rather divide once and fuse with the hypodermal syncytium (S); this fate is designated 3°. While the location of the anchor cell is not fixed during the induction period, it is generally centered over P6.p. In 1° lineages, the first two divisions are longitudinal in the ventral cord producing four progeny all of which subsequently divide transversely (T). In 2° lineages, the first two divisions are also longitudinal. The four progeny then execute three different patterns of divisions: longitudinal (**L**, bold face indicates adhesion to the cuticle), transverse (T), and no division (N). For more details see STERNBERG and HORVITZ (1986). The designation 1°, 2° and 3° arise because either P5.p or P7.p, which normally execute a 2° lineage, will execute a 1° lineage if P6.p has been ablated in an otherwise wild-type animal (SULSTON and WHITE 1980; STERNBERG and HORVITZ 1986). Similarly, P4.p will execute a 2° lineage if P5.p has been ablated and P8.p will execute a 2° lineage if P7.p is ablated. This "hierarchy" of fates is also indicated in experiments in which all but one VPC is ablated. This isolated VPC will execute a 1°, 2° or 3° fate depending on its distance from the anchor cell. The closer the cell is to the anchor cell, the higher the fate it will execute (STERNBERG and HORVITZ 1986). **B**, In completely Vul *let-23* animals, all VPCs execute 3° or non-vulval fate. Percent induction is 0 (0/3 • 100). **C**. In partially Vul animals, some vulval differentiation occurs, but less than in wild-type. In these animals, hybrid lineages often occur. In the example given here, P5.p executes half a 3° and half a 2° fate, and percent induction in this animal is 50 (1.5/3 • 100). Partial induction can range from 17% (0.5/3 • 100) to 83% (2.5/3 • 100). **D**, In hyperinduced animals, induction in greater than 100%. In this example, percent induction is 133 (4/3 • 100).

TABLE 1

Penetrance of different *let-23* phenotypes for different allele combinations

	♂								
♀	+	*sy1*	*n1045* (20°)	*sy97*	*sy10*	*sy12*	*sy15*	*Df*	*mn224*
A. Percent vulval induction									
+	100							100	100[a]
	(21)							(29)	(34)
sy1	100	14	33	36	8.7	6.5	0	0	101[a]
	(20)	(30)	(32)	(31)	(21)	(36)	(14)	(23)	(30)
n1045 (20°)	100		44	8.7	23	18	1.9	4.4	105[a]
	(20)		(42)	(25)	(26)	(30)	(26)	(30)	(21)
sy97	100			0	0	0	0		
	(20)			(21)	(19)	(21)	(1)		
sy10	100				1.6	0	—[b]		
	(20)				(21)	(24)			
sy12	100					0.6	—[c]		
	(20)					(31)			
B. Percent survival									
+	100							92	ND
	(334)							(257)	
sy1	103	108	100	106	94	104	ND	118	100
	(271)	(243)	(161)	(263)	(232)	(349)		(170)	(214)
n1045 (20°)	97		42	37	56	49	18	25	15
	(341)		(502)	(238)	(303)	(241)	(332)	(211)	(305)
sy97	97			11	21	19	0.4		
	(289)			(389)	(302)	(379)	(453)		
sy10	105				14	17	0		
	(257)				(373)	(442)	(344)		
sy12	106					19	0.3		
	(354)					(280)	(301)		
C. Percent wild-type spicules									
+	100							100	ND
	(20)							(20)	
sy1	100	100	96	90	95	92	ND	95	95
	(21)	(22)	(24)	(20)	(20)	(24)		(20)	(21)
n1045 (20°)	100		24	0	52	59	4.6	0	92
	(21)		(38)	(22)	(21)	(22)	(22)	(17)	(20)
sy97	100			0	5.6	0	0		
	(20)			(19)	(18)	(21)	(2)		
sy10	100				4.8	0	—[b]		
	(20)				(21)	(20)			
sy12	100					0	0		
	(21)					(21)	(1)		
D. Percent fertile hermaphrodites									
+	ND							100[d]	ND
								(18)	
sy1	100	100		100	100	100	100	100	ND
	(23)	(24)		(30)	(30)	(31)	(18)	(30)	
n1045 (20°)	100	100	100	100	71	100	36	0	8
	(23)	(27)	(26)	(36)	(34)	(30)	(42)	(30)	(13)
sy97	100			95	100	100	100		
	(22)			(22)	(31)	(31)	(1)		
sy10	100				0	15	0		
	(23)				(45)	(53)	(1)		
sy12	100					28	0		
	(24)					(47)	(1)		

20° displays the most vulval induction followed by *sy1*, *sy10*, *sy12*, *sy97* (except in *trans* to *sy1*; see below), and then *sy15*. The allele *sy97* has 0% induction in *trans* to *sy10*, *sy12*, or itself. The data also indicates that a reduction but not elimination of *let-23* function can lead to a partly Vul phenotype.

Characterization of the phenotype of *let-23(n1045)* at 25°: As mentioned in the introduction, *let-23(n1045)* hermaphrodites grown at 25° display a phenotype reminiscent of multivulva mutations (FERGUSON and HORVITZ 1985). To characterize this phenotype further, we analyzed vulval induction patterns

TABLE

♀	♂ +	*sy1*	*n1045* (20°)	*sy97*	*sy10*	*sy12*	*sy15*	*Df*	*mn224*
E. Percent wild-type P12									
+	100							100	ND
	(21)							(29)	
sy1	100	100	88	94	95	97	ND	100	97
	(20)	(30)	(32)	(31)	(20)	(36)		(23)	(30)
n1045 (20°)	100		91	92	80	86	77	97	90
	(20)		(44)	(25)	(25)	(29)	(22)	(30)	(21)
sy97	100			95	95	76	100		
	(20)			(20)	(20)	(21)	(1)		
sy10	100				90	80	—[b]		
	(20)				(21)	(25)			
sy12	100					83	0		
	(20)					(29)	(1)		

Each row represents the *let-23* chromosome inherited from the mother and each column represents the *let-23* chromosome inherited from the father. See MATERIALS AND METHODS for details. A, The percent vulval induction and the number of hermaphrodites scored (in parentheses) for each heterozygote. B, The percent survival of each *trans*-heterozygote and the number of Dpy plus Rol males divided by three (in parentheses) as a measure of the sample size (see MATERIALS AND METHODS). Survival greater than 100% is statistically possible due to the method of calculation. C, The percentage of males with both spicules wild type. It is possible for a given male to have one wild-type spicule and one mutant spicule, and we classify these as mutant. The number in parentheses is the number of males scored. D, The percentage of hermaphrodites for a given allele combination that are fertile. The number of hermaphrodites scored is given in parentheses. See MATERIALS AND METHODS for a description of *sy15* and *mn224* fertility. E, The percent wild-type P12 scored for different allele combinations. Number in parentheses is the number of hermaphrodites scored. ND = not determined; *Df* = *mnDf68*.

[a] For *mn224*/+, *mn224*/*sy1* and *mn224*/*n1045* 0/34, 3/30 and 3/21 hermaphrodites were hyperinduced, respectively.

[b] Cross was carried out but no *let-23*/*let-23* cross-progeny survived. Since *sy97*, *sy10* and *sy12* are nearly inviable in *trans* to *sy15*, *mn224* and *mnDf68*, only *sy15* *trans*-heterozygotes were made with these three alleles.

[c] The one hermaphrodite that survived was picked up as an adult and not scored for this phenotype.

[d] Two of these 18 hermaphrodites were sickly with low broods. This is probably a function of the deletion and not the *let-23* locus since lethal alleles in *trans* to a wild-type chromosome are healthy.

and cell lineages in *n1045* hermaphrodites grown at 25° with and without an anchor cell (Table 2). In the presence of an anchor cell, induction is often greater than wild type; more than the three wild-type VPCs can differentiate into vulval fates, and the VPCs that take on 2° fates in wild-type hermaphrodites, P5.p and P7.p, can take on the "higher" 1° fate. These induction patterns are distinct from the induction patterns in Muv hermaphrodites in two ways (*e.g.*, STERNBERG 1988). First, there is often a breakdown in the lateral inhibition that normally prevents the formation of adjacent 1° fates. Second, in *n1045* hermaphrodites grown at 25°, induction is generally centered around the anchor cell: VPCs that execute 3° fates are farther away from the anchor cell than VPCs that execute 2° fates, which are in turn farther away than VPCs that execute 1° fates. However, in Muv hermaphrodites, the most distal VPCs often execute higher fates than their more anchor cell-proximal neighbors (*e.g.*, from anterior to posterior 2° 1° 2° 1° 2° 1°). Thus in *n1045* hermaphrodites, VPCs seem to still respond to the anchor-cell signal in a graded fashion. This conclusion is supported by the finding that in *n1045* animals grown at 25°, there is no induction in the absence of the anchor cell. Again, this is unlike induction in Muv hermaphrodites, which occurs even in the absence of an anchor cell (STERNBERG 1988). We designate this vulval phenotype as the "*h*yperinduced" or "Hin" phenotype. This *n1045* 25° phenotype appears to result from hypersensitive VPCs since we do not believe that the *let-23* gene acts in the anchor cell. In particular, the *let-23* gene is unlikely to act in the anchor cell because of its epistasis to the Muv gene *lin-15* (see MATERIALS AND METHODS). A *lin-15(n309)* hermaphrodite lacking an anchor cell still has a Muv phenotype, but a *let-23(sy97); lin-15(n309)* hermaphrodite, which retains only slight *let-23* vulval activity, is most often Vul. Thus, lack of the *let-23* product is not the same as a lack of the anchor cell, and we infer that the *let-23* gene does not act in the anchor cell. Since *lin-15* might act in cells other than the VPCs or the anchor cell (HERMAN and HEDGECOCK 1990), it is also possible that the *let-23* gene may act in cells other than the VPCs, such as those of the surrounding hypodermis.

Genetic basis for hyperinduction: Hyperinduction in *n1045* hermaphrodites is recessive and dosage-sensitive (Table 3). Heterozygote *n1045*/+ hermaphrodites have wild-type vulvae at 25°. In addition, whereas hermaphrodites with two copies of *n1045* grown at 25° often have more than wild-type induction (phenotypically Hin), hermaphrodites with only one copy of *n1045* (*n1045*/*mnDf68*) grown at 25° have less than wild-type induction (phenotypically Vul or partly Vul).

Hyperinduction appears to result from a partial

TABLE 2

Hyperinduction in *n1045* hermaphrodites grown at 25°

	AC						
	P3.p	P4.p	P5.p	P6.p	P7.p	P8.p	No.
A. Wild-type	S or S S	S S	LLTN	TTTT	NTLL	S S	
n1045/n1045 (25°)	S	LLON	OTTT	TTTT	NTLL	S S	1
	S	S OO	LLTN	TTTT	NTLL	S S	1
	S	LLON	OOTT	TTTT	NTOL	NOLL	1
B. Wild-type	3°	3°	2°	1°	2°	3°	
n1045/n1045 (25°)	3°	2°	1°	1°	1°	2°	1
	3°	2°	1°	1°	2°	3°	7
	3°	2°	2°	1°	2°	3°	7
	3°	3°	2°	1°	2°	2°	2
	3°	3°	2°	1°	2°	3°	6
	3°	2°	1°	2°	3°	3°	2
C. *n1045/n1045* (25°) no anchor cell	3°	3°	3°	3°	3°	3°	7

The relative placement of the anchor cell (AC) during the induction period (late L2-early L3 stage) is indicated. Below each VPC is the fate executed in *n1045* 25° hermaphrodites. The right-most column (No.) indicates the number of hermaphrodites seen with a given induction pattern. Nomenclature is as given in Figure 2 except where noted. A, The lineages of three *n1045* hermaphrodites grown at 25°. Bold-face indicates adhesion to the cuticle and is diagnostic of 2° lineages, as is the presence of an N cell. Oblique axes of nuclear division (O) are acceptable as part of either 1° (here OOTT or OTTT) or 2° (here **LLON**, **NTOL**, or **NOLL**) lineages (STERNBERG 1988). For clarity, 1° lineages are enclosed in heavy boxes and 2° lineages in thin boxes. The lineage [S OO] is hybrid (see DISCUSSION for more on hybrid lineages). B, The fates of the six VPCs determined by anatomical examination of 25 hermaphrodites. Fates shown here are consistent with those lineages shown in A. When examining many animals, this anatomical method is more practical than lineaging due to ease and speed. It is reliable if performed in early-mid L4 hermaphrodites since all divisions are complete and the progeny retain their relative positions and orientations. Subsequently, scoring induction by anatomy becomes more difficult due to the movement of the progeny cells during vulval morphogenesis. C, Anatomically determined VPC fates in gonad-ablated (and therefore anchor-cell deficient) L1 *n1045* hermaphrodites grown at 25° (see RESULTS and MATERIALS AND METHODS).

decrease in *let-23* function. Since *n1045/mnDf68* animals grown at 25° are partially Vul and *+/mnDf68* animals grown at 25° have wild-type vulvae, one copy of *n1045*(25°) has less vulval function than one copy of a *let-23*(+) chromosome. Extrapolating, we infer that at 25°, two copies of *n1045* has less vulval function than two copies of *let-23*(+), even though the former has more vulval induction than the latter. Hyperinduction therefore correlates with a *reduction* of some aspect of *let-23* vulval function. However, this reduction is only partial since a more severe or complete loss *let-23* function results in a partly or completely Vul phenotype, respectively. That the Hin phenotype correlates with more *let-23* function than the Vul phenotype is further supported by the fact that *n1045/n1045* at 25° is Hin but *n1045/mnDf68*, which should have less function, is Vul.

Hyperinduction is not restricted to *n1045*, suggesting that it is not merely an unusual property of the *n1045* allele. The lethal allele *mn224* displays the Hin phenotype in *trans* to both *syl* and *n1045* at 20° (Table 1A). Both *syl* and *n1045(20°)* have less than wild-type *let-23* vulval function since they display a Vul or partly Vul phenotype in *trans* to other alleles. The *mn224* Hin phenotype therefore also correlates with lowering *let-23* vulval function since *mn224/+* has wild-type vulval induction. That is, reducing the *let-23* vulval activity from *mn224/+* to *mn224/syl* or *mn224/n1045* can result in Hin vulvae. Unfortunately, we cannot determine the extent of vulval induction of *mn224* in *trans* to other alleles since these combinations are lethal (Table 1B). The *n1045* and *mn224* data are summarized in Figure 3 as a plot of induction versus inferred *let-23* activity.

***let-23* tissue specificity:** The allele *mn224* is defective in an essential function but retains some *let-23* function in the vulva, suggesting that the *let-23* phenotypes in different tissues are separable. Two general models could account for this separability of phenotypes. First, different tissues could be differentially sensitive to *let-23* dosage. For example, the *let-23* product might phosphorylate factor a in tissue A and factor b in tissue B, with factor a being less prevalent in tissue A than factor b in tissue B. Therefore, tissue A would be more sensitive to a decrease in *let-23* dosage than tissue B. This model predicts alleles that reduce *let-23* function would "uncover" mutant phenotypes in a specific order: *let-23* mutations could result in an A^-B^+ or A^-B^- phenotype (depending on the severity of the allele) but not an A^+B^- phenotype. Second, *let-23* itself might have independently mutable domains and encode tissue-specific functions. For

TABLE 3

Genetic basis of *n1045* hyperinduction

let-23 genotype	No. of hermaphrodites at 25° with induction: <100% (phenotypically Vul of partly Vul)	=100% (phenotypically wild-type[a])	>100% (phenotypically Hin)	Average % induction
+/+	0	All	0	100
n1045/n1045	2	12[b]	9	107
n1045/+	0	21	0	100
n1045/mnDf68	19[c]	0	0	15
+/mnDf68	0	21	0	100

All data were generated at 25° using the same complementation scheme as in Table 1. Average induction seen with *n1045* is generally less at all temperatures when generated with this scheme than when using the original *n1045* isolate. For example, induction in the original *n1045* strain at 25° is 127% (data from Table 2) versus 107% here. Induction of *n1045* homozygotes at 15° is 11% using the complementation scheme and 25% in the original strain. Nonetheless, for the data generated by either method we have determined that at 25° *n1045* displays the Hin phenotype, this hyperinduction is recessive, and *n1045/mnDf68* animals are Vul or partly Vul but never Hin. There are three possible explanations for this difference in *n1045* induction. First, since the complementation scheme uses mothers which are belanced over a *let-23*(+) chromosome, it is possible that *n1045* is maternally rescued. However, we were unable to detect differences in vulval induction of *n1045/n1045* progeny of either homozygous (*n1045/n1045*) or heterozygous (*n1045/+*) mothers (see MATERIALS AND METHODS). Second, it is possible that the linked markers (*rol-6* and *dpy-10*) used in the complementation scheme have dominant effects on induction, although such an effect has not been seen with any other allele (see MATERIALS AND METHODS). Last, the difference could be due to the elimination by recombination of a tightly linked modifier during marker addition. Precedent for this may exist with the allele *sy12* (see MATERIALS AND METHODS).

[a] Except where noted, animals with 100% induction displayed wild-type induction patterns.

[b] Two of these 12 hermaphrodites did not have wild-type induction patterns, although the wild-type number of VPCs (*i.e.*, three) divided.

[c] Twelve of these 19 animals had 0% induction.

example, the *let-23* gene might interact differently with factor a and b such that it is possible to disrupt the interactions of the *let-23* product with factor a only (A^-B^+ phenotype), factor b only (A^+B^- phenotype), or with both (A^-B^- phenotype).

We distinguished between these models by quantitating the penetrances of five *let-23* phenotypes associated with the allele combinations used to measure vulval induction (see above) and ask whether or not these phenotypes are separable by *let-23* dosage. The penetrances of the five phenotypes are given in Table 1 such that 100% is wild type in all cases: percent vulval induction (Table 1A); percent survival (Table 1B); percent wild-type male spicules (Table 1C); percent fertile hermaphrodites (Table 1D); and percent wild-type P12 (Table 1E).

We find that for any given phenotype but P12, we can determine a loss of function phenotype and can order the alleles from most to least *let-23* activity (Table 4A). All alleles were recessive for all phenotypes. These results suggest that the *let-23* mutant phenotypes arise from reduction or elimination of *let-23* function and that the *let-23* gene is needed for the wild-type development of these different tissues. Only a few allele combinations are inconsistent with the ordering of alleles given in Table 4A. The vulval phenotype of the *sy1/sy97* heterozygote is less severe than expected (Table 1A). This is perhaps best demonstrated as percent of hermaphrodites which can lay eggs: 8% of *sy1/sy1* ($n = 98$) and 0% of *sy97/sy97* ($n > 100$) hermaphrodites are Egl^+ whereas 53% of *sy1/sy97* hermaphrodites are Egl^+ ($n = 30$). The simplest interpretation of this intragenic complementation is that the *let-23* product acts as a multimer and that these mutations are in protein coding sequence (reviewed in WHITEHOUSE 1969). Also, homozygous *n1045* hermaphrodites at 20° display more penetrant essential and male spicule defects than expected based on other *trans* heterozygous combinations (Table 1, B and C). This increased penetrance might result from some slight dominant negative effects of *n1045* for these phenotypes, or from *n1045* partially complementing the defects in *sy10*, *sy12* and possibly *sy97* for these two phenotypes .

Although our mutations display tissue-specific effects, we cannot order the phenotypes with respect to their sensitivity to *let-23* dosage (Table 4B). Rather, the phenotypes are independently mutable and the *let-23* gene appears to encode tissue-specific functions. The allele *sy1* would indicate that the vulva is the most sensitive tissue to *let-23* dosage. This conclusion, however, is contradicted by *n1045* and *mn224*. The allele *n1045* is less severe than *sy1* in the vulva but more severe in all other tissues. The allele *mn224* is less severe than *sy1* in the vulva but displays fully penetrant larval lethality and sterility. Unpredictably, *mn224* is also nearly wild type for spicule function. This allele therefore suggests that it is possible to eliminate *let-23* function in some tissues and not others. Lastly, the allele *sy97*, which is wild type for hermaphrodite fertility but severe in all other tissues, would suggest that defects in fertility are the least sensitive phenotype to *let-23* dosage and contradicts sensitivities inferred from *n1045* and *sy10*. These results indicate that the tissue-specific effects associated with different *let-23* alleles result not from differences in dosage sensitivity but from independently mutable domains.

Although this conclusion is based primarily on three alleles (*sy1*, *sy97* and *mn224*), we believe these findings are significant. First, these alleles represent half of our non-null alleles (14 out of 20 alleles are nulls). Second, with few exceptions (see above), these alleles consistently and quantitatively behave as outlined, despite the fact that we can otherwise assign loss of function phenotypes. Third, these results are taken from alleles which were generated in only two types

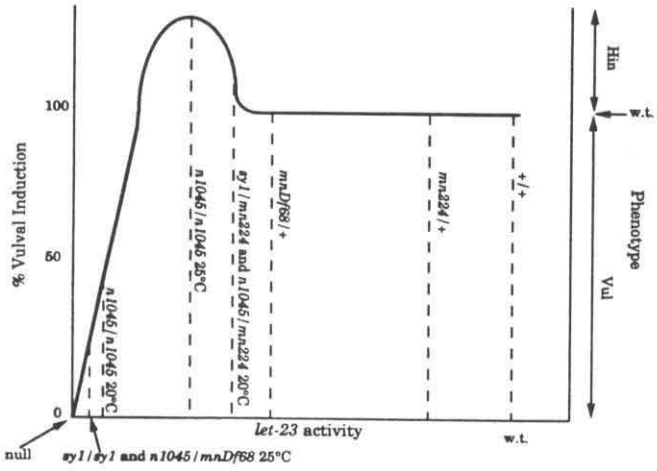

FIGURE 3.—Hypothetical dose response curve for *let-23* vulval induction. Since *let-23(+)/mnDf68* is wild type, we assume that initial decreases in *let-23* do not affect induction. Therefore, starting from wild type, the curve is flat. We infer that homozygous *n1045* at 25° has less function than wild type but is hyperinduced so the curve rises as activity drops. A further decrease in function (*e.g.*, *n1045/mnDf68* at 25°) results in the Vul phenotype so the curve falls off until at no *let-23* activity, induction is 0%. Since *mn224* is lethal, we cannot infer its *let-23* activity as a homozygote relative to the other alleles but it appears to have significantly more activity than *mnDf68*, and has wild-type induction in *trans* to *let-23(+)*. Hence, we put *mn224/+* far to the right of *mnDf68/+*. Both homozygous *sy1* and *n1045* at 20° are Vul, but in *trans* to *mn224* are Hin. As discussed in the text, this is consistent with our *n1045* results, and we have plotted the graph accordingly. w.t. = wild type.

of screens: for vulvaless (17/20 alleles) and lethal (3/20) mutations.

The P12 to P11 transformation in *let-23* mutants differs from the other phenotypes in that it exhibits partial maternal rescue (Table 5). This maternal rescue explains the weak penetrance seen in Table 1E since the data are generated from balanced *let-23(mutant)/let-23(+)* mothers. We have found no instance of completely penetrant transformation, and we cannot assign a loss of function phenotype. The allele *sy1* has 100% wild-type P12 even from a homozygous *sy1* mother (data not shown).

DISCUSSION

We initiated studies of *let-23* to understand the genetic basis for the *hyperinduced* (Hin) phenotype associated with one allele. In Hin hermaphrodites, the VPCs are often induced to a higher fate than in wild-type hermaphrodites (*i.e.*, 3° and 2° cells in wild type can become 2° and 1°, respectively, in a Hin animal). Our results suggest that in a Hin hermaphrodite, the VPCs are hypersensitive to the inductive signal. Furthermore, our data suggest that hyperinduction results from a partial reduction of *let-23* function and not from gain of function or neomorphic function. This conclusion is surprising given that a strong reduction or elimination of *let-23* function results in reduced or no induction of the VPCs. In addition, we measured the penetrance of five *let-23* phenotypes for many allele combinations. These phenotypes show a remarkable degree of independence of mutability (Table 4B). For example, it is possible to preferentially eliminate the *let-23* vulval function alone (allele *sy1*), to preferentially eliminate the essential and fertile functions (allele *mn224*), and to preferentially retain the fertile function (allele *sy97*).

Models for *let-23* function in the vulva: Our data suggest that proper determination of vulval fate requires two opposing pathways, both regulated by the *let-23* receptor tyrosine kinase. Since a complete loss of *let-23* function leads to no induction of vulval fate, the *let-23* gene is required for a *stimulatory* pathway essential for any vulval differentiation to occur. On the other hand, since some mutations that reduce *let-23* function result in greater than wild-type induction of vulval fate (Hin phenotype), the *let-23* gene is also apparently involved in an *inhibitory* pathway that normally acts negatively to limit the amount of induction that occurs. This inhibitory pathway seems to modulate the stimulatory pathway and not vice versa since a loss of *let-23* function leads to no induction and since VPCs in a Hin animal require the anchor cell signal and respond to it in a graded fashion, suggesting that stimulation of induction itself is functional but not properly regulated. These two pathways may or may not be separately controlled by the *let-23* gene itself. For example, the *let-23* product might activate two cascades, one that results in induction and the other that results in negative regulation. Alternatively, the *let-23* product might activate only one cascade that later splits into two pathways, stimulatory and inhibitory. In addition, other genes are likely to act with *let-23* in both these pathways since, for example, mutations in *lin-2* and *lin-7* can result in both the Vul and Hin phenotypes (FERGUSON and HORVITZ 1985; G. JONGEWARD and P. STERNBERG, in preparation).

We propose that hyperinduction results from *let-23* mutations that compromise the inhibitory pathway but not the stimulatory pathway. In these animals, induction occurs but is not properly limited or regulated, resulting in higher than wild-type VPC fates. We envision two models for how *let-23* mutations can lead to hyperinduction (Figure 4A). These models are parallel to the possible models for separable tissue-

TABLE 4

Summary of complementation analysis

A. Ordering of *let-23* alleles for different *let-23* phenotypes

	let-23 phenotype			
	Defective vulval induction	Lethality	Defective male spicules	Hermaphrodite sterility
Increasing severity ↓	*mn224* *n1045* (20°) *sy1* *sy10* *sy12* *sy97* *sy15, mnDf68*	*sy1* *n1045* (20°) *sy10, sy12, sy97* *mn224, sy15, mnDf68*	*sy1, mn224* *n1045* (20°) *sy10* *sy12* *sy97, sy15, mnDf68*	*sy1, sy97* *n1045* (20°) *sy12* *sy10* *mn224, sy15* *mnDf68*
Null phenotype	Vulvaless	Larval lethal	Crumpled spicules	Sterile

B. Inferred defects in *let-23* functions for different alleles

	let-23 function				
Allele	Vulval induction	Essential	Male spicules	Hermaphrodite fertility	P12
Wild type	+	+	+	+	+
n1045	(+)	(+)	(+)	(+)	(+/−)
sy10	(−)	(−)	(−)	(−)	?
sy15, mnDf68	−	−	−	−	?
sy1	(−)	+	+	+	+
mn224	(+)	−	+	−	?
sy97	(−)	(−)	(−)	+	(+/−)

A, For each *let-23* phenotype, we have ordered the alleles from least severe to most severe and have assigned a complete loss of function phenotype as follows. Complete loss of vulval function correlates with no vulval induction (see text). A complete loss of *let-23* essential function likely results in larval lethality. First, both the deletion *mnDf68* and the canonical null *sy15* in *trans* to all alleles but *sy1* decrease survival. In particular, the alleles *sy10*, *sy12* and *sy97* are virtually inviable in *trans* to a null. There is thus a critical threshold of *let-23* dosage for survival since any combination of two of these alleles results in 10–20% survival, but only one copy of any of them is <1% viable. Second, in an allelic series, 0% survival is consistent with least *let-23* essential function (e.g. the canonical lethal *sy15* and the lethal *mn224* behave like *mnDf68* for essential function and are inviable). As discussed above, a *let-23* null also results in larval lethality. This distinction between loss of essential function and the null phenotype is necessary because of the allele *mn224*, which is a loss-of-function for essential activity but not a loss-of-function for the entire gene, since it retains substantial vulval and male spicule activities. Complete loss of *let-23* spicule activity likely results in a completely penetrant crumpled spicule phenotype. First, *n1045* at 20° in *trans* to null alleles has a higher penetrance of mutant phenotype. Second, in an allelic series, low function levels correlate with a highly penetrant crumpled spicule phenotype (see especially *sy97*). We have found that *let-23* males with defective spicules cannot mate (see MATERIALS AND METHODS). Complete loss of *let-23* fertile activity likely results in a completely penetrant sterile phenotype. First, *n1045* at 20° in *trans* to null alleles has a higher penetrance of the mutant phenotype. Second, in an allelic series, low activity levels correlate with complete sterility (see especially *sy10*). B, This table summarizes inferred impairment in the different *let-23* tissue-specific functions associated with each *let-23* allele. Inferred impairment for each entry is based on the severity of the defects seen in that allele relative to wild-type and the other alleles within that phenotype. From least to most impaired: + refers to wild-type or close to wild-type function; (+) refers to reduced function; (−) refers to low function; − refers to no function as defined by a deficiency.

specific phenotypes (see RESULTS), except that the separable phenotypes (Hin and Vul) now affect the same tissue. If the inhibitory pathway is more sensitive to *let-23* dosage than the stimulatory pathway, then a moderate reduction in *let-23* function would preferentially affect the inhibitory pathway, resulting in a higher than wild-type induction (model I). Alternatively, the inhibitory pathway could be controlled by a region of *let-23* distinct from the stimulatory pathway; mutations that have a Hin phenotype could then result from a mutation preferentially affecting this inhibitory pathway region (model II). This latter possibility is intriguing given that the *let-23* protein is a member of the EGF receptor tyrosine kinase subfamily (AROIAN *et al.* 1990) and that one of our Hin alleles, *n1045*, is amber-suppressible (FERGUSON and HORVITZ 1985). This mutation may therefore result in a truncated receptor lacking some C-terminal sequence (a truncation starting too far from the C terminus, such as in the kinase domain, would presumably have more severe phenotypes than seen for *n1045*). It is known that the C terminus of the EGF receptor is needed for its proper negative regulation because of the presence of autophosphorylation sites (BERTICS and GILL 1985) and sequences required for down-regulation of receptor (RIEDEL *et al.* 1989; CHEN *et al.* 1989). Furthermore, both mutations that eliminate autophosphorylation sites (HONEGGER *et al.* 1988) and truncations of the receptor C terminus (WELLS *et al.* 1990) can result in cells hypersensitive

TABLE 5

Maternal rescue of P12 phenotype

let-23 genotype of mother	*let-23* genotype	Percent wild-type P12
sy97/sy97	*sy97/sy97*	40 ± 9
sy97/+	*sy97/sy97*	78 ± 8
sy12/sy12	*sy12/sy12*	39 ± 18
sy12/+	*sy12/sy12*	83 ± 14
n1045/n1045 15°	*n1045/n1045* 15°	44 ± 8
*n1045/+*15°	*n1045/n1045* 15°	55 ± 7

We scored P12 phenotype in hermaphrodites from mothers that were homozygous and heterozygous for *let-23*. Given is the percent wild-type P12 and two standard deviations. The maternal rescue with the amber allele *n1045* is less than in *sy97* or *sy12* hermaphrodites. The incomplete penetrance of the rescue might indicate a need for some *let-23* zygotic product.

to ligand. Thus, for example, the *n1045* mutation might result in a receptor competent to transduce signal but defective in down-regulation. As a consequence of receptors recycling to the cell surface, the VPCs might become hypersensitive to the inductive signal. The absence of hyperinduction in *n1045* hemizygotes could be explained if one copy of defective receptor provided insufficient stimulatory function. Similarly, the recessive nature of this mutation may be due to the limiting effects of one copy of defective receptor or restoration of proper regulation by the wild-type copy.

The inferred inhibitory pathway might operate intercellularly or intracellularly (Figure 4B). The anchor cell inductive signal might act to stimulate a VPC, which then negatively signals its neighbors to inhibit their induction (intercellular inhibition; model III). If the inhibitory signal transmitted by a VPC was proportional to the inductive signal the VPC received, then this would serve to reinforce the graded anchor cell signal: P6.p would inhibit P5.p and P7.p from executing too high a fate, and P5.p and P7.p would similarly inhibit P4.p and P8.p respectively. Precedent for intercellular VPC interactions (*e.g.*, 1°–1° lateral inhibition) exists (STERNBERG 1988; THOMAS, STERN and HORVITZ 1990). However, since the Hin phenotype can include the execution of 2° fate by VPCs that normally execute a 3° fate in addition to the presence of adjacent 1° cells, then this inter-VPC inhibition would lower the extent of induction in general and not only prevent the formation of adjacent 1° cells. Alternatively, both the stimulatory and inhibitory pathways regulated by *let-23* could operate within a given VPC (model IV). For example, the inhibitory pathway could involve intracellular down-regulation of the receptor for inductive signal, which might be *let-23* itself. Failure of this process could result in VPCs that do not properly negatively regulate the signal transduction, resulting in the hyperinduced phenotype.

***let-23* tissue specificity:** Mutations in the *let-23* gene can, to a large extent, independently affect the different *let-23* phenotypes, suggesting that the *let-23* gene behaves differently in different tissues and that the *let-23* gene has tissue-specific functions. Several possible mechanisms could account for this tissue-specificity: promoter elements specific for different tissues, tissue-specific alternatively spliced transcripts, or differential interactions with tissue-specific factors. There is some evidence that three of our *let-23* mutations are in protein coding sequence and not promoter elements. First, the *n1045* mutation is amber-suppressible. Second, the alleles *sy1* and *sy97* show striking allele-specific complementation for the vulval function. In addition, although several alternatively spliced transcripts could account for independent mutability, initial molecular characterization of the *let-23* gene suggests that there are not enough transcript species (AROIAN *et al.* 1990).

Another possibility is that there is one *let-23* product that interacts with different factors in different tissues. These factors could act to modify, stabilize, or destabilize the *let-23* product in different tissues, or they could be tissue-specific ligands or substrates of the *let-23* receptor itself. The independent mutability of the *let-23* functions might then be due to differential interactions of the *let-23* product with these tissue-specific factors. As noted above, a multiple substrate mechanism might be responsible for the stimulatory and inhibitory pathways regulated by the *let-23* gene in the vulva. That tissue-specificity is independently mutable as opposed to strictly dosage-sensitive prompts us to at least consider that the vulval stimulatory and inhibitory pathways are directly and separately controlled by the *let-23* gene.

The multiplicity of *let-23* function is perhaps not surprising given what is known about the mammalian EGF receptor. The receptor has diverse effects in the cell (reviewed in ULLRICH and SCHLESSINGER 1990), and it and its subfamily members are believed to behave differently in different cell types (KHAZAIE *et al.* 1988; DIFIORE *et al.* 1990). Moreover, the Drosophila EGF receptor locus (DER), known by *faint-little-ball*, *torpedo*, and *Ellipse* mutations (SCHEJTER and SHILO 1989; PRICE, CLIFFORD and SCHUPBACH 1989; BAKER and RUBIN 1989), displays a similar multiplicity of phenotypes and partial independence of mutability of the gene functions associated with the phenotypes (CLIFFORD and SCHUPBACH 1989). As with the *let-23* gene, there probably are too few DER transcripts for an alternative splicing model to account for the differential mutability.

Role of the *let-23* gene in determination and possibly proliferation of vulval fate: That the *let-23* gene encodes an EGF receptor-like molecule raises the question of how such a molecule might function in nematode development. In mammalian systems,

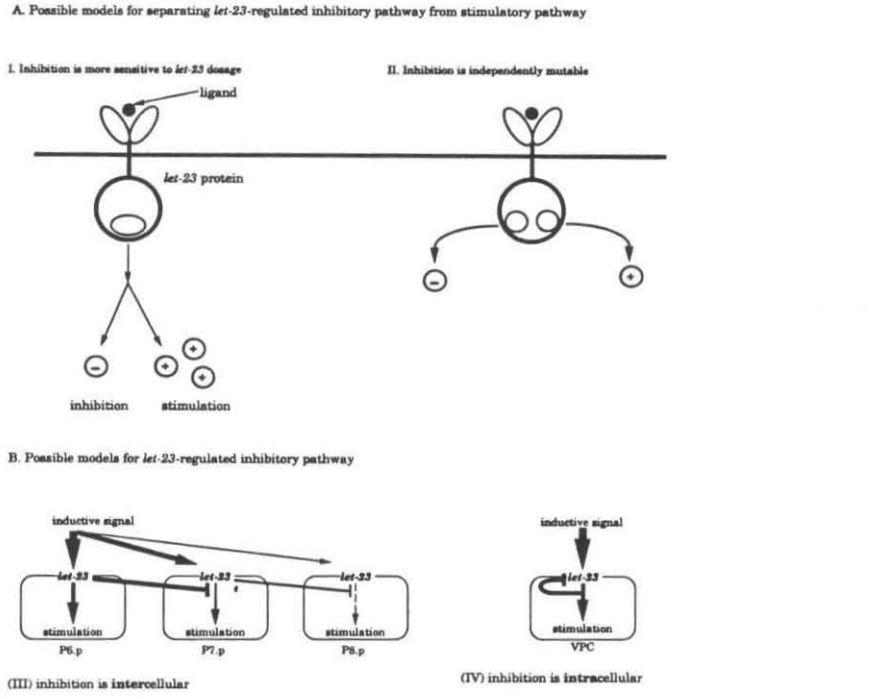

FIGURE 4.—Models for *let-23* function during vulval induction. **A**, Model for the separability of the stimulatory and inhibitory pathways regulated by *let-23* . This separation can be achieved by either assuming that the inhibitory pathway is more sensitive to *let-23* levels than the stimulatory pathway (model I) or that the stimulatory pathway and the inhibitory pathway are separately controlled by *let-23* and are somewhat independently mutable (model II). In model I, the two pathways may or may not be separately controlled by the *let-23* gene; they could branch at or downstream of the *let-23* product and still have different dosage sensitivities. As noted in the text, other genes, such as *lin-2* and *lin-7* which also display the Vul and Hin phenotypes, are likely to act with *let-23* in both of these pathways (G. JONGEWARD and P. STERNBERG, in preparation). **B**, Models for *let-23*-regulated inhibitory pathway. The inhibitory pathway which is defective in hyperinduced animals could act intercellularly (model III) or intracellularly (model IV). In model III, *let-23* is needed for stimulation of vulval fates and negative signalling between VPCs. If the *let-23*-regulated inhibitory pathway is compromised, then a given VPC would become hypersensitive to inductive signal because it is not inhibited by neighboring VPCs. This neighboring VPC inhibition may either be passive (i.e., VPCs proximal to the anchor cell normally prevent high signal levels from reaching more distal VPCs by simply removing signal from the extracellular space between the anchor cell and the VPCs) or active. In model IV, hypersensitivity results from alleviation of internal inhibition. Normally, the *let-23* gene acts to signal a VPC to adopt vulval fate and also negatively regulates transduction in the same VPC to ensure proper response levels. In the example given, negative regulation occurs upstream of *let-23*, but it may occur downstream. For simplicity, we assume that both of the *let-23*-regulated pathways act in the VPCs, but, until we know where *let-23* is expressed, we cannot exclude that the stimulatory and/or inhibitory pathway originate in other cells, such as the surrounding hypodermis.

ligands of EGF receptor, EGF and TGF-α, can elicit proliferation, can inhibit proliferation, or can have other unrelated effects (reviewed in SPORN and ROBERTS 1988). Examination of vulval development in *let-23* mutants indicates that the *let-23*-directed pathways are involved in cell-type determination and possibly also cell proliferation.

Evidence for a proliferative role comes from hybrid vulval-hypodermal lineages found in some *let-23* mutant animals (see Figure 2C). These non-wild-type lineages result in one daughter of a VPC executing a vulval fate and the other daughter executing a hypodermal fate; these lineages are not unique to *let-23* (SULSTON and WHITE 1980; KIMBLE 1981; SULSTON and HORVITZ 1981; GREENWALD, STERNBERG and HORVITZ 1983; STERNBERG and HORVITZ 1986, 1989). Our *let-23* data suggest that hybrid lineages appear to arise from VPCs in which there are lower than wild-type levels of signal transduction. We also found that the polarity of hybrid lineages correlates with the location of the anchor cell. For either P5.p or P7.p, the VPC daughter closer to the anchor cell has a nine times greater chance of executing the vulval fate than the daughter farther from the anchor cell, whereas for P6.p, whose daughters are equidistant from the anchor cell, the chance is significantly less biased. In addition, the decision by a given VPC to execute a hybrid lineage does not show any obvious

correlation with the fate of the neighbor(s) of that VPC (see MATERIALS AND METHODS for data on hybrid lineages). Based on these data, we speculate that the anchor-cell signal is causing a bias in the distribution of some factor in the VPCs necessary for their daughters to divide. Therefore, in VPCs with lowered levels of induction, for example due to reductions in *let-23* or *let-60 ras* activity (both of which can result in hybrid lineages; this paper and M. HAN, personal communication), the anchor-cell distal daughter is less likely to divide than the anchor-cell proximal daughter. This model suggests that hybrid lineages arise from a defect in proliferation of some VPC daughter cells and is consistent with a growth factor receptor-directed pathway. A specific version of this model is that the *let-23* product coupled to its hypothetical ligand itself might be the factor whose distribution is influenced by the anchor-cell signal, since in mammalian systems ligand induces clustering of receptor (reviewed in SCHLESSINGER *et al.* 1983). Other models unrelated to proliferation are also possible to explain hybrid fates; for example, these lineages may represent determination of a novel VPC fate.

Nonetheless, the *let-23* gene does appear to function directly in cell-type determination independent of proliferation. This conclusion is best demonstrated by the transformation of 2° fate to 1° fate in some hyperinduced animals in which there is no change in the number of rounds of mitosis involved (except for the "N" cell; see Figure 2A).

The overall functioning of the *let-23* gene is therefore intricate. The *let-23* gene appears to function differently in the different tissues where it acts. In the vulva, the data further suggest that *let-23* functions in two antagonistic pathways (indeed, mutations in the gene provided the opportunity to discern these different pathways), and these pathways seem to control cell-type determination and possibly also proliferation.

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Chapter III

The *let-23* gene necessary for *Caenorhabditis elegans* vulval induction encodes a tyrosine kinase of the EGF receptor subfamily.

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The *let-23* gene necessary for *Caenorhabditis elegans* vulval induction encodes a tyrosine kinase of the EGF receptor subfamily

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The *let-23* gene is required for induction of the *Caenorhabditis elegans* vulva. It is shown that *let-23* encodes a putative tyrosine kinase of the epidermal growth factor receptor subfamily. Thus, *let-23* might encode the receptor for the inductive signal required for vulval development. Because *let-23* acts upstream of *let-60 ras* in the vulval determination pathway, the identification of the *let-23* product provides support for a link *in vivo* between tyrosine kinase growth factor receptors and *ras* proteins in a pathway of cell-type determination.

DURING *Caenorhabditis elegans* vulval induction, the combined action of several intercellular signals specifies the precise pattern of fates of the six precursor cells (VPCs)[1–4]. The main determinant of this pattern is an inductive signal from the gonadal anchor cell that stimulates the nearest three VPCs to proliferate and generate vulval tissue; the remaining three cells generate nonspecialized hypodermis[5–7]. A 'lateral signal' acts between VPCs to ensure the proper pattern of cell fates[3]. Genes necessary for vulval induction have been identified by 'Vulvaless' mutations that prevent induction[4,8–13,14]. One such gene, *let-60*, encodes a *ras* protein[15]. Other genes, identified by 'multivulva' mutations, prevent vulval differentiation in the absence of inductive signal[3,4,8–11,16]. One such gene, *lin-15*, seems to act in cells other than the anchor cell or VPCs[17], suggesting the presence of a negatively acting signal from a third tissue such as the surrounding hypodermal syncytium hyp7.

The *let-23* gene, originally identified by a larval lethal mutation[18], has a pivotal role in *C. elegans* vulval determination: loss of *let-23* function leads to none of the VPCs having vulval fates, but some mutations in *let-23* result in too many VPCs having vulval fates (refs 10 and 11; and R.V.A. and P.W.S., manuscript in preparation). Here we report that *let-23* encodes a protein of the epidermal growth factor (EGF) receptor tyrosine kinase subfamily. On the basis of its molecular structure and previous genetic data, we propose that *let-23* normally functions to receive an inductive signal, presumably from the anchor cell, and acts to specify cell type. With previous data indicating that *let-23* acts upstream of the *let-60 ras* gene in the vulval determination pathway[13,15], our results provide evidence for a link *in vivo* between a growth factor receptor and *ras*.

Genetic and physical maps of *let-23* region

We localized *let-23* on the *C. elegans* physical map[19,20] by identifying physical markers that map genetically to the left and right of the gene (Fig. 1). Three-factor mapping positioned the previously identified restriction fragment length polymorphism (RFLP) *Tc5A* roughly 80 ± 70 kilobases (kb) to the left of *let-23* (see Fig. 1 legend). We found the left breakpoint of the deficiency *mnDf67*, cloned the junction fragment, and 'jumped' to the right breakpoint, which then defined a right-most boundary for *let-23* (see Fig. 1 legend). Thus, the 200-kb region of DNA between *Tc5A* and the *mnDf67* right breakpoint contains the *let-23* gene and centres around the cosmid T08E2. Three-factor mapping data with the *Tc5A* RFLP suggested that *let-23* is in the cosmid T08E2 (Fig. 1), which contains a tyrosine kinase gene (see below). Efforts to detect allele-specific RFLPs in this 200-kb region were thwarted by the presence of extensive areas of repetitive DNA (data not shown).

Isolation of a tyrosine kinase gene near *let-23*

In parallel, a wild-type *C. elegans* genomic library was screened with a 0.75-kb *Eco*RI–*Pvu*II fragment of the oncogene v-*ros*[21] encoding the tyrosine kinase domain. Six classes of hybridizing clones were obtained. The hybridizing regions in these clones were partially sequenced. A coding sequence for a putative tyrosine kinase catalytic domain was found in each of the clones, and the sequences were found to all be different (M.K. and Y.O., unpublished observations). The clones were placed on the *C. elegans* physical map. One clone, NGros213-13.3, was mapped to linkage group II near *let-23* and is completely contained in the cosmid T08E2. The cloned gene was designated *kin-7*. A restriction map of the *kin-7* gene is shown in Fig. 2, as are various constructs referred to later.

The kinase gene rescues *let-23* defects

To determine whether *let-23* and *kin-7* are the same gene, we performed germline transformation experiments (Fig. 2). Cosmid and plasmid DNA were injected into the germ line of a balanced *let-23* lethal strain to test for rescue of the *let-23* lethal phenotype. Because the *let-23* allele used also gives rise to defects in the vulva and in fertility, we could assay rescue of these phenotypes as well.

The overlapping cosmids T08E2 and ZK1052, both of which contain *kin-7*, rescue mutants from *let-23* defects, whereas the neighbouring but nonoverlapping cosmid W07A12 does not (data not shown; see Fig. 1 for cosmid locations). The subclone pK7-13.8, which contains the entire kinase gene and several kilobases on either side, rescues mutants from *let-23* defects (Fig. 2). But 5′ and 3′ deletion derivatives of pK7-13.8 (subclones pK7-5.5 and pK7-ΔTK respectively) that both truncate within the gene and share about 1 kb of overlap, fail to rescue (Fig. 2). Therefore, the rescuing activity results from the kinase gene and not another gene on either the 5′ or 3′ end of pK7-13.8. Furthermore, failure of both pK7-5.5 and pK7-ΔTK to rescue is not due to any abnormal properties of these plasmids (such as accidental point mutations), because we could achieve rescue by coinjecting the two plasmids (Fig. 2). Homologous recombination *in vivo* would allow these two plasmids to generate an intact *kin-7* gene. The plasmid pK7-11.5 does not rescue *let-23* mutants from lethality (Fig. 2). As this construct retains the entire *kin-7* coding sequence and about 1 kb of upstream sequence, sequences essential for rescue lie more than 1 kb upstream of the initiator methionine codon. The plasmid NGros213-13.3 (Fig. 2), which deletes the 3′ end of pK7-13.8, including about 600 base pairs (bp) of C-terminal complemen-

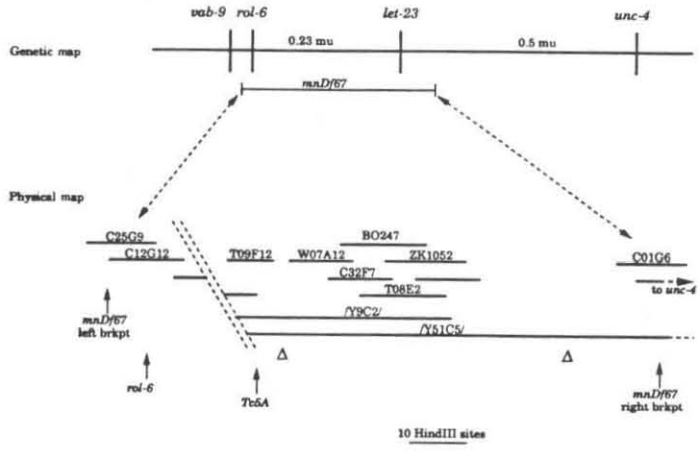


FIG. 1 Genetic and physical maps of the *let-23* region. A continuous stretch of cosmid DNA extends from the cosmid containing *rol-6* to the cosmid T09F12 (data from the *C. elegans* physical map[19,20] and A. Coulson, personal communication). Between T09F12 and *unc-4* are numerous gaps in cosmid DNA that are spanned by yeast artificial chromosome clones (YACs)[20]: T08E2, cosmid T08E2; Δ, location in gaps in cosmid stretches; /Y9C2/, YAC Y9C2; brkpt, break-point; arrows point to location of cosmid that contains stated molecular markers; mu, map units.

METHODS. (1) Tc5A three-factor mapping: We mapped the previously identified Bergerac/Bristol polymorphism, *Tc5A* (J. Park, personal communication), relative to *rol-6* and the *let-23* recessive lethal allele *sy15* (R.V.A. and P.W.S., manuscript in preparation; for details of mapping with Bergerac-specific polymorphisms, see ref. 44). We constructed the strain *rol-6(e187)let-23(sy15)/ vab-9(e1744) Tc5A* from the strain *vab-9(e1744) Tc5A rol-1(e91)* (provided by A. Papp; *rol-1* maps to the right of *unc-4*). The *rol-6 let-23* chromosome is from the Bristol strain and lacks the Bergerac-specific polymorphism *Tc5A*. The *vab-9* chromosome has DNA sequences between *vab-9* and *unc-4* derived from the Bergerac strain and contains the *Tc5A* polymorphism (a novel 3.2-kb *Eco*RI fragment that appears when a genomic Southern blot is probed with the T09F12 subclone pAH1). We selected 20 Roller (Rol) non-lethal recombinants, made the recombinant *rol-6* chromosome homozygous, and isolated genomic DNA from these recombinants using standard protocols[45]. We digested the DNAs with *Eco*RI, ran them on a 1% Tris–borate-EDTA polyacrylamide gel, blotted the gel onto a nylon membrane, and probed the blot with plasmid pAH1. Sixteen recombinants displayed the Bergerac pattern, indicating that *let-23* is to the right of *Tc5A* and is 1/4 (4/16) the distance from *rol-6* to *Tc5A*. Because the interval between *rol-6 and Tc5A* contains 113 *Hin*dIII sites (the metric used in the *C. elegans* physical map data base), our three-factor data suggests *let-23* is 28 ± 25 *Hin*dIII sites (assuming the average *C. elegans* G–C content of 36%, one *Hin*dIII site occurs about every 3,000 base pairs). To locate *let-23* on the molecular map, we estimated the size of the gap in the cosmid map to the right of the cosmid T09F12. We sized the YAC Y9C2 that spans this gap on a pulsed field gel[46] as 180 kb, and, subtracting for the amount of cosmid DNA covered by this YAC, estimated the gap to be about 10 kb or 3 *Hin*dIII sites. The next gap to the right was similarly estimated to be 24 *Hin*dIII sites by sizing the YAC Y51C5. Our best estimate of 28 *Hin*dIII sites from *Tc5A* therefore positioned *let-23* in the cosmid T08E2. (2) *mnDf67* breakpoint mapping: To define a marker on the right side of *let-23*, we mapped the right breakpoint of the deficiency *mnDf67* (ref. 47) that lies to the right of the *let-23* gene. The left breakpoint of this deficiency lies between two cloned markers, *rol-6* (ref. 48) and a candidate for *vab-9* (J. Kramer, personal communication). DNA from a cosmid, C25G9, detected a novel 10-kb *Sal*I fragment in DNA isolated from animals heterozygous for *mnDf67*. This fragment was cloned from a size-selected λ phage library of *mnDf67* DNA and was used to probe the *C. elegans* physical map. In addition to hybridizing to the cosmid C25G9, the *mnDf67* junction fragment hybridized to the cosmid C01G6 which lies to the right of Tc5A and left of *unc-4*.

tary DNA sequence, but retains the kinase domain and 200 bp downstream, rescues *let-23* lethality, suggesting, together with the pK7-ΔTK results described above, that the kinase domain is important for rescue. Our conclusion that *let-23* is *kin-7* was confirmed by the localization and sequencing of a point mutation associated with a *let-23* null allele in the region corresponding to the kinase domain (see below).

Wild-type mRNA and cDNA Clones

A 4.9-kb strong band and a 3.5-kb faint band were detected on northern blot hybridizations (Fig. 3) of poly(A)$^+$ RNA prepared from a mixed population of wild-type N2 strain probed with a 2.0-kb nonrepetitive *let-23* genomic fragment (Fig. 2). The 4.9-kb major band corresponds to the *let-23* cDNA (see below). The faint band could represent a cleavage product of the 4.9-kb messenger RNA, a minor form of the *let-23* transcript or a transcript of another kinase gene with similarity to *let-23*.

Roughly 7×10^5 plaques of a cDNA library from mRNA of a mixed population of *C. elegans* were screened with the 2.0-kb *let-23* genomic fragment. Only one clone, Cros331, was obtained. This clone had a 2-kb cDNA for the C-terminal half of *let-23*. To obtain the entire coding sequence, 13 oligonucleotide primers were prepared, based on the genomic sequence and its predicted exon–intron structure. Poly(A)$^+$ RNAs were reverse-transcribed using oligo 1 and oligo 5 as the primers (Fig. 4), and amplified by PCR using oligonucleotide primers. The amplified sequences were cloned into plasmids. Because all the primer pairs lay across at least one intron, the possibility of amplifying genomic DNA could be excluded.

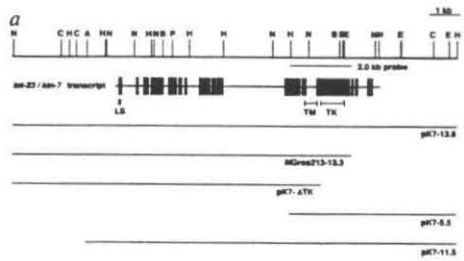
The assembled *let-23* cDNA sequence contains a single open reading frame that encodes 1,323 amino acids with ATG (position 80) as the initiation codon (Fig. 4). There is a poly(A) tail at the 3′ end of the cDNA. This cDNA seems to lack a few hundred bases corresponding to the 5′ end of the mRNA, because it is 4.3-kb long without poly(A) and the mRNA size was estimated to be 4.9 kb. The entire coding sequence, however, is covered as there is an in-frame stop codon (position 22) upstream of the putative initiator methionine codon.

The *let-23* allele *sy5*, which behaves genetically like a null mutation and is a homozygous larval lethal (R.V.A. and P.W.S., manuscript in preparation), has a point mutation in the region corresponding to the conserved kinase subdomain IX (ref. 22), which changes the TGG codon encoding Trp 1,078 to an opal codon (TGA) (Fig. 4). This mutation would result in a truncated protein lacking some of kinase subdomain IX and all C-terminal sequences. That this allele results in a null phenotype is consistent with studies of tyrosine kinases that suggest that the C terminus of the catalytic domain resides close to, but downstream of, the conserved arginine in subdomain XI (ref. 22). Furthermore, such a truncation lacks rescuing activity: injection of plasmid pK7-13.8 digested by *Sal*I (*Sal*I cuts once in genomic sequence; see Fig. 2) to give a kinase gene deleted for kinase subdomains X and XI and all sequences 3′ of this, fails to rescue (data not shown).

let-23 is a member of the EGF receptor subfamily

The predicted product of the *let-23* gene has several remarkable primary structures: two hydrophobic stretches, a putative tyrosine kinase domain, and two cysteine-rich motifs. The general architecture (Fig. 5) is the same as that of human EGF receptor[23], *Drosophila* EGF receptor[24] (abbreviated DER), and the *Xiphorphorus Tu* locus[25] (not shown).

One of the two hydrophobic stretches follows the initiator methionine and a basic residue (Arg) and seems to be a leader sequence for insertion into the membrane[26]. The other hydrophobic stretch, which lies in the middle, is most probably a



a

b

A Rescue?	B No. rescued animals	C No. control transformants	D No. stable lines showing rescue
Yes	38	92	7/7
Yes	*	*	2/2
No	0	70	0/6
No	0	133	0/5
No	0	37	0/2
Yes	5	39	1/1

FIG. 2 Genomic organization and germline transformation. *a*, Restriction map of the *kin-7* genomic region is shown at the top. Location of the *let-23/kin-7* transcript (cDNA) is shown in the next row down, with amino-acid-encoding regions shown as solid boxes. The 5′ end of the transcript is not determined. Locations of the genomic regions in the subclones used are depicted below. LS, leader sequence; TM, transmembrane domain (interrupted by an intron); TK, tyrosine kinase catalytic domain. Restriction enzyme sites: *N*, *Nsi*I, C, *Sac*I, H, *Hin*dIII, A, *Apa*I, B, *Bam*HI, P, *Pst*I, S, *Sal*I; E, *Eco*RI. *b*, Results of germ-line transformation experiments for each plasmid. The bottom row are the results for coinjection of pK7-ΔTK and pK7-5.5 (see text and below). Column A: 'Yes', given plasmid rescues the lethality associated with a *let-23* null allele; 'No', plasmid does not rescue. Column B: number of animals rescued in the first generation (F1) after injection. Column C: number of animals showing expression of a coinjected dominant marker and serves as a control for successful injection. Column D: number of stably transformed lines displaying rescue relative to the total number of stably transformed lines tested. Concentrations of plasmid DNAs (ng μl^{-1}): 50, 35, 18, 50 and 80 + 40 for pK7-13.8, pK7-ΔTK, pK7-5.5, pK7-11.5 and pK7-ΔTK + pK7-5.5, respectively. All plasmids were used at nearly uniform molarity except for pK7-ΔTK and pK7-5.5, which were coinjected at about twice the relative molarity of the others. Our results are insensitive to *let-23* dosage: we have separately injected pK7-ΔTK and pK7-5.5 at 50 ng μl^{-1} and pK7-13.8 at 10 ng μl^{-1} and 250 ng μl^{-1} with no change in results. We have also coinjected pK7-ΔTK and pK7-5.5 at 40 and 20 ng μl^{-1} respectively and found F1 rescue (two Rol Unc animals). Asterisk denotes that rescued F1 animals were found, but total numbers of rescued and control animals were not determined.

METHODS. Genomic clones: 3.9×10^4 plaques of an EMBL4 library constructed from an *Mbo*I partial digest of *C. elegans* N2 genomic DNA (gift from C. Link) were screened with the 0.75-kb *Eco*RI–*Pvu*II fragment of p-*ros*-1, a plasmid clone (gift from K. Shimizu) of the v-*ros* oncogene[23] at a low-stringency hybridization. Nylon membranes (Biodyne A) were prehybridized at 42 °C for 3 h in 20% formamide, 6 × SSC, 5 × Denhardt's solution, 0.2% SDS, 500 μg ml^{-1} of salmon sperm DNA. About 0.5 μg ^{32}P-labelled probe DNA (specific activity $>1 \times 10^8$ c.p.m. μg^{-1}) was added and hybridization was performed under the same condition for about 24 h. The filters were washed at 42 °C for 3 h with 3 × SSC/0.1% SDS, and exposed to an X-ray film (Kodak X-OMAT AR) for 24–40 h with an intensifying screen. The 11.5-kb *Eco*RI insert DNA of NGros113, a phage clone carrying *kin-7* gene, and 5.5-kb *Hin*dIII fragment of a cosmid clone B0247 (Fig. 1) (gift from J. Sulston and A. Coulson) were subcloned into plasmid pBluescript SK(+) to make NGros213-13.3 and pK7-5.5, respectively. These two plasmids were the sources of *kin-7* gene clones for later experiments. Germ-line transformation: We injected plasmids/clones into the strain *let-23(mn23) unc-4(e120)/mnC1[dpy-10(e128) unc-52(e444)]*. The *let-23* allele *mn23* (ref. 17) results in 100% penetrant larval lethality and behaves like a genetic null (R.V.A. and P.W.S., manuscript in preparation). The balancer *mnC1* inhibits recombination in the region around *let-23* (ref. 18). We injected into the distal arm of the gonad syncytium of 1- to 2-day-old adult hermaphrodites according to the method of C. Mello, V. Ambros, J. Kramer and D. Stinchcomb (manuscript in preparation). The plasmid DNA of interest was coinjected with a plasmid (pRF4, courtesy of J. Kramer) bearing a dominant *rol-6* mutation (*rol-6(d)*; concentration of pRF4, 50 ng μl^{-1} for all experiments). Expression of pRF4 in an otherwise wild-type worm results in a Roller (Rol) phenotype. Appearance of F1 Rol progeny after injection therefore indicates successful injection and expression of injected DNA. Previous experiments have shown that homologous, coinjected plasmid DNAs form large linear arrays containing both DNAs (C. Mello, personal communication). All plasmids used in our experiments contain large regions with homology to pRF4 owing to the presence of vector sequences. About 10% of F1 Rol animals could stably transmit the arrays to some of their progeny, thereby resulting in some F2 Rol animals (and therefore F3 and so on). If a plasmid rescues *let-23* lethality, then these stable lines will segregate viable Unc-4 (abbreviated Unc) animals, which if not suppressed would die as young larvae owing to the presence of the linked *let-23(mn23)* mutation. The number of stable lines showing rescue out of the total number of stable lines generated for a given experiment is displayed in column D. In all cases where we had stable lines but no rescue, we verified the presence of the *unc-4* chromosome by mating Rol animals with *rol-6(e187) unc-4(e120)/mnC1* males and looking for Unc progeny. This confirms that the failure to see Unc animals was not due to a rare recombination event that eliminated the *unc-4* marker. Rescued Unc animals are often Rol, indicating that the plasmid and *rol-6(d)* DNA are coexpressed; sometimes they are not. Nonetheless, in the next generation (if fertile) some Rol animals always segregate, suggesting that the *rol-6(d)* DNA is present in these non-Rol Unc animals but that the expressivity of the *rol-6(d)* phenotype is weaker than *let-23* rescue. In extensive negative control experiments with DNA from other chromosomes, stable Rol lines never segregate Unc or Rol Unc worms, consistent with lack of *let-23* rescuing activity. Rescued Unc animals are often sterile, indicating that the lethality, but not the sterility, associated with loss of *let-23* function is rescued (R.V.A. and P.W.S., manuscript in preparation). This partial rescue could be due to requirements for higher dosage for overcoming *let-23* sterility and/or mosaicism of the array in a given animal: the array may be lost in lineages where it is required to rescue sterility. Fertile Rol Unc and Unc animals are often capable of laying eggs, indicating rescue of the *let-23* vulval defect. The presence of F1 Rol Unc and Unc animals from injected mothers is an even more sensitive assay than segregation of stable Rol lines for *let-23* rescuing activity. In this assay, *rol-6(d)* DNA is a control for successful injection and the presence or absence of Unc animals indicates rescue or lack of rescue of *let-23* lethality. The number of Unc animals (Rol and not Rol) is given in column B; the number of Rol (not Unc) animals is given in column C). There is perfect correlation between the two assays: DNA which results in F1 Unc and Rol Unc animals also gives rise to stable Rol lines which segregate Unc and Rol Unc animals, and DNA which does not result in F1 Unc and Rol Unc animals gives rise to stable Rol lines which do not segregate Unc and Rol Unc animals. Rescue in the pK7-ΔTK and pK7-5.5 coinjection experiments (at both concentrations) is less efficient than rescue by pK7-13.8, but this is not unreasonable given that rescue requires *in vivo* recombination between the two plasmids to create a functional gene. Although the two plasmids overlap by about 1 kb within the kinase gene, they also share about 3 kb of identical vector sequence; homologous recombination in this region would not reconstruct a functional kinase. Three of the five F1 Unc animals in the coinjection experiment shown were also Rol, indicating the presence and expressivity of injected DNA. Lastly, we have also rescued the lethal allele *mn216* (ref. 47) and the Vulvaless allele *sy97* (R.V.A. and P.W.S., manuscript in preparation) (data not shown).

transmembrane domain because its 23 amino acids have a hydrophobicity index[27] of 2.6 and make it long enough to span a membrane; also, this sequence is flanked by charged residues.

Figure 6*a* shows the alignment of the kinase domains of the *let-23* product and representative tyrosine kinases of five subfamilies[22]. The *let-23* domain shows highest similarity (44.0% identity) to the human EGF receptor. It also shares 40.6% identity with DER, 31.6% with the human cellular *src* product, 32.0% with the human cellular *abl* product, 28.6% with the human insulin receptor, and 32.0% with the mouse PDGF receptor. The *let-23* domain has 36 of 39 consensus amino-acid residues thought to be involved in specific aspects of kinase activity[22]. For example, the invariant lysine in subdomain II seems to be directly involved in the phosphotransfer reaction[28]. The *let-23* putative kinase is likely to be tyrosine-specific as subdomains VI(DLATRN in the single-letter amino-acid code) and VIII(AIKWLAIE) are more similar to the tyrosine kinase consensus (DLAARN or DLRAAN in subdomain VI and PI/VK/RWT/MAPE in VIII) than to the serine/threonine kinase consensus (PLKPEN in VI and GT/SXXY/FAPE in VIII)[22]. But the *let-23* domain has no tyrosine residues within 20 residues upstream of AIE in subdomain VIII, whereas many tyrosine kinases have an autophosphorylation site in this region[29].

The alignment of the extracellular domain of the *let-23* product with those of human and *Drosophila* EGF receptors is shown in Fig. 6*b* with respect to cysteine residues and the amino-acid spacings between them. There are two cysteine-rich motifs (see also Fig. 5) where cysteine residues occupy identical positions with interspersion of other amino acids. The amino-acid identity between the *let-23* product and DER or the human EGF receptor is 33.2% or 28.8% in motif I, and 33.9% or 35.2% in motif II-1. Cysteine-rich motif II-2 is found in the *let-23* product and DER (22.0% identity) but not in the human EGF receptor. There is also limited similarity in the presumed ligand-binding domain located between cysteine-rich motifs I and II-1 (26.6% identity

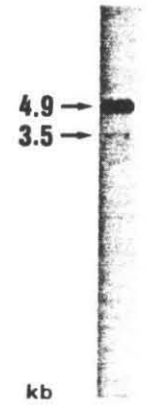


FIG. 3 Northern blot hybridization.
METHODS. Poly(A)$^+$ RNA (10 μg) from a mixed population of wild-type strain N2 was analysed on 1.0% agarose–formaldehyde gel. The sizes of RNA were estimated by comparison with the contaminating 28S and 18S ribosomal RNA. A mixed population of *C. elegans* (N2) was cultured in a liquid medium[45]. From these worms, poly(A)$^+$ RNA was prepared by the guanidinium–CsCl method and one cycle of chromatography on oligo(dT)–cellulose[49]. Poly(A)$^+$ RNA (10 μg) was size-fractionated on a 1.0% agarose–formaldehyde gel and transferred to nylon membrane filter (Biodyne A). The filter was hybridized at 42 °C in 50% formamide, 4xSSC, 0.1% SDS, 1xDenhardt's solution and 200 μg ml^{-1} salmon sperm DNA overnight, and washed with 2xSSC and 0.1% SDS for 1 h. An exposure to X-ray film was performed for 72 h with an intensifying screen.

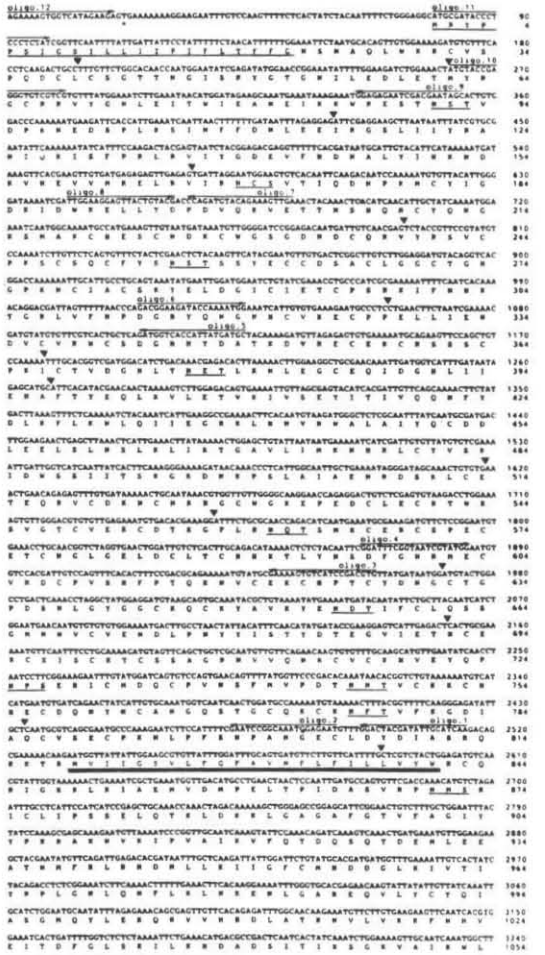


FIG. 4 Nucleotide sequence of the wild-type cDNA and the deduced amino-acid sequence (single-letter code). Several structural features are underlined: putative leader sequence by a heavy line; putative transmembrane domain by a solid bar; consensus sequence for *N*-linked glycosylation by a thin line. Arrows above the sequence show the position and orientation (5′ to 3′) of oligonucleotide primers for PCR amplification. The positions of intron insertions are marked by solid triangles. The position of the *sy5* point mutation is at W1,078 (see text). Asterisks, stop codons.
METHODS. Cloning of cDNA and PCR amplification: A cDNA clone was isolated from a λ ZAP library[50] with the 2.0-kb *kin-7* genomic fragment as the probe (Fig. 2). Cros331, a recombinant pBluescript SK(−) plasmid was excised from the phage cDNA clone using protocols supplied by Stratagene. For PCR amplification of mRNA sequence, we used oligos 2 and 4, 3 and 6, 5 and 8, 7 and 10, 9 and 11, 9 and 12, respectively, as the primers. Poly(A)$^+$ RNA (5 μg) and 0.5 pmol of an oligonucleotide primer in 36.8 μl water were heated at 65 °C for 5 min, and left at room temperature until cooled. Two microlitres of 10xRTC buffer (0.5 M Tris–HCl, pH 8.3 at 42 °C, 0.5 M NaCl, 80 mM $MgCl_2$, 5 mM EDTA, 5 mM dithiothretiol), 5 μl of 10 mM dNTP, 0.25 μl (50 U) RNasin, and 2 μl (39 U) of avian myeloblastosis virus reverse transcriptase (Life Sciences) were added to the mixture, which was then incubated for 2 h at 42 °C. The nucleic acids were twice precipitated in ethanol under 2.14 M ammonium acetate, and then dissolved in 20 μl water. An aliquot (1 μl) of the cDNA and a pair of primers (20 pmol each) in 84.5 μl of water were boiled for 2 min, quenched on ice, mixed with 10 μl of 10xPCR buffer (0.5 M Tris–HCl, pH 8.3 at 20 °C, 15 mM $MgCl_2$, 0.25 M KCl, 0.5% Tween-20, 1 mg per ml of BSA), 5 μl of 1 mM dNTP and 0.5 μl (2.5 U) *Taq* DNA polymerase (Promega), and overlaid with 30 μl mineral oil. Thirty cycles of amplification (94 °C, 0.5 min; 50 °C, 1 min; 70 °C, 2 min) were carried out and followed by a 10-min final extension at 70 °C. The amplified cDNAs were cloned into the *Eco*RV site of plasmid pBluescript SK(+). Sequencing: The sequence from 1 to 2,391 is mainly based on the genomic sequence, because PCR often produces base substitutions. Three substitutions, A(751) to G, G(1,351) to A, and T(2,166) to C were probably due to an error in PCR. The sequence from 2,392 to 4,340 is that of the original cDNA clone Cros331. Exon sequences of the genomic DNA of Ngros213-13.3 and Cros331 cDNA were sequenced completely on both strands. PCR-amplified and cloned cDNAs and genomic DNA derived from pK7-5.5 corresponding to the C terminus were sequenced on one or both strands mainly to identify exon–intron boundaries. DNAs were subcloned into pBluescript, and deleted templates for sequencing were generated by the exonuclease III sequential digestion method[51]. Sequenase (USB) and dGTP were used principally in the sequencing reactions. For several templates, combinations of sequenase and dITP or *Taq* DNA polymerase (Promega) and 7-deaza-dGTP were used to avoid compression and pausing artefacts due to secondary structures.

Localization of the *sy5* mutation: We localized the *sy5* point mutation by hydroxylamine mismatch detection[52] (R. Barstead, personal communication). We PCR-amplified 750 bp of the kinase domain from homozygous wild-type and *sy5* genomic DNA (isolated from dead larvae), hybridized the mutant and wild-type DNAs, subjected the hybrids to hydroxylamine and piperidine reactions, and gel-electrophoresed the reaction. A 170-bp cleavage fragment was detected in three independent PCR reactions, and the PCR product was sequenced from a gel slice without subcloning (D. Nickerson, personal communication). The location of the *sy5* mutation is consistent with the 170 bp fragment generated.

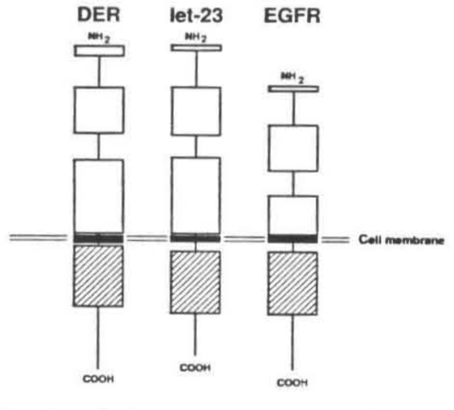


FIG. 5 Comparison of schematic structures of *let-23* with human and *Drosophila* EGF Receptors. Cysteine-rich regions, open boxes; transmembrane domains, solid boxes; tyrosine kinase catalytic domains, hatched boxes; leader sequences, dotted boxes. There are three strongly homologous regions (see text and Fig. 6 for details).

with DER and 28.7% with the human EGF receptor). We conclude that *let-23* is a *C. elegans* member of the EGF receptor subfamily.

let-23 may encode the inductive-signal receptor

As mentioned above, the *let-23* gene is necessary for the induction of VPCs by the gonadal anchor cell and is thought to have a pivotal role in vulval determination. Furthermore, our analysis suggests that *let-23* does not act in the anchor cell, because a mutation that severely reduces *let-23* activity is epistatic to the gonad-independent multivulva mutation *lin-15(n309)* (R.V.A. and P.W.S., manuscript in preparation). That is, a lack of (or strong reduction in) *let-23* activity is not equivalent to a lack of the anchor-cell signal in a *lin-15(n309)* background, and we infer that *let-23* does not act in the anchor cell. Although *let-23* could act in other cells (see Fig. 7 legend), the simplest interpretation is that *let-23* is required for VPCs to respond to the anchor-cell signal.

On the basis of this genetic information and the molecular structure of the gene product, we propose that *let-23* encodes the receptor for the vulval inductive signal. In various tissue culture systems, EGF receptor elicits a mitogenic response in epithelial cells after binding of ligand (EGF or transforming growth factor-α (TGF-α))[30,31]. We propose that the *let-23* product binds the anchor-cell signal molecule and subsequent transduction of that signal is required for VPCs to adopt vulval fates. Another receptor-like molecule acting during vulval development, the *lin-12* protein, has EGF-like (that is, ligand) repeats in its putative extracellular domain[32,33]. But the phenotypes and genetic properties of *lin-12* mutants suggest that the *lin-12* product is unlikely to be a receptor for the inductive signal, although it might be a receptor for a signal acting between VPCs[4,34].

let-23 acts by way of *let-60 ras*

Genetic analyses have enabled the identification and ordering of the actions of many genes involved in *C. elegans* vulval determination[4,11,13]. The finding that *let-23* encodes a growth factor receptor tyrosine kinase is relevant to the identification of another component of the vulval induction pathway, the *let-60* gene, which encodes a *ras* protein[15]. Because increased activity and increased dosage of *let-60 ras* suppresses *let-23* loss-of-function vulval defects, *let-60 ras* is believed to act downstream of *let-23* in the vulval determination pathway[13,15]. Also, loss of

a

```
        Subdomain      I                              II               III              IV
                     ** *  * *                      ** *               *    *            +
let-23  (883) LQTKLDKKLGAGAFGTVFAGIYYPKRAKNVKIPVAIKVPQTDQSQTD--EMLEEATNMFRL-RHDNLLKIIGFCMHDDGL
EGFR    (686) TEF.KI.V..S......YK.LWI.EGE.-........ELREAT.PKANK.I.D..YV.ASV-DNPHVCRLL.I.LT-STV
DER     (857) AELRKGGV..M....R.YK.VWV.EGEN-........ELLKSTGAESSE.F.RE.YI.ASE-E.V....LLAV..S-SQM
SRC     (265) ESLR.EV...Q.C..E.WM.TWNG------TTR....TLKPGTM--SPEAF.Q..QV.KK.-..EK.VQLYAVVSE-EPI
Abl     (240) TDITMKH...G.QY.E.YE.VWKK-----YSLT..V.TLKE.TM--EVE.F.K..AV.KEI-K.P..VQLL.V.TREPPF
INSR    (982) EKIT.LRE..Q.S..M.YE.NARDIIKGEAETR..V.TVNESA.LRERI.F.N..SV.KGF-TCHHVVRLL.VVSKGQPT
PDGFR   (566) D.LVIGRT..S....Q.VEATAHGLSHSQATMK..V.MLKSTARSSEKQALMS.LKI.SH.GP.L.VVNLL.A.TKGGPI

        Subdomain      V                                          VI                    VII
                      * *                                       *     **** + *          ***
let-23        KIVTIYRPLGNLQNFLKLHKEN-------LGAREQVLYCYQIASGMQYLEKQRVVHRDLATRNVLVKKFNHVEITDFGLS
EGFR          QLI.QLM.F.C.LDYVRE..D.-------I.SQYLLNW.V...K..N...DR.L......A......TPQ..K......A
DER           MLI.QLM...C.LDYVRNNRDK-------I.SKALLNWST...K..S...EK.L......A.....RLL-AG.DH....A
SRC           Y...E.MSK.S.LD..KGETGKY------.RLPQL.DMAA......A.V.RMNY....RAA.I..GENLVCKVA....A
Abl           Y.I.EFMTY...LDY.RECNRQE-----VN.VVLLYMAT..S.A.E....KNFI.....A..C..GENHL.KVA.....
INSR          LV.MELMAH.D.KSY.RSLRPEA(-8-) PTLQEMIQMAAE..D..A..NAKKF......A..CM.AHDFT.K.G...MT
PDGFR         Y.I.E.CRY.D.VDY.HRN.HTP(-97-).SYTDL.GFS..V.N..DF.ASKNC......A....ICEGKL.K.C....A

        Subdomain          VIII                   IX                       X
                          +  * * *          ** * *    *       *                         *
let-23        KILKHDADSITIKSGKVAIKWLAIEIFSKNCYTHASDVWAFGVTCWEIITFGQSPYQG-MSTDSIHNFLKDGNRLSQPPNC
EGFR          .L.GAEEKEYHAEG...P...M.L.SILHRI...Q....SY...V..LM...SK..D.-IPASE.SSI.EK.E..P...I.
DER           .L.SS.SNEYKAAG..MP.....L.CIRNRVF.SK.........I..LL....R.HEN-IPAKD.PDLIEV.LK.E..EI.
SRC           RLIEDNEYTAR-QGA.FP...T.P.AALYGRF.IK....S..ILLT.LT.K.RV..P.-.VNREVLDQVER.Y.MPC..E.
Abl           RLMTG.TYTAH-AGA.FP...T.P.SLAYNKFSIK.......LL...A.Y.MS..P.-IDPSQVYEL.EKDY.MKR.EG.
INSR          RDIYETDYYRKGGK.LLPVR.M.P.SLKDGVF.TS..M.S...VL...TSLAEQ....-L.NEQVLK.VM..GY.D..D..
PDGFR         RDIMRDSNY.SKG.TYLPL..M.P.SIFWSL..TL....S..ILL...F.L.GT..PELPMN.QFY.AI.R.Y.MA..AHA

         Subdomain      XI
                     **     ** *
let-23        SQDLYQELLRCWMADPKSRPGFEILYERFKEFCKVP (1148)
EGFR          TI.V.MIMVK...I.AD...K.RE.IIE.SKMARD. ( 951)
DER           .L.I.CT..S..HL.AAM..T.KQ.TTV.A..ARD. (1121)
SRC           PES.HDLMCQ..RKEPEE..T..Y.QAFLEDYFTST ( 523)
Abl           PEKV.ELMRA..QWN.SD..S.AEIHQA.ETMFQES ( 500)
INSR          PERVTDLMRM..QFN.NM..T.LEIVNLL.DDLHPS (1258)
PDGFR         .DEI.EIMQK..EEKFET..P.SQ.VLLLERLLGEG ( 933)
```

b

```
                                       Cysteine-rich motif I
let-23   31C5C1C25C114   C28C9C3C3C7C7C3C2C10CC3C3C7C2C8C3C26C3C10C3C14C2C4C3C16C   66C27
DER          52C26C98    C29C2C3C3C7C7C3C4C7 CC3C3C7C2C12 C26C3C10C3C10C2C3C3C16C   116
EGFR      18C11C26C98    C29C2C3C4C7C7C3C3C7 CC3C3C7C2C8C3C26C3C11C3C14C2C3C3C24C   107

              Cysteine-rich motif II-1
let-23   C32C6C3C4C7C2C8C3C13C2C3C2C7C2C15C3C11C2C3C4C11C2
DER      C28C6C3C4C7C2C8C3C13C2C3C2C7C2C8 C3C10C2C3C2C11C2
EGFR     C28C6C3C4C7C2C8C3C15C2C3C8C7C2C8 C3C21C2C3C3C7 C6     MB

              Cysteine-rich motif II-2
let-23   C12C9C22C2C3C2C11C1C13C3C14C2C3C5C7C2C12C3C13C13      MB
DER      C13C6C25C2C3C2C9 C2C8 C3C12C6  C2C7C2C21C3C17C9       MB
```

FIG. 6 Comparison of amino-acid sequences (single-letter code) of *let-23* and other tyrosine kinases. *a*, Alignment of the amino-acid sequence of the putative tyrosine kinase catalytic domain of *let-23* and those of six other tyrosine kinases: EGFR, human EGF receptor; DER, *Drosophila* EGF receptor[24,53]; SRC, human cellular homologue of the oncogene product from Rous sarcoma virus[54]; Abl, human cellular homologue of the oncogene product from Abelson murine leukaemia virus[55]; INSR, human insulin receptor[56]; PDGFR, mouse platelet-derived growth factor receptor[57]. Asterisks, invariant residues; +, invariant except *let-23*; dots, identical to *let-23*; dashes, space for the alignment. *b*, Alignment of *let-23*, DER and EGFR extracellular cysteines. Numbers indicate the number of amino acids between cysteines. Regions with identical amino-acid spacing between cysteines among two or three of the kinases are boxed. MB, membrane.

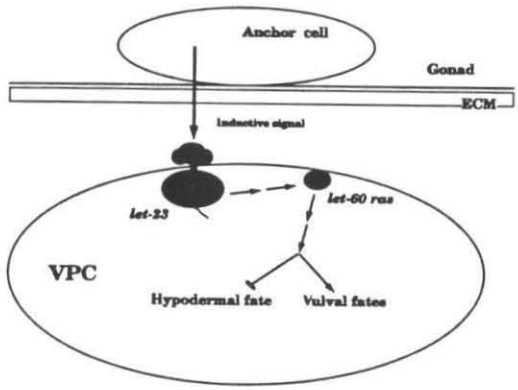


FIG. 7 Hypothetical function for *let-23* and *let-60 ras* during vulval induction. We envisage that *let-23* is embedded in the plasma membrane of the VPCs and that *let-60 ras* is associated with the membrane. The *let-23* receptor binds the inductive signal, which apparently diffuses from the anchor cell[6,7], and subsequently activates *let-60 ras*. After activation by *let-23*, *let-60 ras* causes VPCs to execute vulval fates instead of hypodermal fates[13,15]. ECM, extracellular matrix. (Evidence suggests that *lin-15* acts in cells other than VPCs or anchor cell[17]. Therefore *let-23* and/or *let-60 ras* might operate in cells other than VPCs. Nonetheless, all data so far are compatible with *let-23* and *let-60 ras* acting in the VPCs and *lin-15* acting in other cells to prevent the action of *let-23*/*let-60* in some VPCs by way of an intercellular signal.)

let-60 ras function leads to the same phenotype as loss of *let-23* function, that is, a complete lack of vulval induction.

Several studies have suggested a connection between *ras* proteins and tyrosine kinase growth factor receptors. The effects of serum, EGF and platelet-derived growth factor (PDGF) on cell growth are inhibited by injection of monoclonal antibodies directed against *ras* proteins[35] and the infection of cell lines with a Ki-*ras* oncogene abrogates growth requirements for EGF[36]. A biochemical link between growth factor receptors and *ras* proteins has been suggested by studies of the GTPase activating protein GAP[37]. GAP associates with and is phosphorylated by the receptor for PDGF[38,39], and phosphorylation of GAP can be stimulated by EGF in a cell line overexpressing EGF receptor[40]. Furthermore, GAP has been implicated in regulating *ras* proteins because it catalyses the conversion of Ras-GTP to Ras-GDP[41,42] and can inhibit morphological transformation by normal Ha-*ras*[43]. So it has been hypothesized that GAP links growth factor receptors with *ras* proteins. These studies are consistent with genetic studies (see above) that conclude that the chief effect of the *let-23* kinase is exerted through *let-60 ras*.

Our model for *let-23* and *let-60 ras* action during vulval induction is summarized in Fig. 7. Genetic analysis of the vulval pathway in combination with the molecular characterization of two of the genes involved has provided support for a link *in vivo* between a protein of the growth factor receptor tyrosine kinase family, *let-23*, with a *ras* protein, *let-60*. Because mutations in *let-60 ras* lead to similar lethal and male tail phenotypes as *let-23* mutations, and because an increase in *let-60* activity suppresses *let-23* lethality (ref. 13 and R.V.A. and P.W.S., manuscript in preparation), the proteins encoded by these two genes most probably operate together in more than one *C. elegans* developmental pathway. □

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Chapter IV

Localization and sequencing of *let-23* point mutations: structure/function analysis of an EGF receptor-like gene

Introduction

Mutations in the *let-23* epidermal growth factor receptor (EGFR)-like gene can be classified into four groups: null alleles, hypomorphic alleles, tissue-specific alleles, and Hin alleles (Aroian and Sternberg, 1991). The largest group of alleles (14/20) result in early larval lethality and behave like they eliminate *let-23* activity. Three subviable alleles, *n1045*, *sy10*, and *sy12*, behave like they reduce *let-23* function for all tissues where *let-23* is known to act. Three other alleles, *mn224*, *sy1*, and *sy97* behave like they reduce *let-23* function in specific tissues only. Finally, two of the alleles already mentioned, *n1045* and *mn224*, also appear to be defective in a vulval inhibitory pathway. These mutations can give rise to the hyperinduced or Hin phenotype in the vulva.

Although EGFR-like genes and receptor tyrosine kinases have been the subject of numerous studies, only limited, and sometimes contradictory, information exists about how alterations in EGFR might give rise to the above classes of mutations. Apart from changes in the tyrosine kinase domain (see, for example, Honegger *et al.*, 1987; Raz *et al.*, 1991), it is not clear what specific alterations might result in a null allele. To my knowledge, hypomorphic alleles of EGFR have not been characterized. Tissue-specificity has been the subject of a few EGFR studies with mixed results. One study suggested that the C-terminus provided tissue-specific oncogenic capacity, although this result depended on using a cell type that does not normally express EGFR (Khazaie *et al.*, 1988). Another study suggested that tissue-specific oncogenic capacity resided in the kinase domain (DiFiore *et al.*, 1990a). Alterations, such as C-terminal truncations

or mutations in autophosphorylation sites, can result in a gain of function phenotype parallel to the Hin phenotype. However, results and interpretations from similar experiments contradict each other, and the exact role of the C-terminus and autophosphorylation sites are unclear (for examples, see Discussion sections in Akiyama *et al.*, 1991; Margolis *et al.*, 1990; Felder *et al.*, 1990).

The development of the EGFR field has been influenced by an emphasis of EGFR as an oncogene and by the difficulty of dissecting the structure/function relationships of a large, multifunctional protein in vertebrate systems. As a consequence, many of the vertebrate EGFR structure/functional studies have concentrated either on very broad regions [*e.g.*, defining the ligand binding domain or the effects of N-terminal and C-terminal truncations (Lax *et al.*, 1989; Khazaie *et al.*, 1988; DiFiore *et al.*, 1990b)] or on a very narrow range of residues [e.g., autophosphorylation sites, Thr654, and Val664 (Honegger *et al.*, 1988; Schlessinger, 1988; Weiner *et al.*, 1989; Akiyama *et al.*, 1991). Attempts at making novel mutations, even obvious ones, have not always proven successful. For example, significant alterations in the transmembrane domain, such as deleting ten amino acids or introducing prolines, still result in a functional EGFR (Carpenter *et al.*, 1991).

Genetically tractable organisms, like *C. elegans* and *Drosophila*, can potentially provide valuable insights into how EGFR-like molecules function because: (1) it is possible to isolate novel mutations based on functional/phenotypic criteria, as opposed to trying to choose a specific mutation and then deciphering the effects; (2) mutations can be studied in the context of the organism, as opposed to in tissue-culture cells; (3)

mutations can be studied in an appropriate physiological context, as opposed to relying on data gathered from abnormal gene expression levels or relying on data gathered from transformed cells or cells which do not normally express the receptor; and (4) the effects of mutations can be evaluated at the cellular level.

I decided to tie together my genetic and molecular studies of the *let-23* gene by sequencing the mutations associated with various *let-23* alleles. This information serves to correlate how alterations in *let-23* primary structure relate to *let-23* function and should assist in understanding both the role of *let-23* in *C. elegans* development and the functioning of EGFR and receptor tyrosine kinases. One promising application of this genetic/molecular approach using the *Drosophila* EGFR gene has recently been reported (Raz *et al.*, 1991). For another promising approach using scanning mutagenesis in yeast to study structure/function of kinases, see Gibbs and Zoller (1991).

Approach

The general approach used to localize point mutations is shown in Figure 1. Genomic DNA isolated from a strain heterozygous for a *let-23* mutation (*i.e.*, *let-23(allele1) / let-23(+)* or simply a1 / +) is amplified using the polymerase chain reaction (PCR) so as to produce a fragment representing a 500 - 1000 base pair (bp) region of the *let-23* coding sequence. The fragment is end-labelled with γ^{32}P-ATP and heated to melt both the wild-type duplexes and mutant duplexes. The single-stranded DNA is then allowed to reanneal. As a result, both homoduplexes (+ / + and a1 / a1) and

heteroduplexes (a1 / +) form. These duplexes are treated with hydroxylamine under conditions which modify any mismatched cytosine (C) residue. The DNA is then subjected to piperidine, which cleaves at these modified C's, and the entire reaction is electrophoresed on a denaturing acrylamide gel. The presence of a polymorphic fragment suggests the presence of a point mutation in this region for the allele a1. Mutant a1 DNA is then sequenced in this region.

This technique has several limitations which will not allow it to detect all mutations. First, only mismatched C's are modified. If mutations are randomly generated, this limitation to cytosine leaves 1/6 mutations undetected (*i.e.*, an AT-to-TA transversion). However, this limitation should not be too constraining since the mutagen ethylmethanesulfonate (EMS), which was used to generate all *let-23* alleles to date, favors GC-to-AT transitions (8/8 mutations sequenced to date result from a G-to-A transition). Such a mutation is detectable by hydroxylamine modification. Second, hydroxylamine detects C mismatches in most, but not all, sequence contexts (Bhattacharyya and Lilley, 1989). Third, this technique will not detect two mutations in the same PCR fragment that result in mismatched C's on the same strand; only the mismatched C closest to the labelled end will be detected (30% chance given a double hit in the same region). However, since the mutagenesis conditions used favors introduction of only a single point mutation in a given gene, the probability of such an undetectable double-hit mutation is very low.

Materials and Methods

General Methods

Methods for culturing, handling, and genetic manipulation of *C. elegans* were as described by Brenner (1974). Strains were grown at 20°C unless otherwise noted. The only *rol-6* and *unc-4* alleles used were *rol-6(e187)* and *unc-4(e120)*, respectively.

Genomic DNA was isolated using a standard *C. elegans* protocol (Sulston and Hodgkin, 1988; A. Fire, personal communication). DNA was isolated from the following strains: MT2729 [*let-23(mn224) unc-4 / mnC1*], PS58 [*let-23(mn23) unc-4 / mnC1*], PS104 [*rol-6 let-23(sy8) / mnC1*], PS105 [*rol-6 let-23(sy9) / mnC1*], PS109 [*rol-6 let-23(sy11) / mnC1*], PS116 [*rol-6 let-23(sy14) / mnC1*], PS119 [*rol-6 let-23(sy5) / mnC1*], PS122 [*rol-6 let-23(sy18) / mnC1*], PS228 [*rol-6 let-23(sy6) / mnC1*], PS229 [*rol-6 let-23(sy16) / mnC1*], PS230 [*rol-6 let-23(sy17) / mnC1*], PS272 [*rol-6 let-23(sy15) / mnC1*], PS275 [*rol-6 let-23(sy7) / mnC1*], PS288 [*rol-6 let-23(sy13) / mnC1*], SP680 [*let-23(mn216) unc-4 / mnC1*] (all fifteen of these *let-23* alleles are homozygous lethal), PS20 [*let-23(sy1)*], PS48 [*let-23(n1045)*], PS107 [*rol-6 let-23(sy10) / mnC1*], PS227 [*rol-6 let-23(sy12) / mnC1*], PS294 [*let-23(sy97) / mnC1)*], and wild type *C. elegans* (N2) (Herman, 1978; Sigurdson *et al.*, 1984; Ferguson and Horvitz, 1985; Aroian and Sternberg, 1991). Note that all strains are heterozygous for *let-23* except for the strains bearing the alleles *sy1* and *n1045*.

Localization and sequencing of point mutations

For a given set of PCR primers (see below), genomic DNAs from different strains was amplified under standard conditions, and the PCR fragments were gel purified using GENECLEAN II (Bio 101 Inc.). For

most alleles, this purified DNA was directly used in the hydroxylamine reactions. Since the genomic DNA for *sy1* and *n1045* were isolated from homozygous mutant strains, purified PCR products from these alleles were first mixed 1:1 with wild-type DNA amplified in the same region.

Hydroxylamine mismatch detection was carried out as using a protocol provided by Bob Barstead (adapted from Cotton *et al.*, 1988). About 20ng of each purified PCR product was end labelled with γ^{32}P-ATP and purified once down a G25 column. A positive control was included to ensure all steps of the reaction were working. The labelled DNAs were brought up to 300mM NaCl, 100mM Tris-HCl pH 8, and 0.1 mM EDTA, heated to 90°C to denature all duplexes, and allowed to hybridize overnight at 60°C. After precipitation, the resulting pellets were brought up in 0.1X TE. Hydroxylamine was added to a final concentration of 2.4 M and the reactions were incubated at 37°C for 2 hours. The reactions were again precipitated, brought up in 1 M piperidine, and then submerged in a 90°C water bath for 0.5 hour. Following this, the piperidine was removed by lyophylization, the dried reactions were resuspended in gel loading buffer (40% Formamide, 8 mM EDTA, 0.02% Bromophenol Blue and 0.02% Xylene Cyanol FF), and each were electrophoresed on a 5% denaturing acrylamide gel. The presence of a polymorphic fragment in any lane indicated the presence of a point mutation, and the size of that fragment was estimated. Figure 2 shows a sample autoradiogram following electrophoresis of hydroxylamine reactions.

The mutations were sequenced as follows. Nematodes homozygous for a given mutation were picked into an eppendorf tube containing 100µl dH_20, 100µl phenol:chisam (chloroform:isoamylalcohol), and 50µl of acid rinsed

beads. For lethal alleles, approximately 50 dead larvae were picked. For the subviable alleles *n1045*, *sy97*, and *sy12*, 10 adults were used. The eppendorf tube was vortexed for 1 minute and spun in a microcentrifuge. The aqueous phase was extracted with chisam and then PCR amplified with the set of primers known to span a polymorphic hydroxylamine fragment. The resulting PCR-amplified fragment was directly sequenced from both ends after gel purification (Kretz *et al.*, 1989; adapted by D. Nickerson, personal communication). Sequencing reactions from wild-type DNA were also included. The sequencing reactions were electrophoresed such that the middle of the gel contained the size region where the polymorphism was found. At least 200 bases of each fragment were sequenced (±50 bases around the polymorphic distance from either end). To date, only one sequence alteration has been found for all alleles sequenced and all alterations found are consistent with the respective sizes of the hydoxylamine polymorphic fragments. Thus, it is unlikely that the identified mutations are PCR artifacts since the two independent PCR reactions (*i.e.*, the one used for hydroxylamine reactions and the one used for sequencing reactions) indicate a polymorphism in the same location.

Analysis of *n1045* RNA

The strain PS48(*let-23(n1045)*) was grown at 15°, 20°, and 25°C and harvested as above. RNA was extracted by crushing the nematode pellets, followed by homogenization with a polytron in guanidinium thiocyanate, and then pelleting through a CsCl cushion (protocol from Paul Kayne and Russell Hill, personal communication). Both wild-type and mutant RNA were reverse transcribed with random hexamers using standard

conditions (wild-type RNA provided by Jane Mendel). PCR amplification was performed using primers that amplify between exon 16 and the 3' untranslated region. PCR amplified fragments from mutant and wild-type were gel purified, blunt-end ligated into Bluescript, and sequenced. RNAse protection was performed using the Ambion RPA-II kit. Normalization between RNA preps for wild-type and *n1045* was achieved using a probe for an actin gene (*act-1*, provided by M. Krause). The *let-23*-specific protection resulted in some full length protected probe and also significant degradation products of lower molecular weight. Since all bands appeared in the wild-type and *n1045* lanes and were absent in the yeast RNA control, these bands are probably protection-specific and could be due to secondary structure in the probe-RNA hybrids. No such degradation products were evident in the *act-1* protections. Quantitation of band intensities was carried out using a phospho-imager (with assistance from Sean Tavtigian). Quantitative PCR was carried out by end labelling the PCR primers with γ^{32}P-ATP and amplifying reverse-transcribed RNA for 15, 20, 22, and 25 rounds. The PCR reactions were electrophoresed on a denaturing acrylamide gel and band intensities were quantitated using a phospho-imager. PCR products are evident at 20 but not 15 rounds of amplification (data not shown). Since the relative intensities of the three prominent bands were unchanged at 20, 22, and 25 rounds, it is likely the PCR reaction is still in the linear range of amplification.

Results

Mismatch detection

The *let-23* coding sequence covers over 8.5 kilobases (Kb) of genomic DNA (Aroian *et al.*, 1990), and I had nine sets of PCR primers synthesized that span the entire coding sequence. Each set of PCR primers results in a 500 - 1000 bp fragment (called PCR Domains 1-9 or PD1-9) upon amplification of genomic DNA (Figure 3).

Since polymorphisms within 50 bp of either end of the fragment may be hard to resolve on a sequencing gel, most of the primers were selected so that there is 50 - 100 bp of intron sequence at both ends of the amplified product. This strategy should allow us to resolve any detectable mutation in coding sequence. However, I could not use this strategy in the C-terminus where their is a lack of large introns. This problem was circumvented by constructing the C-terminal PDs so that they share about 100 bp of overlap in coding sequence. For example, the 3' end of PD7 overlaps the 5' end of PD8 by about 130 bases. Any polymorphism close to the the 3' end of PD7 can therefore be detected easily by PD8.

One limitation to the strategy just described is that I will miss mutations in intron sequences and in upstream and downstream regulatory domains. I expect that mutations in intron sequences will only be interesting in the event of alternative splicing, for which at present there is no evidence. Mutations in regulatory domains, while potentially interesting, do not focus on elucidating the relationship of structure to function of the EGFR protein.

The results of mismatch detection to date are shown in Table 1. Most of the protein has been tested with the results that roughly half (11/20) alleles have been definitively localized (see Discussion). The sequence of eight of these is presented below

Sequence of lethal alleles *sy5*, *sy7*, *sy11*, *sy16*, and *sy17*

Three lethal alleles, *sy5*, *sy7*, and *sy16*, alter sequence in the kinase domain (Figure 4). All these alleles alter sequence in kinase subdomain IX [(Hanks *et al.*, 1988); There are a total of 11 kinase subdomains. For *let-23*, subdomain IX is located about 75% of the way through the kinase domain]. The sequence of the *sy5* mutation (Aroian *et al.*, 1990), which inserts a stop codon at W1078 and would result in a protein truncated in the kinase domain, is consistent with the null phenotype conferred by this allele. The alleles *sy7* and *sy16* alter residues conserved among kinases and tyrosine kinases, respectively (Figure 4).

The lethal allele *sy17* alters the 5'-most base of intron 4 from a G to an A (wild-type genomic sequence from M. Koga, unpublished data). Since this initial G is conserved in all *C. elegans* and eukaryotic introns (refer to Figure 6 for the *C. elegans* consensus splice site), this allele presumably confers a null phenotype because intron 4 is not spliced or because a mis-splice occurs. Insertion of intron 4 into the *let-23* coding sequence would place an in-frame stop codon nine amino acids downstream of the normal exon 4/intron 4 boundary. The resulting *let-23* protein would lack 85% of the wild-type protein, including the entire cytoplasmic domain.

The lethal allele *sy11* probably alters an aspartic acid in exon 14 (D758) to an asparagine residue. (I say probably because the sequencing gel was slightly ambiguous and needs to be repeated. Nonetheless, this result is almost certainly accurate). This aspartic acid resides in cysteine-rich motif II-2 (Figure 5) and is 61 amino acids upstream of the transmembrane

domain. This residue does not appear to be conserved among any of the EGFR family.

The lethal mutations *sy15*, *mn23* and *mn216*, although localized, have not yet been sequenced.

Sequence of the hypomorphic allele *sy12*

The *sy12* mutation alters a cysteine residue in the extracellular domain (C368 in exon 8) to a tyrosine (Figure 5). The location of this cysteine is conserved in *let-23*, the *Drosophila* EGFR, and human EGFR. This cysteine is close to the 5' end of the EGFR ligand binding domain.

Sequence of the hyperinduced allele *n1045*

The reduction of function allele *n1045* alters the final base of intron 16 from the normally invariant G to an A (Figure 6). This intron is about 300 bases downstream of the kinase domain.

To understand the effects of this mutation, I isolated RNA from *n1045* at 15°, 20°, and 25° and extensively analyzed the effect of the mutation on the RNA product at 20°. I isolated RNA at the three temperatures since the strain PS48 *let-23(n1045)* results in a Vul phenotype at 15°, a Hin phenotype at 25°, and a mixture of phenotypes at 20° (Ferguson and Horvitz, 1985; Aroian and Sternberg, 1991; Appendix I; R.V.A. and P.W.S., unpublished data).

Reverse transcription and PCR amplification of 20° RNA using C-terminal specific primers indicates two prominent and one minor transcript species (Figure 7). A control using wild-type RNA displays only one transcript. Approximately 52% of the *n1045* transcripts are the same

size as the wild-type control (Band II), 42% are smaller than wild type (Band I), and 5% are larger than wild type (Band III). I subcloned and sequenced the PCR fragments from the single wild-type and the three mutant bands: 4/4 subclones from the wild-type control were wild type (exons 16-17-18), 2/2 subclones from Band III displayed an insertion of intron 16 (exon 16-intron 16-exons 17-18), and 4/4 subclones from Band I displayed use of the intron 17 3' acceptor site by the intron 16 5' donor site (exons 16-18). All these products might be expected by the *n1045* mutation. Protein translated from Band I RNA would result in a TAG (amber codon) at the exon 16-18 boundary (the sequence of exon 18 beyond this stop codon is in-frame relative to wild-type exon 18; see discussion). Protein translated from Band III RNA would result in a stop codon (TAA) in intron 16, five amino acids after the last amino acid of exon 16.

Sequencing of the wild-type sized band, Band II, indicated (Figure 8): 5/11 transcripts made the wild-type splice and 6/11 transcripts chose five different 3' splice acceptor sites close to the wild-type splice site. Only one of these sites contained a cryptic AG acceptor sequence, although the other four acceptor sites contained either an A in the penultimate position or a G in the ultimate acceptor position. Both the wild-type splice at the mutated AA acceptor site and the four cryptic splices not using a cryptic AG are unprecedented in eukaryotic splicing literature (see Discussion). Of the non-wild-type splices, all but one result in a stop codon close to the end of exon 16 (the splice which removes four bases of exon 17 encodes a long, nonsense protein starting at the end of exon 16). It is certainly possible that I have not yet sequenced all the splicing products produced. As a result of the various mix of spliced products, approximately one quarter of all RNA

species present in *n1045* at 20° are wild type (52% of the RNA is in Band II and 5/11 of the Band II transcripts are wild type).

I believe these results are real and are not, for example, an artifact of PCR amplification or contamination. First, amplification of reverse-transcribed (RT) wild-type RNA results in only one band, and sequence data indicates this band is homogeneous and wild type. Second, all bands are dependent on reverse transcription, since non-RT RNA yields no bands upon amplification. Third, the presence of the three bands is not affected by pre-treatment of the RNA with DNAse. Finally, I have also amplified RT RNA between exons 13 and 15 and found that introns 13 and 14 are correctly spliced out, suggesting that the *n1045* mutation is not affecting upstream splicing events.

In order to ascertain what the total levels of *let-23* RNA levels are in *n1045* versus wild-type, I performed RNAse protection experiments using a probe which protects about 160 bases of the 3' end of the kinase domain. This probe should be common to all the transcripts. The results indicate that the overall *let-23* transcript levels are the same in both the mutant and wild-type (data not shown), a result supported by quantitative PCR results (Figure 7).

Lastly, I have also begun to analyze the *n1045* RNA products at 15° and 25°. All three bands are present at these temperatures. However, preliminary results suggest that at 15°, Band I (exon 16-18) is more prevalent than Band II (wild-type sized) relative to what was found at 20°. Conversely, at 25°, Band II is more prevalent than Band I relative to what was found at 20° (data not shown).

Sequence of the *sy97* mutation

The tissue-specific *sy97* mutation has also been sequenced. It alters the 3' end of intron 17 from G to A, analogous to the *n1045* alteration at intron 16 (Figure 9). RNA analysis has not yet been performed. If intron 17 is not spliced, then the altered open reading frame results in a stop codon three amino acids downstream of the end of exon 17.

Discussion

General considerations

I have demonstrated that it is possible to localize and sequence mutations throughout the *let-23* gene, even given its large size. Furthermore, most of the mutations are interesting in terms of protein structure and function. Even though two of the five lethal mutations sequenced (*sy5* and *sy17*) resulted in probable protein truncations, the other three resulted in single, missense mutations. Of the non-lethal alleles, one resulted in a missense mutation. The other two, although giving rise to apparent splicing defects, provide potentially valuable information about structure/function relationships (see below).

One potential criticism of the present study is that I did not test the entire protein for hydroxylamine polymorphisms with the lethal alleles and *n1045*. It is unlikely that EMS would cause multiple mutations in the same gene under the conditions used to generate these alleles. Nonetheless, now that the materials, techniques, and procedures for localizing *let-23* mutations have been developed, it should be readily possible to answer whether or not any given allele shows multiple polymorphisms.

I am encouraged by the fact that mutations were found throughout the entire portion of the gene. This finding suggests that the *let-23* gene provides an excellent opportunity to study many parts of the EGFR protein. Furthermore, I am encouraged by the fact that some of the mutations sequenced are novel in the EGFR field.

I do note, however, that only eleven of twenty alleles have been localized, even though most of the protein has been tested. There are several explanations. First, neither PD1 nor upstream control sequences have been tested for polymorphisms. Some lethal alleles could have mutations here. Second, it was obvious that some DNAs and some reactions, especially those for PD3, did not work well (*i.e.*, scarce counts were present at the end of the reaction) and should be repeated. Third, in several instances, faint polymorphic bands were noted but did not repeat. These may result from mismatched T-Ts (which hydroxylamine modifies inefficiently) or from contexts not good for modification by hydroxylamine. Fourth, a strong polymorphism was noted in PD4, but, due to a tube mix up, the allele associated with that polymorphism is not known. This polymorphism raises the number localized to twelve.

Analysis of lethal mutations

Our results with the alleles *sy5*, *sy7*, and *sy16* suggest that: (1) the *let-23* gene is a kinase, since alterations which affect amino acids conserved among the kinase family severely affect *let-23* function, and (2) although previously not the subject of study, subdomain IX serves some important kinase function. Based on studies involving other kinases (reviewed in Hanks *et al.*, 1988), the truncation of *let-23* by the *sy5* allele in kinase

subdomain IX probably results in an inactive kinase. This alteration is consistent with the null phenotype of this allele. The lethal allele *sy7* alters a glycine found in virtually all kinases to a glutamic acid. This mutation significantly alters both the size of the side chain and net charge. Although no previous studies involving this residue are known, the *sy7* allele suggests that altering this residue significantly affects protein kinases either by disrupting protein folding or by directly hindering kinase activity. The lethal allele *sy16* alters a threonine in subdomain IX to an isoleucine. This threonine is either a threonine or a serine in tyrosine kinases, but not serine/threonine kinases. The *sy16* mutation changes a polar amino acid to a hydrophobic amino acid and may therefore disrupt folding in the area or may disrupt kinase activity by altering an important residue. The only other study involving a mutation in subdomain IX is with the DER protein (Raz *et al.*, 1991). In that study, alteration of a different threonine residue severely (but not completely) disrupted protein function.

The mutation associated with the lethal allele *sy11* (aspartic acid to asparagine) alters net charge, but is otherwise conservative. It is possible that this aspartic acid residue interacts with a positive charge somewhere else on the protein. Interestingly, this mutation resides in the additional cysteine-rich domain present in the *C. elegans* and *Drosophila*, but not vertebrate, EGFRs. The mutation suggests that this additional cysteine-rich domain is important for protein function in the nematode. There is no obvious similarity between *C. elegans* and *Drosophila* sequences at or around this aspartic acid residue.

n1045

Important results of *n1045* genetic analysis are: (1) the *n1045* mutation is recessive at all temperatures for all phenotypes and appears to reduce *let-23* function in all tissues where *let-23* acts; (2) at 25°, *n1045/n1045* displays more than wild-type induction (hyperinduced or Hin phenotype) but at 25° *n1045/Df* is Vul; (3) the vulval defect displays a cold-sensitive phenotype -- *n1045/n1045* at 15° is Vul [at 20°, *n1045/n1045* displays a mixture of Vul/Hin/wild-type vulval phenotypes]; (4) neither viability, male spicules, nor hermaphrodite fertility show significant temperature sensitivity; (5) unlike for the vulval function, *n1045/n1045* at 25°, 20°, and 15° displays the loss of function phenotype for viability and male spicule functions; and (6) *n1045* vulval and lethal defects are partially amber suppressible (Aroian and Sternberg, 1991; Appendix I; Ferguson and Horvitz, 1985). I interpreted these results to suggest that *n1045* reduces both stimulatory and inhibitory *let-23*-controlled pathways in the vulva (Aroian and Sternberg, 1991). Furthermore, at 25°, two *n1045* copies do not significantly activate the inhibitory pathway but do significantly activate the stimulatory pathway. As a result, the Hin phenotype ensues. One copy of *n1045* (*i.e.*, *n1045/Df*) does not activate enough of either pathway, and the Vul phenotype ensues.

We hypothesize that this preferential defect in inhibition results from the fact that either: (A) *n1045* does nothing more than reduce *let-23* **dosage** levels and the inhibitory pathway is more sensitive to a reduction in *let-23* dosage than the stimulatory pathway; or (B) physical characteristics of the protein have been altered (*i.e.*, the protein is **neomorphic** in that it displays some characteristics of a gain of function mutation and some characteristics of a loss of function mutation). In support of A, for example, *let-23* might activate some substrates that eventually lower the

level of vulval induction, but these substrates or proteins are very sensitive to a decrease in *let-23* activity or *let-23* protein levels. In support of B, *n1045* might result in unusual activation of the receptor by the removal of autophosphorylation sites or of sequence elements that result in the recycling of receptors, while, in addition, the receptor itself has kinase lowered activity (see Chapter I).

The results of *n1045* molecular analysis are unexpected. [To assist in this discussion, I approximate the effects of the *n1045* mutation such that it produces only two transcript species: (1) mutant transcripts, or MTs, representing 75% of the total and producing a protein that truncates at the 3' end of exon 16; and (2) wild-type transcripts, or WTs, representing 25% of the total.] One result of the mutation is to produce a truncated receptor via MTs. The presence of MTs immediately evokes a neomorphic model in which the receptors lack autophosphorylation sites or elements responsible for receptor degradation (see Discussion in Chapter II). The presence of MTs is also consistent with a dosage model since the truncated products might have lower *let-23* activity (DiFiore *et al.*, 1990b). However, the surprise with the *n1045* mutation is the significant levels of WTs -- 25% the level of WTs in *let-23(+)/let-23(+)* animals. How can the *n1045* mutation be recessive while at the same time the homozygous mutation gives rise to significant levels of WTs?

I envision three models that unite the molecular and genetic data (Figure 10). Model I: the WTs do not matter. The MTs produce a neomorphic protein, and the vulva is sensitive to its dosage levels. One copy of *n1045* does not produce enough MTs to cause a gain of function phenotype, but two copies do. The WTs produced by *n1045* alone are not

sufficient for proper levels of *let-23* activity. Model II: the MTs do not matter and are essentially inactive. The 75% reduction in *let-23* WTs affects *let-23*-directed inhibition more than *let-23*-directed stimulation (dosage model). This model suggests that there is a significant difference in most tissues between *let-23* reductions of 75% and 50% because *let-23(-)*/+ is wild type. Model III: both MTs and WTs matter. The MTs, produce not gain of function but rather poison proteins. The MTs produce receptor that, upon dimerization with WT-produced receptors, inactivate the complex. This inactivation could occur, for example, because the heterodimer cannot efficiently interact with substrate. As a result, *let-23* levels are significantly lowered. With two copies of *n1045*, the effective WT-produced protein levels are low enough to affect inhibition but not stimulation (dosage model). This model is unanticipated but may be the best since it explains the data while invoking the least stringent dosage requirements.

To test these models, I plan to construct a *let-23* genomic clone that results in a protein truncated after exon 16 and inject a high copy number of this construct into animals wild-type for *let-23*. Model I predicts the transformed animals will be Hin, model II predicts they will be wild type, and model III predicts they will be Vul. In parallel, I will inject a wild-type *let-23* genomic clone into *n1045* animals. Model I predicts this clone will not rescue the Hin phenotype, Models II and III predict it will.

Amber suppression, temperature sensitivity, and tissue-specificity of *n1045* are compatible with all three models. Amber suppression could come about via the Band I transcript (exon 16-18). In a wild-type background, this transcript results in the receptor truncated after exon 16. However, in an amber suppressor background, this transcript encodes a

protein that is essentially wild-type except that it lacks the 35 amino acids encoded by exon 17. The amber suppressor might therefore result in more "wild type-like protein" relative to truncated protein. Temperature sensitivity could occur because the truncated receptor is more hyperactive at higher temperatures (Model I), because the wild-type protein is more active at higher temperatures (Model II), or because the truncated, dominant negative protein is more stable at lower temperatures and thus there is stronger dominant negative effect (Model III). Initial analysis of RNA extracted from *n1045* hermaphrodites at different temperatures tends to support model III since at 15° there seems to be more truncated and less wild-type sized transcripts. All models are compatible with the fact that *n1045* at 25° shows a loss of function phenotype in tissues other than the vulva if we assume that *let-23* behaves differently in the vulva than other tissues (*e.g.*, autophosphorylation sites are only important in the vulva or the dosage-sensitive inhibitory pathway is only present in the vulva). An intriguing possibility is that this inhibitory pathway involves the *lin-12* pathway (see Chapter I) since, of the five *let-23* decisions/tissues characterized, *lin-12* functionally overlaps only in the vulva. Lastly, I note that our genetic data suggests *n1045* might have a slight dominant negative effect as a homozygous mutation then when heterozygous with *sy10*, *sy12*, and *sy97* (Aroian and Sternberg, 1991). This result is consistent with model III.

The variety and sequence of 3' splice acceptor sites, including the altered wild-type site (from AG to AA), used as a consequence of the *n1045* mutation are unprecedented in the splicing field. Mutations which cause splicing at cryptic AG donor sites of at 3' donor sites downstream of the

original 3' donor site (*i.e.*, exon skipping) have been reported (Treisman *et al.*, 1982; Treisman *et al.*, 1983; Mitchell *et al.*, 1986; Aebi *et al.*, 1986; Tromp and Prockop, 1988). In one study, the same mutation as *n1045* (AG to AA) was tested along with various other mutations at the 3' splice site (AG to TG, CG, or AT): in all cases, no splicing occurred *in vitro* while use of a cryptic AG 3' splice site was seen *in vivo* (Aebi *et al.*, 1986). In other studies, the effect of 3' acceptor site mutations from AG to AA, AC, or GG was to cause exon skipping, splicing to a cryptic AG site, and/or low levels of unspliced intron (Mitchell *et al.*, 1986; Su and Lin, 1990; Cladaras *et al.*, 1987; Tromp and Prockop, 1988; all these effects are also seen with *n1045*). For comparison, the unusual 3' sites used in *n1045* are TG, AT (twice), GG, and AA. One common feature of these unusual *n1045* sites is the presence of either the penultimate A or the ultimate G of the wild-type AG site.

Analysis of non-lethal mutations *sy12* and *sy97*

The non-lethal mutations *sy12* results in a strong, but incomplete reduction of *let-23* function in all tissues where *let-23* is known to act (Aroian and Sternberg, 1991). This reduction of function is more severe in all tissues than more for *n1045*. The *sy12* allele alters a cysteine to a tyrosine in cysteine-rich domain I (Figure 4). This cysteine is also just upstream of the ligand binding domain, and the spacing of cysteine residues in this region is fairly well conserved among the EGFR family. The EGFR cysteine-rich regions are hypothesized to form a network of cysteine bridges in order to provide scaffolding for a conformation that mediates receptor-receptor interaction and/or for the transduction of the ligand-binding signal across the plasma membrane (Yardin and Ullrich,

1988). The *sy12* mutation might result in a disruption of this scaffolding due to either steric alterations caused by the tyrosine residue or, more likely, by the lack of an important cysteine bridge. As a result, protein dimerization or signal transduction might be severely, but not completely, impacted. As far as I know, there has been no other direct study of the role of the EGFR cysteine-rich regions or individual cysteine residues.

The tissue-specific allele *sy97* shows strong defects for all *let-23* activities but fertility, for which it is wild type (Figure 9). Although I do not yet know the effect of the *sy97* intron mutation on RNA processing, the result of this mutation might be to disrupt the amino acid sequence encoded by the last exon, exon 18.

In combination with genetic data, this result would suggest that: (1) the *let-23* carboxy-terminus is important for functioning in all tissues except for the tissue required for fertility (on the other hand, it is conceivable that alternative splicing is used in that tissue such that the normal exon 18 splice site is unimportant); and (2) exon 17 is important for fertility. This last suggestion is based on the fact that *n1045*, which results in truncation of both exons 17 and 18 shows a defect in fertility, wheres *sy97*, which might result in a truncation of exon 18 alone, does not. The fact that *sy97* shows a more severe defect than *n1045* in all other phenotypes is probably due to the presence of some wild-type transcript associated with the *n1045* mutation. The sequencing of the *sy97* mutation provides strong evidence that receptor tyrosine kinase tissue-specificity can be contained in the C-terminus. Next steps in understanding the *sy97* mutation include RNA analysis and possibly construction of various genomic constructs to test the hypothesis that exon 17 is important for activity in one tissue.

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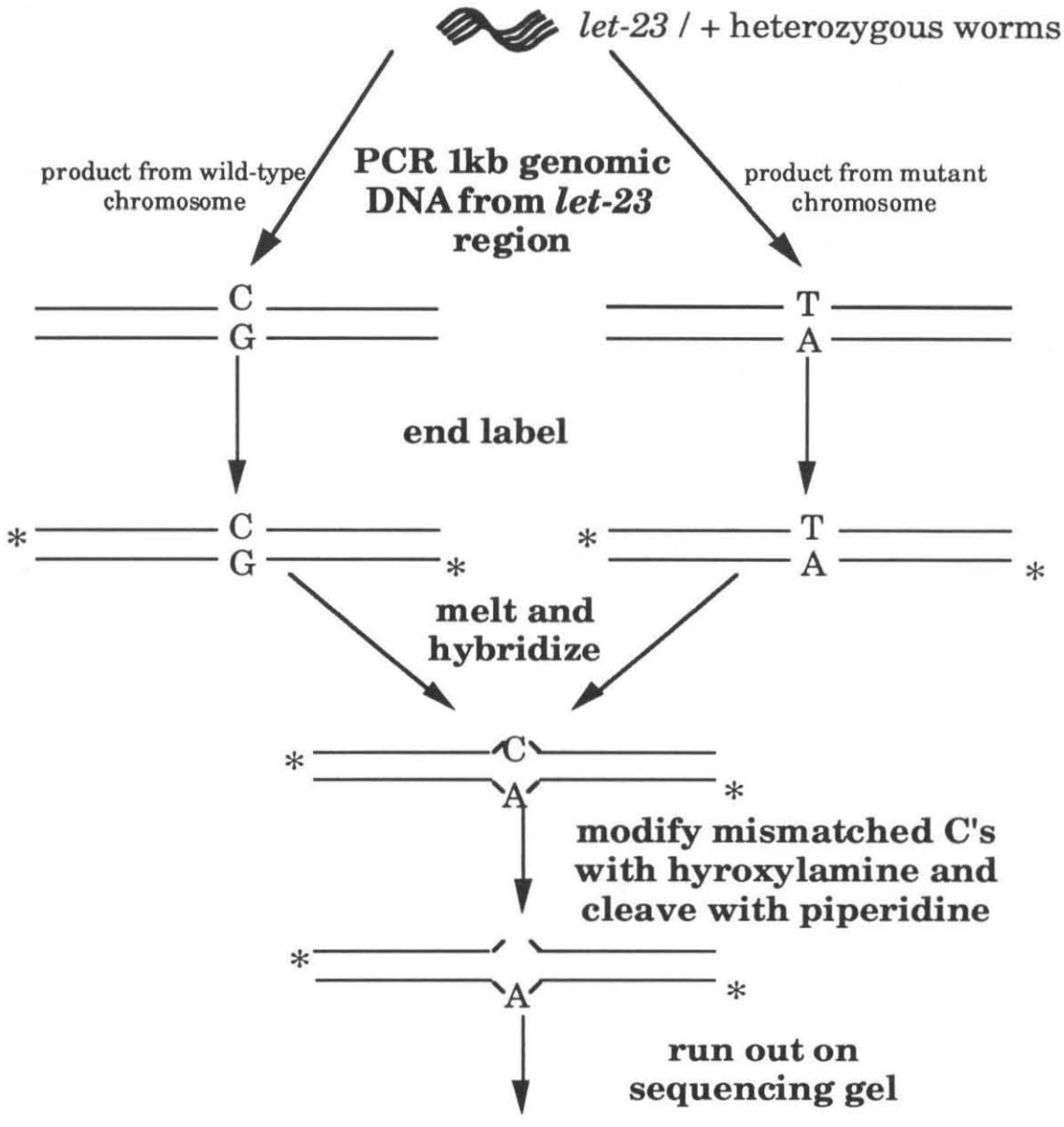
Table 1. Results are shown for hydroxylamine mismatch detection experiments for twenty *let-23* alleles. The null *let-23* alleles are listed above the heavy horizontal line, the non-null alleles are listed below it. A "+" indicates that a polymorphic fragment was found for that allele in a given PCR domain (PD). A "-" indicates a negative result. No alleles have yet been tested in PD1. However, since this fragment contains only 61/1322 amino acids, it is unlikely that many alleles have polymorphisms here. For null alleles, once a polymorphism was found, that allele was not tested in subsequent reactions. It is possible, although unlikely, that these alleles contain more than one mutation. Also, null alleles were not tested in PD9 since germ line transformation experiments show that this region of the protein is not required for rescue of lethality. Non-null alleles, except for *n1045*, were tested in all PDs. There are two "+" results for *n1045* because the *n1045* polymorphism resides in the overlap of two PDs.

	PD1	PD2	PD3	PD4	PD5	PD6	PD7	PD8	PD9
mn23					+		-	-	
mn216				+	-		-	-	
sy5							+		
sy6		-	-	-	-	-	-	-	
sy7							+		
sy8		-	-	-	-	-	-	-	
sy9		-	-	-	-	-	-	-	
sy11					+		-	-	
sy13		-	-	-	-	-	-	-	
sy14		-	-	-	-	-	-	-	
sy15		+	-	-	-		-	-	
sy16							+		
sy17		+	-	-	-		-	-	
sy18		-	-	-	-	-	-	-	
mn224		-	-	-	-	-	-	-	-
n1045							-	+	+
sy1		-	-	-	-	-	-	-	-
sy10		-	-	-	-	-	-	-	-
sy12		-	+	-	-	-	-	-	-
sy97		-	-	-	-	-	-	-	+

Figure 1. Strategy to localize *let-23* point mutations.



point mutation involving a mismatched C will result in a polymorphic fragment

Figure 2. Example of hydroxylamine mismatch detection. Full length PCR fragment for 12 alleles is at the top of each lane. White arrowheads point to polymorphisms in two lanes. The left-most lane shows a 123 bp ladder.

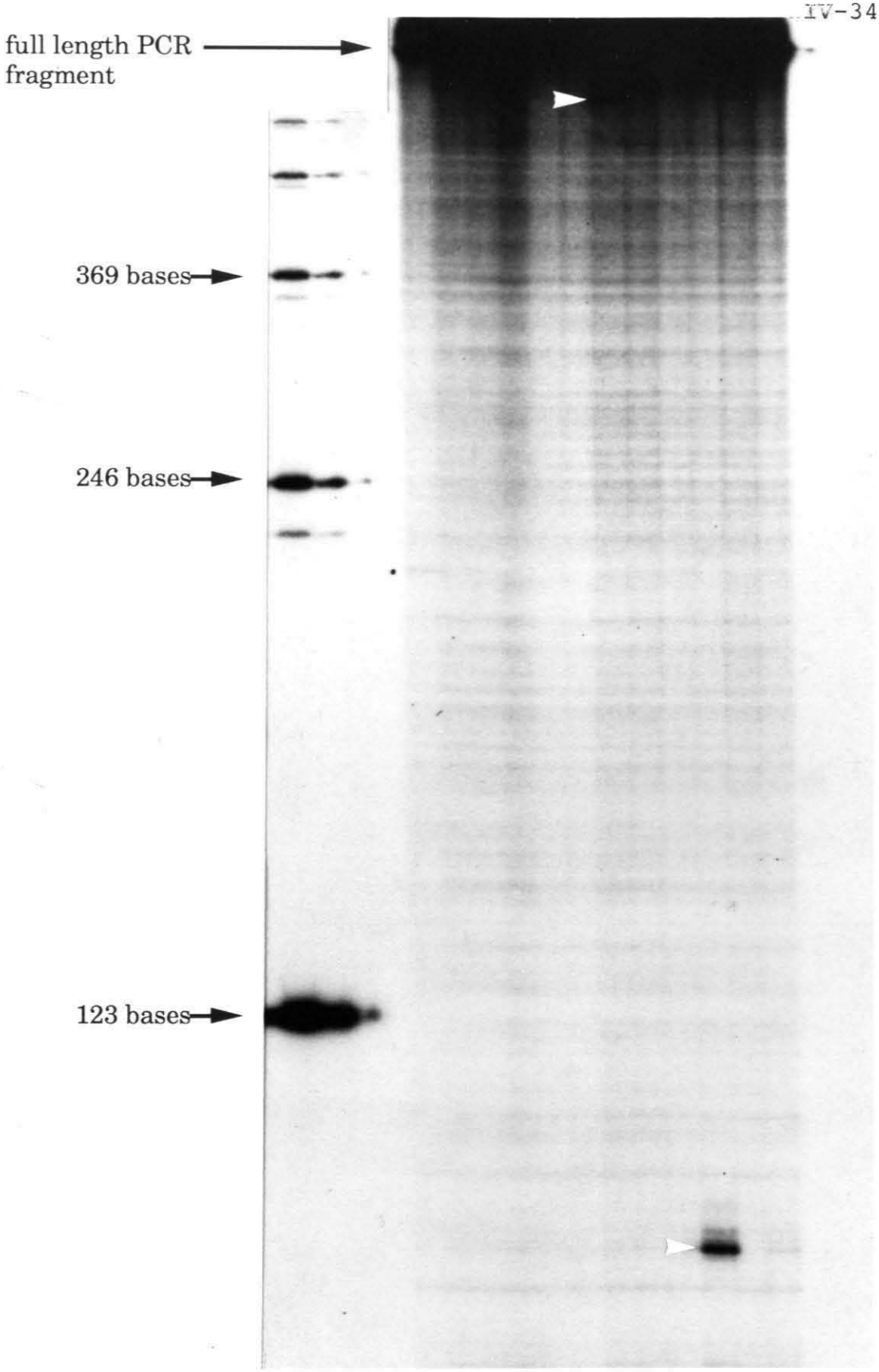

full length PCR fragment
369 bases
246 bases
123 bases

Figure 3. Location of nine PCR domains (PDs) that span the entire *let-23* coding sequence. Boxes represent exons, lines represent introns. The known structural and functional domains of EGFR are shown above the exon/intron schematic.

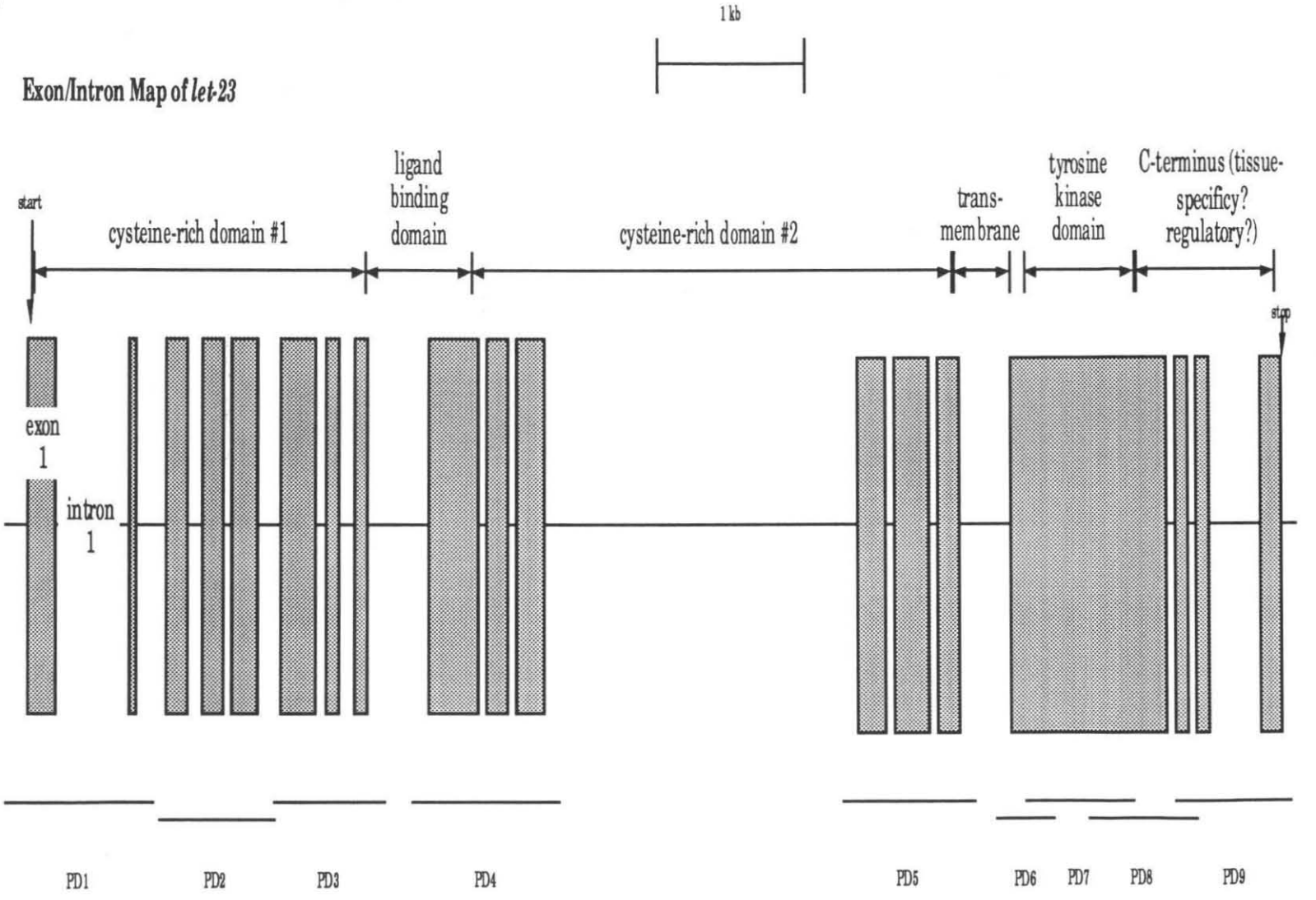

1 kb
Exon/Intron Map of *let-23*
start
cysteine-rich domain #1
ligand binding domain
cysteine-rich domain #2
trans-membrane
tyrosine kinase domain
C-terminus (tissue-specificy? regulatory?)
stop
exon 1
intron 1
PD1
PD2
PD3
PD4
PD5
PD6
PD7
PD8
PD9

Figure 4. Sequence of kinase domain mutants. Part of the wild-type kinase subdomain IX sequence is shown. The location and alterations associated with three lethal mutations is indicated along with their amino acid location (Aroian *et al.*, 1990). For comparison are shown the equivalent protein sequences from: (1) cAMP-dependent protein kinase catalytic subunit, α form from bovine (cAPKa); (2) *Schizosaccharomyces pombe* cdc2; (3) human fetal liver *raf*; (4) human fetal liver *src*; (5) *Drosophila* EGF receptor (DER); and (6) human placenta EGF receptor (EGFR) (Hanks *et al.*, 1988). The first three are serine/threonine kinases. The last three are tyrosine kinases.

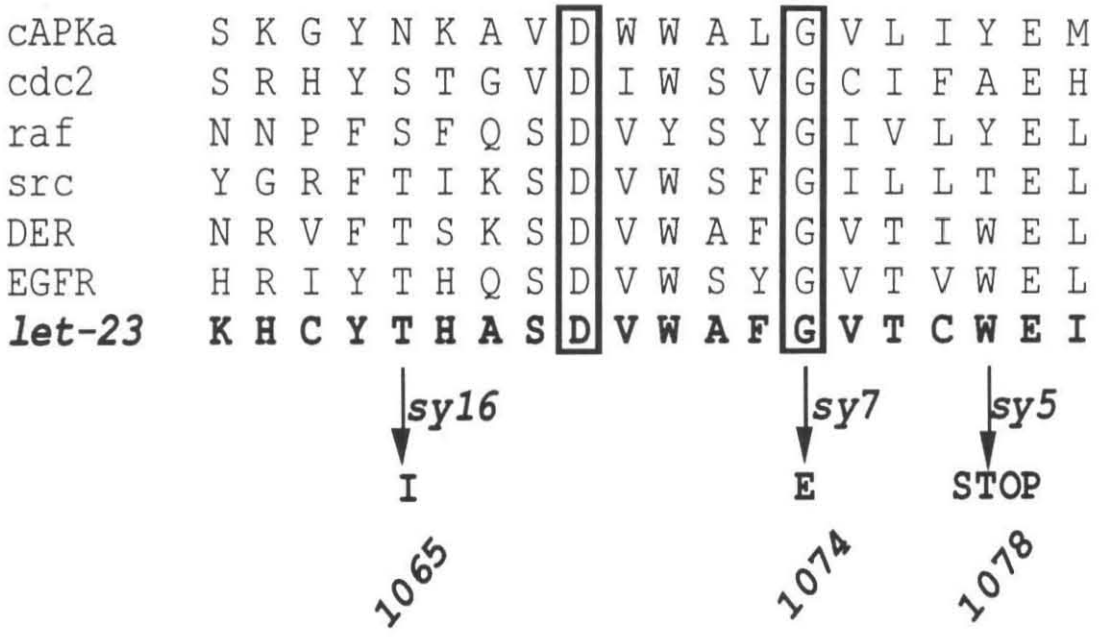

cAPKa S K G Y N K A V D W W A L G V L I Y E M
cdc2 S R H Y S T G V D I W S V G C I F A E H
raf N N P F S F Q S D V Y S Y G I V L Y E L
src Y G R F T I K S D V W S F G I L L T E L
DER N R V F T S K S D V W A F G V T I W E L
EGFR H R I Y T H Q S D V W S Y G V T V W E L
let-23 K H C Y T H A S D V W A F G V T C W E I
sy16
sy7
sy5
I
E
STOP
1065
1074
1078

Figure 5. Location of the *sy11* and *sy12* mutations relative to the conserved cysteine spacing of the EGFR family (adapted from Aroian *et al.*, 1990 Figure 6b). The numbers represent the number of amino acid residues between cysteines for *let-23*, DER, and human EGFR. The cysteine mutated in the allele *sy12* is underlined and in bold; the alteration to tyrosine is shown. The stretch of five amino acids between two cysteines that harbors the *sy11* mutation is indicated by an underlined, bold-faced "5". The sequence of these five amino acids is shown, along with the *sy11* alteration.

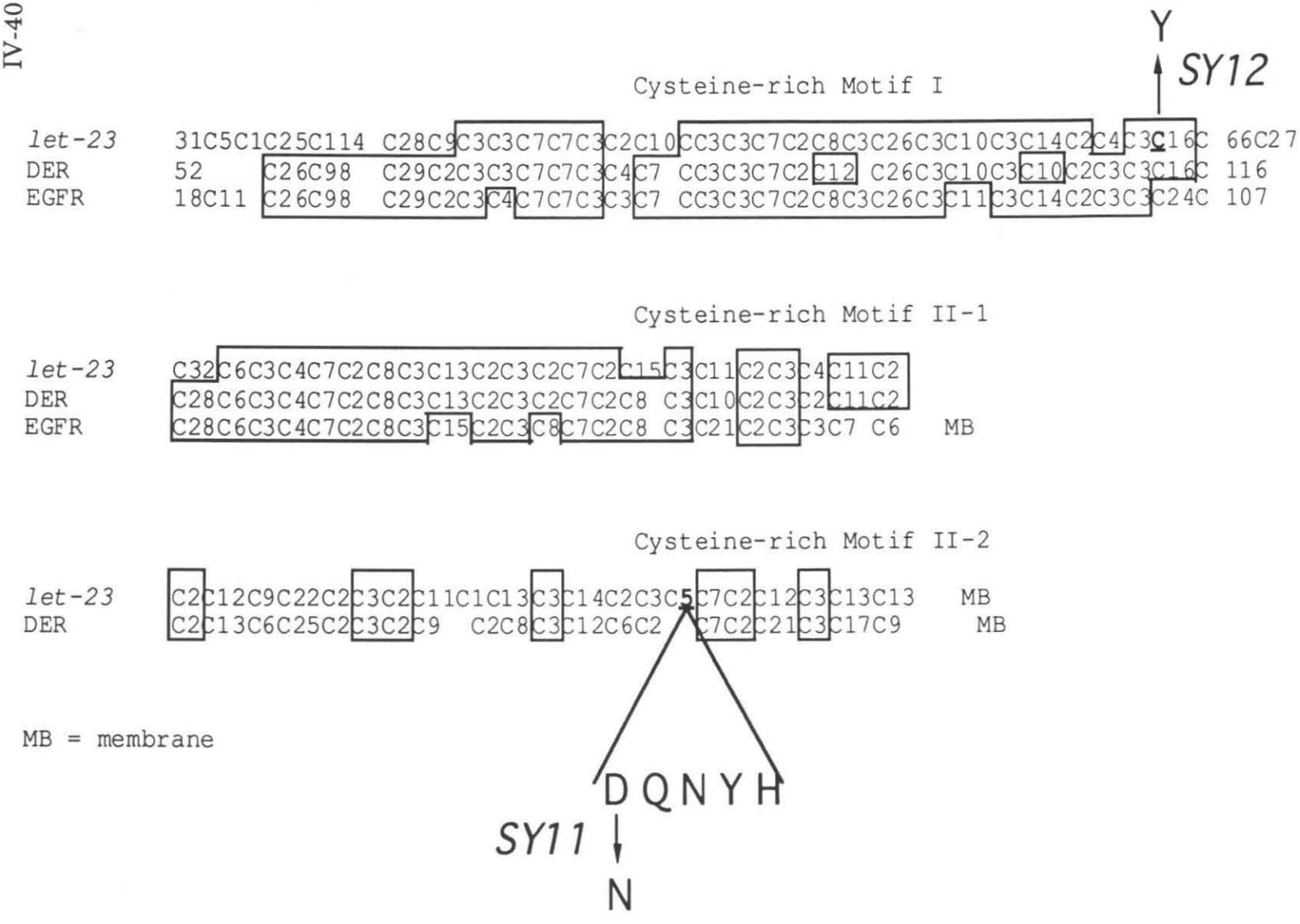

Y
SY12
Cysteine-rich Motif I
let-23 31C5C1C25C114 C28C9C3C3C7C7C3C2C10CC3C3C7C2C8C3C26C3C10C3C14C2C4C3C16C 66C27
DER 52 C26C98 C29C2C3C3C7C7C3C4C7 CC3C3C7C2C12 C26C3C10C3C10C2C3C3C16C 116
EGFR 18C11 C26C98 C29C2C3C4C7C7C3C3C7 CC3C3C7C2C8C3C26C3C11C3C14C2C3C3C24C 107
Cysteine-rich Motif II-1
let-23 C32C6C3C4C7C2C8C3C13C2C3C2C7C2C15C3C11C2C3C4C11C2
DER C28C6C3C4C7C2C8C3C13C2C3C2C7C2C8 C3C10C2C3C2C11C2
EGFR C28C6C3C4C7C2C8C3C15C2C3C8C7C2C8 C3C21C2C3C3C7 C6 MB
Cysteine-rich Motif II-2
let-23 C2C12C9C22C2C3C2C11C1C13C3C14C2C3C5C7C2C12C3C13C13 MB
DER C2C13C6C25C2C3C2C9 C2C8C3C12C6C2 C7C2C21C3C17C9 MB
MB = membrane
DQNYH
SY11
N

Figure 6. Sequence of *n1045* mutation in intron 16. An expanded view of the exon/intron structure of the C-terminus is shown along with approximate exon/intron sizes in bp. Wild-type genomic sequence from the end of exon 16 to the beginning of exon 17 (including intron 16) is shown. The *n1045* alteration is indicated. Below is the *C. elegans* consensus splice site (Emmons, 1988).

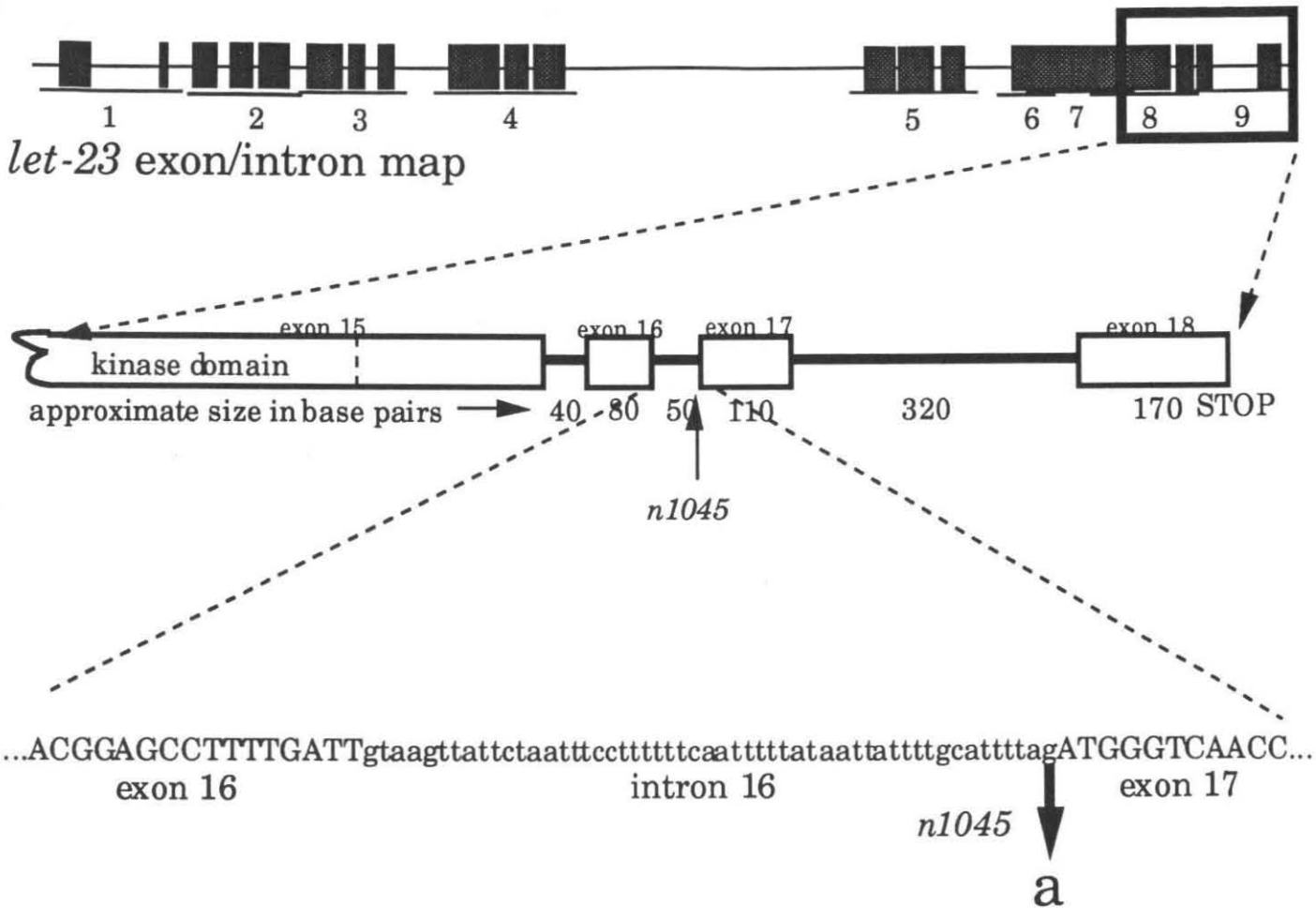
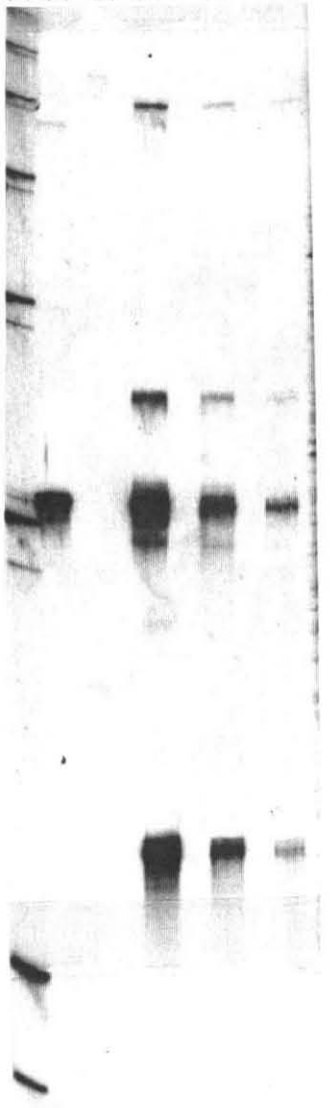

1
2
3
4
5
6
7
8
9
let-23 exon/intron map
exon 15
exon 16
exon 17
exon 18
kinase domain
approximate size in base pairs
40
80
50
110
320
170 STOP
n1045
...ACGGAGCCTTTTGATTgtaagttattctaatttccttttttcaattttttataattattttgcattttagATGGGTCAACC...
exon 16
intron 16
exon 17
n1045
a
exon/intron consensus
% occurence
47
25
63 67
100 100 73 77 75 62 60
91 98 70 70 100 100
45
36

Figure 7. Three major RNA species associated with the *n1045* mutation. The lanes represent (1) 123 bp ladder; (2) reverse-transcribed (RT) wild-type RNA (0.24μg) amplified with C-terminal primers for 25 rounds; (3) negative control using same primers but no RT RNA added; (4) RT *n1045* 20° RNA (0.6μg) amplified with same primers for 25 rounds; (5) 22 rounds; and (6) 20 rounds. The sizes of the bands are shown along with the relative abundance and a schematic representation of the exon-intron structure. A faint fourth band at 741 bases is the expected size for genomic DNA or unspliced message. This band could be due to a slight genomic DNA contaminant or to inefficient splicing.



size (bases)	% of total transcript	# of subclones sequenced	*n1045* RNA product
←741	1.5	0	
←425 (Band III)	4.5	2	16 — 17 18
←372 (Band II)	52	11	16 17 18 and others
←264 (Band I)	42	4	in frame amber codon at this junction: 16 18

Figure 8. Wild-type-sized *n1045* RNA band is a mix of at least six different RNA species. A schematic showing the splicing products found upon sequencing eleven different subclones of Band II. The number in parentheses is the number of subclones displaying given splice.

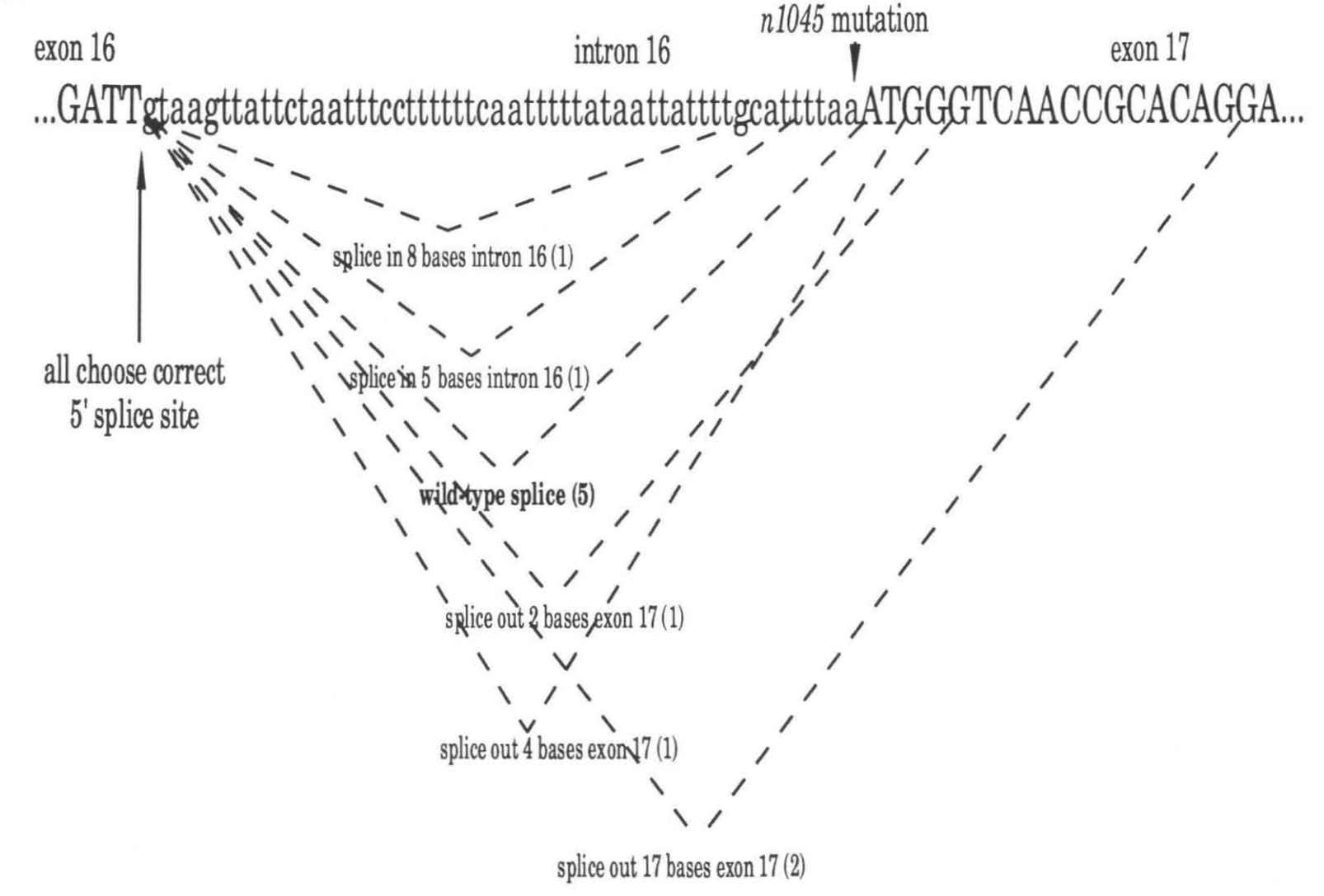
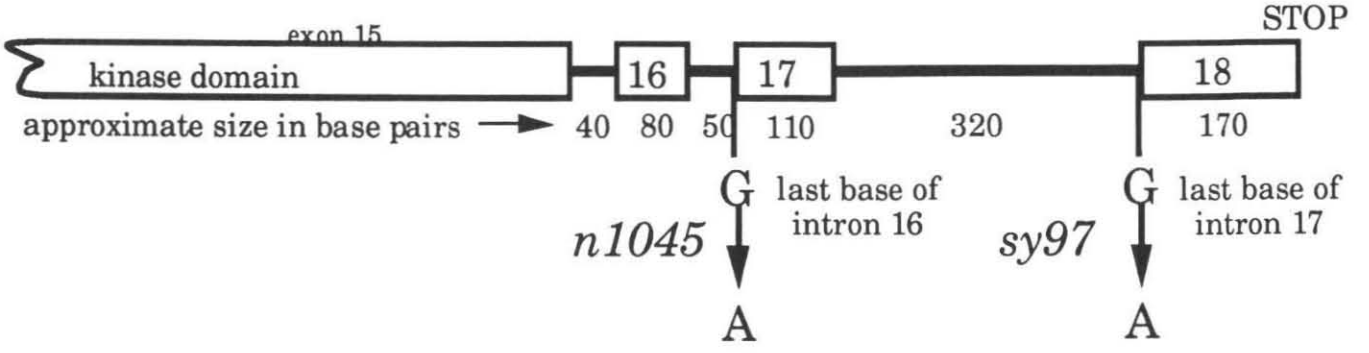
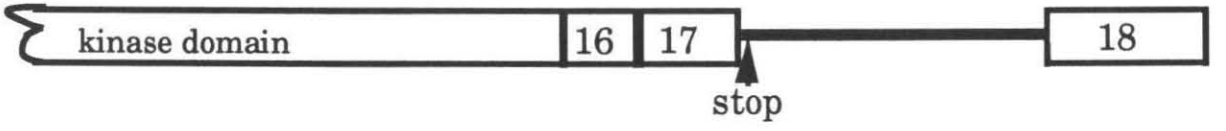

exon 16
intron 16
n1045 mutation
exon 17
...GATTgtaagttattctaatttccttttttcaattttataattattttgcattttaaATGGGTCAACCGCACAGGA...
all choose correct
5' splice site
splice in 8 bases intron 16 (1)
splice in 5 bases intron 16 (1)
wild-type splice (5)
splice out 2 bases exon 17 (1)
splice out 4 bases exon 17 (1)
splice out 17 bases exon 17 (2)

Figure 9. *sy97* sequence. The location of the *sy97* mutation at the end of intron 17 is shown along with the location of the analogous alteration in intron 16 associated with *n1045*. A summary of the genetic data comparing *sy97* with *n1045* is also shown.



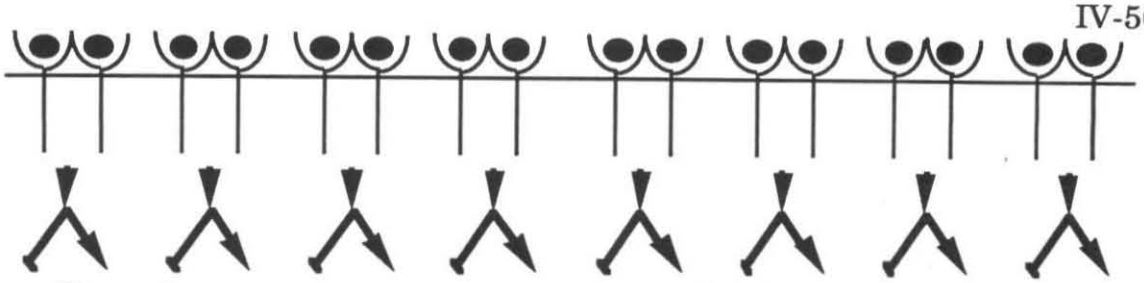
Putative *sy97* transcript



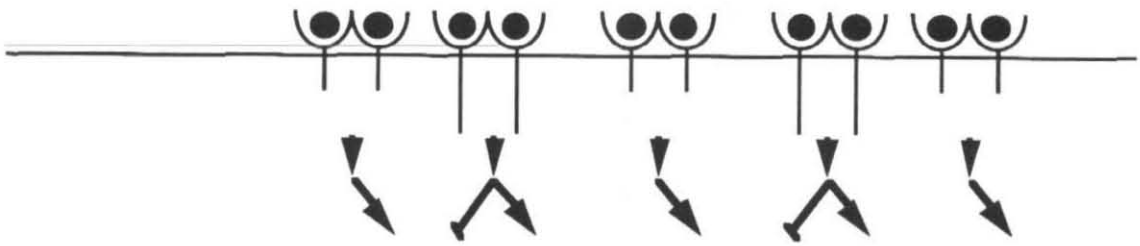
Genetically inferred activity in different tissues

Allele	Essential	Vulva	Male Tail	Fertile	P12
wild-type	NORMAL	NORMAL	NORMAL	NORMAL	NORMAL
n1045	REDUCED	REDUCED	REDUCED	REDUCED	REDUCED
sy97	V. LOW	V. LOW	V. LOW	**NORMAL**	V. LOW

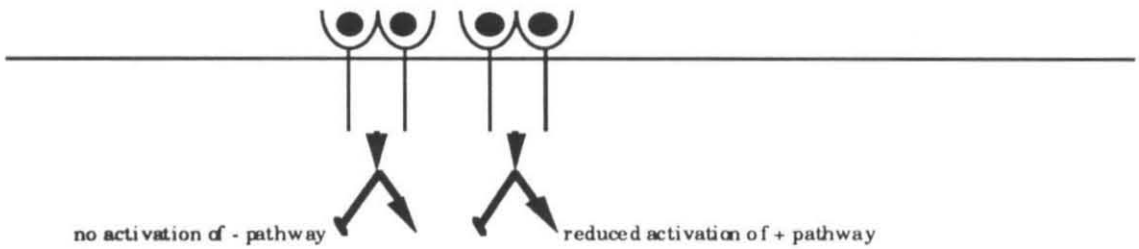
Figure 10. Three possible models to account for the behavior of the *n1045* mutation.



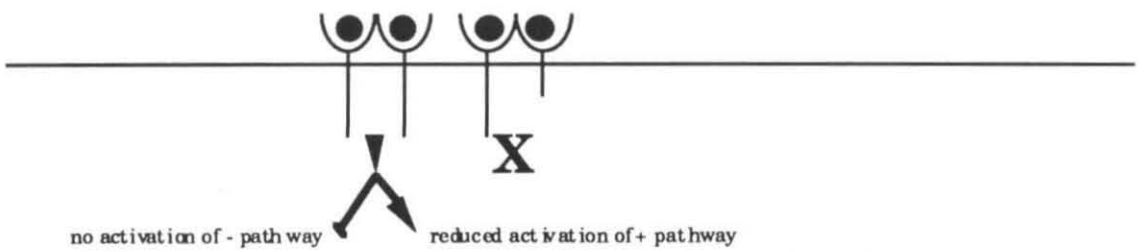
wild-type *let-23*: receptors activate stimulatory and inhibitory vulval pathways



Model I: *n1045* truncated receptors are activated at higher levels than wild-type and result in too much stimulation. The length of the truncation is exaggerated.



Model II: the truncated receptors are unimportant. The Hin phenotype results from the 75% reduction in wild-type *let-23* transcripts. This model assumes that the inhibitory pathway is more sensitive to a decrease in *let-23* function than the stimulatory pathway.



Model III: the truncated receptors have a dominant negative effect. Heterodimers between wild-type and mutant receptors are inactive and differential activation of the two pathways occurs. This model differs from Model II in that the reduction of activity is more severe than 75%.

Appendix I

Effect of the temperature sensitive *let-23(n1045)* mutation on different phenotypes at 15°, 20°, and 25°

Effect of the *let-23(n1045)* mutation at three different temperatures for five phenotypes are shown. This appendix provides supplemental data to Chapter II (Aroian and Sternberg, 1991). For complete figure legend and experimental details, see Table 1, Chapter II. The data for + / + and *n1045* (20°), taken from Table 1, Chapter II, is shown for ease of comparison. *Df* = *mnDf68*.

Aroian, R.V. and Sternberg, P.W. (1991). Multiple functions of *let-23*, a *Caenorhabditis elegans* receptor tyrosine kinase gene required for vulval induction. Genetics 128, 251-267.

♂→ / ♀↓

♀ \ ♂	+	n1045 (25°C)	n1045 (20°C)	n1045 (15°C)	Df
+	100 (21)				
n1045 (25°C)	100 (21)	107 (23)			15 (19)
n1045 (20°C)	100 (20)		44 (42)		4.4 (30)
n1045 (15°C)				11 (29)	

% vulval induction

♂→ / ♀↓

♀ \ ♂	+	n1045 (25°C)	n1045 (20°C)	n1045 (15°C)	Df
+	100 (334)				
n1045 (25°C)	98 (192)	44 (307)			33 (127)
n1045 (20°C)	97 (341)		42 (502)		25 (211)
n1045 (15°C)				31 (247)	

% survival

♂→ / ♀↓

♀ \ ♂	+	n1045 (25°C)	n1045 (20°C)	n1045 (15°C)	Df
+	100 (20)				
n1045 (25°C)	100 (20)	4 (24)			0 (22)
n1045 (20°C)	100 (21)		24 (38)		0 (17)
n1045 (15°C)				18 (22)	

% wild-type spicules

♂→ / ♀↓

♀ \ ♂	+	n1045 (25°C)	n1045 (20°C)	n1045 (15°C)	Df
+					
n1045 (25°C)	100 (20)	84 (45)			0 (17)
n1045 (20°C)	100 (23)		100 (26)		0 (30)
n1045 (15°C)				100 (45)	

% fertile hermaphrodites

♂→ / ♀↓

♀ \ ♂	+	n1045 (25°C)	n1045 (20°C)	n1045 (15°C)	Df
+	100 (21)				
n1045 (25°C)	100 (21)	100 (22)			94 (18)
n1045 (20°C)	100 (20)		91 (44)		97 (30)
n1045 (15°C)				55 (33)	

% wild-type P12 fate

Appendix II

The *let-60* locus controls the switch between vulval and nonvulval cell fates in *Caenorhabditis elegans*

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The *let-60* Locus Controls the Switch Between Vulval and Nonvulval Cell Fates in *Caenorhabditis elegans*

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ABSTRACT

During induction of the *Caenorhabditis elegans* hermaphrodite vulva by the anchor cell of the gonad, six multipotent vulval precursor cells (VPCs) have two distinct fates: three VPCs generate the vulva and the other three VPCs generate nonspecialized hypodermis. Genes that control the fates of the VPCs in response to the anchor cell signal are defined by mutations that cause all six VPCs to generate vulval tissue (Multivulva or Muv) or that cause all six VPCs to generate hypodermis (Vulvaless or Vul). Seven dominant Vul mutations were isolated as dominant suppressors of a *lin-15* Muv mutation. These mutations are dominant alleles of the gene *let-60*, previously identified only by recessive lethal mutations. Our genetic studies of these dominant Vul recessive lethal mutations, recessive lethal mutations, intragenic revertants of the dominant Vul mutations, and the closely mapping semidominant multivulva *lin-34* mutations suggest that: (1) loss-of-function mutations of *let-60* are recessive lethal at a larval stage, but they also cause a Vul phenotype if the lethality is rescued maternally by a *lin-34* gain-of-function mutation. (2) The dominant Vul alleles of *let-60* are dominant negative mutations whose gene products compete with wild-type activity. (3) *lin-34* semidominant Muv alleles are either gain-of-function mutations of *let-60* or gain-of-function mutations of an intimately related gene that elevates *let-60* activity. We propose that *let-60* activity controls VPC fates. In a wild-type animal, reception by a VPC of inductive signal activates *let-60*, and it generates into a vulval cell type; in absence of inductive signal, *let-60* activity is low and the VPC generates hypodermal cells. Our genetic interaction studies suggest that *let-60* acts downstream of *let-23* and *lin-15* and upstream of *lin-1* and *lin-12* in the genetic pathway specifying the switch between vulval and nonvulval cell types.

VULVAL development in *Caenorhabditis elegans* has been studied intensively as a model system to understand the mechanism by which cell-cell interactions specify the formation of a pattern of cell types during animal development (for recent reviews see HORVITZ 1988; STERNBERG 1990). During postembryonic development of the *C. elegans* hermaphrodite, each of six vulval precursor cells (VPCs) has the potential to generate either vulval cells or hypodermal cells. During vulval induction, however, only three of the six VPCs are specified to become the two VPC types, 1° and 2°, by a graded signal from the anchor cell of the gonad (Figure 1). 1° and 2° precursor cells divide further to form the vulva. The other three VPCs remain in the ground state (3° cell type) and generate progeny that fuse with a large syncytial hypodermal cell (SULSTON and HORVITZ 1977; KIMBLE 1981; STERNBERG and HORVITZ 1986; STERNBERG 1988). The relative positions of the VPCs with respect to the anchor cell determine which of them are induced to 1° or 2° cells (STERNBERG and HORVITZ 1986). Besides the inductive signal from the anchor cell, a "lateral signal" acts between the VPCs to prevent the immediate neighbors of a presumptive 1° cell from also becoming 1° cells (STERNBERG 1988).

Mutations that result in misspecification of VPC fates have defined genes necessary for the normal patterning process (HORVITZ and SULSTON, 1980; SULSTON and HORVITZ 1981; GREENWALD, STERNBERG and HORVITZ 1983; FERGUSON and HORVITZ 1985, 1989; FERGUSON, STERNBERG and HORVITZ 1987). Vulvaless (Vul) mutations cause fewer than three VPCs to generate vulval cells, often resulting in an egg-laying defect (Figure 1). Multivulva (Muv) mutations cause more than three VPCs to generate vulval cells and undergo morphogenesis to produce additional vulval-like structures (Figure 1). These mutations define three major classes of genes: (1) "Vul" genes are necessary for 1° and 2° cell fates. (2) "Muv" genes promote the 3° cell fate. (3) *lin-12* is necessary for determining 2° cell fates. Genetic interactions among these three classes of mutants suggest that there are two interacting genetic pathways that specify the fates of VPCs (STERNBERG and HORVITZ 1989): Vul and Muv genes act in a pathway that determines whether a VPC becomes a 3° (nonvulval) or a non-3° (vulval) cell, and the *lin-12* gene functions in a separate pathway that determines whether a VPC becomes a 2° or non-2° cell. The sites of action of the Muv and Vul genes are not known, but based on

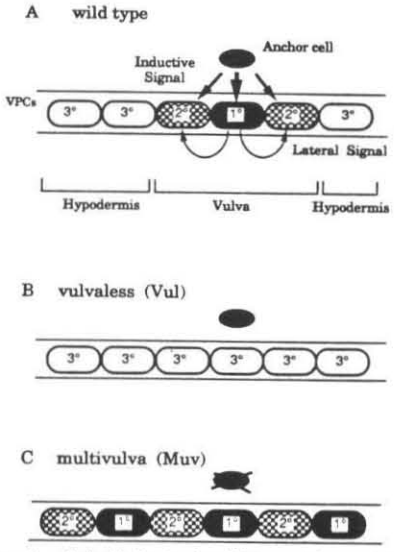

FIGURE 1.—Vulval induction in wild type and mutant *C. elegans* hermaphrodites. (A) The six multipotent vulval precursor cells (VPCs), P3.p, P4.p, P5.p, P6.p, P7.p and P8.p, are located just ventral to the gonad. Their fates are regulated by the anchor cell of the gonad. According to the current model (STERNBERG and HORVITZ 1986; STERNBERG 1988), a graded signal from the anchor cell induces the central three cells (P5.p, P6.p and P7.p) to generate vulval sublineages (1° or 2°) as opposed to a nonvulval sublineage (hypodermal cell, 3°). The 1° VPC (P6.p in wild type) prevents neighboring VPCs from also becoming 1° via a lateral signal. (B) In vulvaless mutant hermaphrodites, all six VPCs adopt the nonvulval fate (3°) even in presence of the inductive signal. (C) In multivulva mutant hermaphrodites, all six VPCs generate vulval sublineages (1° or 2°) even if anchor cell is ablated.

gonadal ablation and epistasis experiments, most do not act only in the anchor cell (FERGUSON, STERNBERG and HORVITZ 1987). Recent genetic mosaic experiments indicate that *lin-15*, a Muv gene, can act in cells other than the VPCs (R. HERMAN and E. HEDGECOCK, personal communication). However, since the outcome of the signaling pathway is the control of VPC fates, many of the Vul and Muv genes are expected to act within the VPCs in the response to the inductive signal. Understanding how these Muv and Vul genes interact with each other to specify VPC fates is the key to understanding the molecular genetics of this process.

Given the expected complexity of such a cellular regulatory pathway, we predicted that all essential components were not yet identified. To further dissect this pathway, we have taken the approach of isolating mutations that suppress existing Muv mutations. This approach might not only improve the efficiency at which the mutations that are directly involved in VPC induction are isolated but also might indicate how the new and existing genes interact in the pathway. In this paper we describe the isolation and characterization of dominant extragenic suppressors of the Muv mutation *lin-15(n309)*. These dominant suppressor mutations result in a dominant vulvaless phenotype, and are dominant negative ("antimorphic") alleles of *let-60*, previously identified only by recessive mutations with a lethal phenotype. We show, by analysis of the dominant and recessive alleles of *let-60*, that *let-60* function is essential for specifying 1° and 2° vulval cell types, since reduction or elimination of the gene activity results in a vulvaless phenotype. We also present suggestive evidence that the *lin-34* Muv mutations are gain-of-function alleles of *let-60*; the Muv phenotype (where more than three VPCs become vulval cell types) might be caused by *let-60* hyperactivity. Our study of the genetic interactions of *let-60* and other genes in the vulval induction pathway indicates that *let-60* acts downstream of *let-23* and *lin-15* but upstream of *lin-1* and *lin-12*.

MATERIALS AND METHODS

General methods: Methods for culturing, handling, mutagenesis, and genetic manipulation of *C. elegans* were as described by BRENNER (1974). All experiments were performed at 20°. The standard *C. elegans* cellular and genetic nomenclature, defined by SULSTON and HORVITZ (1977) and HORVITZ *et al.* (1979), respectively, is followed in this paper. "VPCs" (vulval precursor cells) are the six cells (P3.p, P4.p, P5.p, P6.p, P7.p and P8.p) that have the potential to participate in vulval development.

Strains: The standard wild type strain N2 and most other strains with various genetic markers were from BRENNER (1974) or the Caenorhabditis Genetics Center. The alleles of various mutants used in the paper are listed below. The source of strains other than BRENNER (1974) or the Genetics Center are also indicated.

LGI: *dpy-5(e61)*.

LGII: *rol-6(e187)*; *unc-4(e120)*; *let-23(mn23)* and *mnC1[dpy-10(e128) unc-52(e444)](II)*(HERMAN 1978).

LGIII: single mutations: *unc-36(e251)*; *unc-32(e189)*.

Linked double mutations: *lin-12(n137) dpy-19(e1259)* (FERGUSON and HORVITZ 1985) and *unc-32(e189) lin-12(n676 n909)* (GREENWALD, STERNBERG and HORVITZ 1983).

LGIV: single mutations: *dpy-20(e1282)*; *unc-22(s7)* (MOERMAN and BAILLIE 1979); *nT1(IV;V)* (FERGUSON and HORVITZ 1985); *lin-34(n1046)* (FERGUSON and HORVITZ 1985); *sDf8* (MOERMAN and BAILLIE 1981); *nDf27* (ELLIS and HORVITZ 1986). *lin-1(e1275)* (HORVITZ and SULSTON 1980).

Linked multiple mutations: *unc-24(e138) mec-3(e1338)dpy-20(e1282)* (provided by M. CHALFIE's laboratory), *dpy-20(e1362) unc-31(e169)*, *dpy-20(e1282) unc-22(s7)* (provided by D. BAILLIE's laboratory); *lin-3(n1059) dpy-20(e1282)* (provided by R. HILL); *unc-8(e49) dpy-20(e1362)*; *let-60(s59) unc-22(s7) unc-31(e169)* and *let-65(s254) unc-22(s7)* (ROGALSKI, MOERMAN and BAILLIE 1982), *let-100(s1160) unc-22(s7) unc-31(e169)*, *let-60(s1124) unc-22(s7) unc-31(e169)* and *let-60(s1155) unc-22(s7) unc-31(e169)* (CLARK *et al.* 1988).

LGV: *dpy-11(e224)*; *him-5(e1490)* (HODGKIN, HORVITZ and BRENNER 1979).

LGX: *lon-2(e678)*; *unc-3(e151)*; *lin-15(n765)* and *lin-15(n309)* (FERGUSON and HORVITZ 1985).

Analysis of vulval developmental defects: Criteria for recognition of egg-laying defect (Egl) and multivulva (Muv) phenotype were previously described by HORVITZ and SULSTON (1980). For counting percentage Muv, adult animals with one or more pseudovulvae (ventral protrusions) in addition to a vulva were classified as Muv. The vulvaless (Vul) phenotype is examined by observing L3 and L4 larvae under Nomarski optics. The percentage of VPC induction is determined as the percentage of VPCs induced to vulval cell type relative to wild type. In a completely vulvaless animal, each of the six VPCs divide once to generate daughters that fuse with the syncytial hypodermis (the 3° fate). The induction in these animals is said to be 0%. In a wild-type hermaphrodite, three of the six VPCs are induced to divide further than the first round of division, producing the progeny characteristic of 1° and 2° VPCs (STERNBERG and HORVITZ 1986). The induction of these further divisions is said to be 100%. Animals with fewer than three cells induced to further division have less than 100% induction (Vul); animals with more than three VPCs induced have more than 100% induction (Muv). According to this definition, if only one of the two daughters of a VPC divided further to generate vulval tissue, the induction is one-half-cell. Therefore, an individual animal with 50% VPC induction would have one and "one-half" VPCs induced. See STERNBERG and HORVITZ (1986) for a discussion of such "hybrid" lineages.

To eliminate the signal producing anchor cell, we ablated the somatic gonad precursor cells during the L1 larval stage (SULSTON and WHITE 1980). The laser microbeam system used for ablation was described previously (AVERY and HORVITZ 1987; STERNBERG 1988).

Isolation of *lin-15(n309)* suppressors: Strain MT309 [*lin-15(n309)*] was mutagenized with ethylmethane sulfonate (EMS) and the F_2 progeny were screened for non-Muv revertants. In most cases, candidates were picked with an egg-laying defect (Egl) phenotype. Each candidate was transferred to a new plate and those that gave viable non-Muv progeny were characterized further. Ten revertants were isolated after screening about 100,000 F_1 mutagenized gametes. All revertants have an Egl plate phenotype and are defective in VPC induction. The dominant nature of seven alleles was established as follows. For each of these seven revertants, fewer than one-fourth of the healthy progeny of an individual non-Muv animal were Muv (other Muv progeny exploded during adulthood). Muv animals were individually picked to agar plates, and found to segregate only Muv progeny as the original parent *lin-15* strain, indicating loss of the suppressor. In addition, any suppressed non-Muv animals always segregate a small portion of Muv animals along with the majority of non-Muv progeny. These results indicate (1) the suppressor mutations in these strains are heterozygous; (2) these mutations are recessive lethal; (3) the suppressor and Egl phenotypes are dominant. All seven revertants were crossed with wild-type males and the suppressor mutations were recovered without the *lin-15* mutations in the background. We refer to these alleles as *let-60(dn)*, where *dn* is dominant negative (see RESULTS).

Genetic mapping of the *let-60(dn)* mutations: The seven dominant suppressor alleles were mapped by crossing the hermaphrodite mutants with males carrying genetic markers on different linkage groups and following the Egl phenotype (the plate phenotype of vulvaless animals observable under the dissecting microscope) in the F_1 progeny. All of the dominant alleles proved to be linked to linkage group IV. The results of three point mapping with markers on chromosome *IV* are shown in Table 1. A genetic map with *let-60*, *lin-34* and relevant nearby genes is shown in Figure 2.

TABLE 1

Genetic three-point mapping of *let-60(dn)* on chromosome *IV*

Markers			Recombinants with *let-60*/total Recombinants[a]	
A	B	*let-60* allele	A non-B[b]	B non-A[c]
unc-24	*dpy-20*	*sy100*	6/6	0/8
		sy92	7/7	0/3
		sy93	3/3	0/14
		sy94	7/7	0/3
		sy95	15/15	0/5
		sy99	19/19	0/11
		sy101	11/11	0/6
dpy-20	*unc-31*	*sy99*	0/7	8/8
dpy-20	*unc-22*	*sy100*	0/20	2/2
		sy99	0/4	2/2
let-65	*unc-22*	*sy93*		1/3
lin-3[d]	*dpy-20*	*sy100*		2/129
		sy93		0/45
		sy94		0/30

In each mapping experiment, *let-60(dn)* alleles were placed in *trans* to two linked markers on chromosome *IV*. Recombinants resulting from recombination between the two markers were selected and scored for the *let-60(dn)* phenotype.

[a] Number of recombinant animals that retain the *let-60* alleles out of total recombinants homozygous for one marker gene.

[b] Recombinants with genotype homozygous for marker A but not for marker B.

[c] Recombinants with genotype homozygous for marker B but not for marker A.

[d] The recessive lethal allele *n1059* is used for *lin-3*.

Isolation of intragenic revertants of *let-60(dn)*: The dominant Vul phenotype of *let-60(dn)* was reverted by screening for the appearance of non-Egl F_1 animals following EMS mutagenesis of *let-65* + + *unc-22*/+ *let-60(sy101dn)* *dpy-20* + hermaphrodites. F_2 eggs were picked from each plate with nonEgl F_1 animals. Candidate F_2 non-Egl animals were picked and analyzed further by crossing with marker strains. *sy101 sy163*, isolated by this method, suppresses the dominant Vul phenotype of *sy101* completely. The suppressor is tightly linked to *sy101dn*.

The dominant suppression phenotype of *let-60(dn)* was reverted by screening for the reappearance of the Muv phenotype of *lin-15(n309)* in a *let-60(dn)* background. Two *let-60(dn)* alleles, *sy94* and *sy101*, were used to construct strains with genotypes of the form *unc-24* + *let-60(dn)* +/+ *lin-3* + *dpy-20(e1282)*; *lin-15(n309)*/*lin-15(n309)*. The *lin-3* mutation used (*n1059*) is a recessive lethal allele. These strains were constructed by crossing *lin-3 dpy-20*/++ males to +*let-60(dn)*+/*unc-24*+*dpy-20*; *lin-15* hermaphrodites. F_1 cross progeny and F_2 progeny were picked and the animals with desired genotype were selected. Hermaphrodites homozygous for *lin-15* were identified by the Muv phenotype of the viable Dpy recombinants (resulting from recombination between *lin-3* and *let-60*). The *lin-15* Muv phenotype is completely suppressed in these strains, except for the rare Dpy recombinants, which can be easily distinguished from nonrecombinants. These strains were mutagenized with EMS and F_1 progeny were screened for nonDpy Muv animals resulting from new suppressor mutations. Since the experiment was designed to isolate intragenic loss-of-function mutations, only F_1 animals were screened. *sy127* was isolated from a mutagenized culture containing both the strain with *sy94dn* and the strain with *sy101dn*, which had

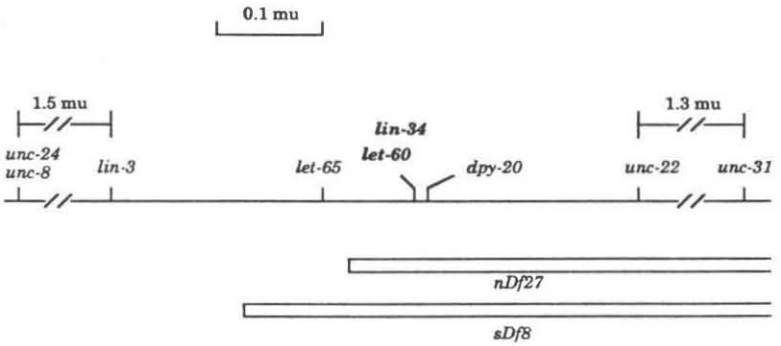

FIGURE 2.—Genetic map of relevant genes near *let-60* on chromosome *IV*. *let-60-lin-34* and seven other genes are shown along with the left breakpoints of two deficiencies. Relative distance between the genes other than *let-60-lin-34* are based on the current genetic map (EDGLEY and RIDDLE 1989). The relative distance between *let-60* and other genes were determined based on the genetic data given in Table 1 and MATERIAL AND METHODS. mu, map unit.

been inadvertently mixed. Therefore, the precise genotype of the new strain is not clear, and is designated as *syXdn sy127*. A dominant Muv mutation, *sy130gf*, was also isolated from the strain with *sy94dn* as the *let-60* allele; *sy130gf* was localized in *trans* on the *lin-3 dpy-20* chromosome. Both *syXdn sy127* and *sy130* were characterized further by crossing out the *lin-15* mutation.

Intragenic revertants should be recovered at similar frequency to that of recovering loss-of-function mutations in a wild-type strain (typically, between 1/2000 and 1/5000 EMS-mutagenized gametes; BRENNER 1974; GREENWALD and HORVITZ 1980). The frequency of isolating intragenic suppressors in this screen is tenfold lower: approximately 1/35,000 EMS-mutagenized gametes. One likely explanation is that *lin-15(n309)* animals are slow growing; the intragenic revertant (*let-60(lf)/+; lin-15*) may be less viable or fecund than the parental strain (*let-60(dn)/+; lin-15*). In this screen, we have picked more than 15 Muv animals as candidates for harboring suppressor mutations, but only two gave viable progeny.

Genetic mapping of *let-60(dn)* revertants: All three new mutations, *sy127*, *sy163* and *sy130*, were mapped with respect to nearby markers. *sy127* was mapped relative to *unc-8*, *unc-31* and *dpy-20* (see Figure 2). All three Unc-8 nonDpy-20 recombinants from a strain of genotype *unc-8 + + dpy-20(e1362)/+ unc-24 sy127* +segregated *sy127*. All four Unc-31 nonUnc-22 nonLet recombinants from *unc-8 + sy127+ +/+ let-100 + unc-22 unc-31* heterozygotes picked up *sy127*. Therefore, *sy127* is located between *unc-8* and *unc-31* and close to *dpy-20* and *unc-22*. The distance between *unc-8* and *unc-31* is approximately 3 map units. *sy163* was mapped relative to *unc-24* and *unc-31*. All nine Unc-24 nonUnc-22 recombinants from a strain of genotype *unc-24 ++ unc-22/ + sy163 dpy-20 +* segregated *sy163* and *dpy-20*. All ten Unc-31 nonLet recombinants from strains of genotype *+let-100 ++ unc-22 unc-31/unc-24 + sy163 dpy-20 ++* and *let-100 ++ unc-22 unc-31/+ sy163 dpy-20 ++* segregated *sy163*. Therefore, *sy163* is located between *unc-24* and *unc-31* and close to *unc-22*.

Four-point mapping for *sy130* was done by constructing a *let-65 ++ unc-22/+ lin-34(sy130gf) dpy-20+* heterozygote and screening for Unc nonLet recombinants. *lin-34(sy130gf)* confers a semidominant Muv phenotype (see Table 2). Among 35 Unc recombinants selected, 23 segregated Dpy and Muv progeny, 12 segregated neither Dpy nor Muv progeny, and none segregated Muv nonDpy progeny. Therefore *sy130* maps between *let-65* and *unc-22* and close to *dpy-20*. We also isolated two animals of genotype *lin-34(n1046gf) dpy-20/let-60(sy100) dpy-20* as recombinants from *lin-34(n1046gf) + unc-22/let-60(sy100) dpy-20 +* heterozygotes, placing *lin-34* to the left of *dpy-20* (Figure 2). Similar data placing *lin-34* just left of *dpy-20* in the region of *let-60* have been obtained by G. BEITEL and R. HORVITZ, and by G. JONGEWARD (both personal communications).

Complementation tests: The following tests were performed.

let-60(dn) with deficiencies: For *sDf8* and *nDf27*, *+let-60(sy100) dpy-20+/lin-34(n1046) ++ unc-22*; *him-5* males were crossed to hermaphrodites carrying deficiencies in *trans* to *nT1* (a chromosomal translocation between linkage groups IV and V that balances the right half of IV). The presence of Unc cross progeny (*Df/unc-22*) indicated that the mating was successful. Since these deficiencies uncover the *dpy-20* mutation, the absence of viable Dpy progeny indicates the failure of *let-60* to complement the deficiencies.

let-60(dn) with let-60(dn): For *sy92* and *sy95*, *++ let-60(sy100) dpy-20/unc-24 lin-34(n1046) ++* males were crossed with *unc-24 + let-60(dn) + /unc-24 mec-3 +dpy-20* hermaphrodites. Only rare nonUnc nonDpy animals are found among the cross progeny (*e.g.*, two nonDpy nonUnc hermaphrodites among more than 20 Dpy nonUnc hermaphrodites from one cross). These rare nonDpy nonUnc F_1 animals were recombinants (*unc-24 + dpy-20(e1282)/+ lin-34(n1046)+*) because they all segregated both Dpy Unc (*unc-24 dpy-20*) and Muv nonUnc (*lin-34(n1046gf)*) F_2 hermaphrodites. Neither *+ sy100 dpy-20/unc-24 sy95 +* nor *+sy100 dpy-20/unc-24 sy92 +* animals were found among the cross progeny, and thus these genotypes were inferred to be lethal. For *sy94*, a similar result was obtained with *unc-24 + let-60(sy100) dpy-20(e1282)/+ lin-34(n1046gf) ++ unc-22* males crossed with *unc-24 ++ let-60(sy94dn) +/+ lin-3 lin-34(sy130gf) +dpy-20* hermaphrodites.

let-60(dn) with let-60(lf): *let-60(s1124)* and *let-60(s59)*, previously isolated in a screen for recessive lethal mutations (CLARK *et al.* 1988), are loss-of-function alleles (see RESULTS). Males of genotype *let-60(s1124) + unc-22unc-31/+ dpy-20(e1282) ++*; *him-5/+* were crossed with *++ let-60(sy100dn) dpy-20/lin-3 lin-34(sy130gf) +dpy-20* hermaphrodites. Phenotypically nonDpy F_1 hermaphrodites were examined for vulval induction and further analyzed. Among more than 50 nonDpy F_1 progeny examined, half were egg-laying competent (nonEgl) and were determined to be *++ let-60(s1124) + unc-22 unc-31/lin-3 lin-34(sy130gf) + dpy-20 ++*. (Their progeny were used to examine the vulval induction of *s1124/s1124* progeny). The other half of the F_1 progeny were Egl and they segregated only dead larvae as the F_2. The genotype of this latter class must be *let-60(s1124) + unc-22 unc-31/let-60(sy100) dpy-20 ++*, and they were rescued by maternal activity of *lin-34(sy130gf)* (see Figure 5). Therefore, *sy100* fails to complement *s1124*. A similar

analysis was carried out for *let-60(s59)*: *let-60(s59) unc-22/ dpy-20(e1362)*; *him-5* males were crossed with *lin-34(sy130gf) dpy-20/let-60(sy100) dpy-20*. As a control, we crossed N2 males with *let-60(sy100dn) dpy-20/lin-34(sy130gf)dpy-20* hermaphrodites and nonDpy F_1 hermaphrodites were examined for vulval induction and their genotype inferred by segregation as above. In addition, *s1124*, *s59* and *s1155*, the third previously isolated *let-60(lf)* allele, were tested for complementation with *sy100* for lethality in separate experiments. *let-60(lf) + unc-22/+dpy-20+* males were crossed with *let-60(sy100dn) dpy-20 +/lin-3(n1046gf) + unc-22* hermaphrodites. F_1 Egl hermaphrodites among nonDpy nonUnc cross progeny were picked and found to generate only dead larvae as F_2 progeny. *s1124* also fail to complement *sy99dn* and *sy101dn* in similar tests. *sy93dn* also failed to complement *let-60(s1124)*. However, from the cross between *let-60(s1124)/+* males and *sy93dn/sy93dn* hermaphrodites, a low percentage of *sy93dn/s1124* animals (4 out of more than 100) have been found among cross progeny. This observation is not surprising since *sy93dn* homozygotes are viable even though they grow slowly and display uncoordinated movement in addition to being vulvaless.

let-60(dn) revertants with let-60(dn): Both *cis*-dominant revertants of *let-60(dn)*, *sy101dn sy163* and *syXdn sy127*, were tested for complementation for viability with *let-60(sy100)*. For *sy101dn sy163*, *let-60(sy-100) dp-20 +/+ lin-34(n1046gf) unc-22* males were crossed with *let-100 ++ unc-22 unc-31/+ sy163 dpy-20 + unc-31* hermaphrodites; no viable Dpy animals were found among cross progeny. For *syXdn sy127, unc-24 let-60(sy100dn)dpy-20 +/+ lin-34(n1046gf) + unc-22* males were crossed with *unc-24 syXdn sy127 +/+ lin-34(n1046gf) unc-22*; *lin-15(n765)* hermaphrodites; only rare recombinant Unc-24 homozygotes were found among the cross progeny. Therefore, *sy101dn sy163* and *syXdn sy127* fail to complement *let-60(sy100)* for viability.

let-60(dn) revertants with deficiency: syXdn sy127 ++/++ dpy-20 unc-22; *him-5* males were crossed with *sDf8/unc-24 mec-3 dpy-20* hermaphrodites. F_1 nonDpy animals were picked and tested for a twitching phenotype in 1% nicotine solution (indicating a genotype of *unc-22/+* or *sDf8/+*). It was found among more than 30 F_1 nonDpy animals tested, only one hermaphrodite shows the twitching phenotype and it is sterile. Therefore, *syXdn sy127/sDf8* is lethal.

***trans*-Heterozygotes:** The following tests were performed.

lin-34 with lin-34: sy130 was isolated as a dominant suppressor of the dominant suppression phenotype of *let-60(dn)*. *sy130* was identified as a putative *lin-34gf* allele by crossing *lin-34(n1046) unc-22(s7)/++* males into the revertant hermaphrodites of genotype *unc-24 + let-60(sy94dn)/+ lin-3 + sy130 dpy-20(e1282)*. Ninety-eight percent of the F_1 progeny with genotype *sy130/n1046* were found to be Muv.

lin-34 with deficiency: lin-34(n1046gf) + unc-31/+ dpy-20+ males were mated with *++ sDf8/unc-24 mec-3 dpy-20* hermaphrodites, which are Dpy. NonDpy F_1 hermaphrodites which should be *lin-3(n1046gf) unc-31/sDf8 +*, were picked for analysis. The percentage of Muv animals among nonUnc-31 adult animals was counted under a dissecting microscope. Seven percent (of 512 animals) were Muv. A strain of genotype *+lin-34(n1046gf) unc-22+ /unc-24 mec-3 + dpy-20+* was constructed for a *lin-34/+* control; 11% (of 467 heterozygous adult animals) were Muv.

lin-34 and let-60(dn): lin-34(n1046gf) was placed in *trans* to each of six *let-60(dn)* alleles (the recessive viable allele *sy93* was not tested). For *let-60(sy100dn)* and *let-60(sy101dn)*, strains with genotype *lin-34(n1046gf) + unc-22/ let-60(dn)dpy-20+* were constructed and analyzed. For *let-60(sy92dn)*, *let-60(sy94dn)* and *let-60(sy95dn)*, strains with genotype *+ lin-34(n1046)unc-22/unc-24 let-60(dn) +* were constructed and analyzed. *lin-34(sy130gf) dpy-20* was placed in *trans* to *let-60(sy100dn) dpy-20*, *let-60(sy99dn) unc-31* and *let-60(s1124) unc-22 unc-31*. When each of three *let-60(dn)* alleles, *sy100*, *sy95* or *sy92* was placed in *trans* to *lin-34*, approximately 1/6 to 1/4 the progeny of the heterozygous parents are homozygous *let-60(dn)/let-60(dn)*. These homozygotes are Vul and segregate only dead larvae as their progeny. We have also constructed similar *lin-34(gf)/let-60(dn)* heterozygotes with *him-5* in background so that we could examine the mating ability of the male animals (HODGKIN 1983). Individual L4 males of these strains was placed in a plate containing three to four hermaphrodites with either Unc (*unc-24*) or Dpy Unc (*dpy-20* and *unc-31*) phenotypes. Among four *let-60(dn)* allele examined, some of the males containing *sy100* (6 of 32), *sy94* (5 of 26), or *sy92* (13 of 22) were able to mate when in *trans* to *lin-34(n1046gf)*; none of the *sy101/n1046* heterozygous males were able to mate (none of 20). By contrast, 12 of 38 *lin-34(n1046gf) + unc-22 +/+ dpy-20 + unc-31*; *him-5* males were able to mate.

Construction and analysis of double mutants: The following methods were used.

let-60(s1124) with lin-15(n309): Heterozygous *+ let-60(s1124) + unc-22(s7) unc-31(e169)/unc-8(e49) + dpy-20(e1362) ++*; *him-5/+* males were mated with *dpy-20(e1282)*; *lin-15(n309)/lin-15(n309)* hermaphrodites. NonDpy F_1 hermaphrodites were picked to new plates. Each F_1 segregated Muv F_2 animals, which continued to propagate all Muv progeny. These Muv animals are heterozygous for the *unc-22* mutation but segregate mostly *unc-22* homozygotes as dead larvae, indicating that the genotype of the Muv animals is *let-60(s1124) + unc-22 unc-31/+ dpy-20 ++*; *lin-15*. Unc-22 animals that survive (L4 larvae or young sterile adults) were examined under Nomarski optics, and no vulval induction were found among 10 animals examined.

let-60(sy100dn) with lin-1(e1275): We first constructed a strain with *lin-1* linked to *unc-24* and *unc-22(s7)*. We then crossed *lin-1 unc-24 unc-22/+++* males with *+let-60(sy100dn) dpy-20 +/lin-34(n1046gf) ++ unc-22* hermaphrodites. The heterozygous cross progeny [*++ let-60(sy100dn) dpy-20/lin-1 unc-24 ++ unc-22*] were individually picked and their progeny were screened for Muv recombinants. Since *let-60(sy100dn)* and *dpy-20* are very tightly linked to each other [about 0.01 map units (m.u.)] and far from *lin-1* (>10 m.u.), nonUnc Muv animals almost exclusively resulted from recombination between *lin-1* and *unc-24*. More than six independent nonUnc Muv recombinants (*lin-1 + let-60(sy100) dpy-20 +/lin-1 unc-24 ++ unc-22*) were picked, and found to segregate Dpy Muv progeny [*lin-1 let-60(sy100) dpy-20*)]. The *lin-1* mutation suppresses both the lethal and Vul phenotype of *let-60(sy100dn)* homozygotes (see RESULTS).

let-60(s1124) with lin-1(e1275): A strain with *lin-1* linked to *dpy-20(e1282)* was constructed. We then crossed *lin-1 dpy-20/++* males with *let-60(s1124) + unc-22 unc-31/+dpy-20(e1362) + unc-31* hermaphrodites. The heterozygous *lin-1 +dpy-20 ++/+ let-60(s1124) + unc-22 unc-31* progeny were picked, and their progeny were screened for nonDpy Muv recombinants. Again, since *let-60* is very close to *dpy-20* and far away from *lin-1*, the nonDpy Muv animals all resulted from recombination between *lin-1* and *let-60* and gave rise to animals of genotype *lin-1 let-60(s1124) + unc-22 unc-31/lin-1 + dpy-20(e1282) ++*. The progeny of these recombinants were examined. Only a small number of Unc-22 animals were found on each plate (about 1/4 of Dpy Muv animals); all these animals were Muv and sterile. *let-*

60(s1124) homozygotes from heterozygous mothers often yield occasional survivors, but these survivors are Vul.

let-60(sy100dn) with lin-12(n137): A strain with genotype *dpy-19 + lin-12(n137)/+ unc-32 lin-12(n676 n909); him-5* (MT2375; P. STERNBERG and R. HORVITZ, unpublished) was used for the construction. *n137* is a dominant allele of *lin-12*. *n676 n909* is a *lin-12(d)* mutant plus an intragenic revertant resulting in loss of *lin-12* function (GREENWALD, STERNBERG and HORVITZ 1983). MT2375 males were mated to *++ let-65 + unc-22/unc-24 mec-3 + dpy-20+* hermaphrodites. The male cross progeny (showing Lin-12(d) phenotype) were picked and mated to *unc-36; + let-60(sy100dn) dpy-20/lin-34(n1046gf)++* hermaphrodites. Hermaphrodite progeny heterozygous for the *unc-22* mutation were selected with 1% nicotine (MOERMAN and BAILLIE 1979). Hermaphrodites with the Lin-12(d) phenotype (Egl with five small ventral protrusions) were picked. Those broods segregating *unc-36; let-60(s100dn) +/let-65 + unc-22* animals were identified, and their genotype was determined to be *+dpy-19 lin-12(n137)/unc-36 ++; +let-60(sy100) dpy-20 +/let-65 ++ unc-22*. Animals with this genotype display the Lin-12(d) phenotype. Analogous experiments were done with *let-60(sy99dn)* and *let-60(sy94dn)* with similar results.

let-60(s1124) with lin-12(n137): MT2375 males (see above) were crossed with *+ let-60(s1124) + unc-22 unc-31/unc-8 + dpy-20 ++* hermaphrodites. F_1 hermaphrodites heterozygous for *unc-22* (show twitching phenotype in 1% nicotine) were picked at the L4 stage. Egl adults with *lin-12(d)* phenotype (with five small ventral protrusions and Egl) were picked. These animals [*dpy-19 lin-12(n137)/++; let-60(s1124) unc-22 unc-31/+++*] segregated a small number of sterile Unc-22 F_2 animals which were homozygous for *s1124* (and had escaped from larval lethality). Visualized under Nomarski optics, seven out of ten of these Unc-22 animals had Lin-12(d) phenotype (all six VPCs are 2°). Only 3/4 of the Unc-22 animals were expected to be either heterozygous or homozygous for *lin-12(d)*.

lin-34(n1046gf) with let-23(mn23): Heterozygous *let-23(mn23)unc-4(e120)/mnC1* males were crossed with *lin-34(n1046gf)* hermaphrodites. F_1 nonMuv hermaphrodites were individually picked. Animals with a genotype of *let-23 unc-4/++; lin-34/+* were identified by analyzing the F_2 progeny of these broods. The F_2 homozygous Unc-4 animals were picked from the above double heterozygous F_1 mothers and examined for phenotypes. Twenty-six nonrecombinant Unc-4 animals were all sterile adults, and 23 of them were Muv. Therefore, the lethal, but not the sterile, phenotype of *let-23(mn23)* is suppressed by *lin-34(n1046gf)*.

RESULTS

Isolation of dominant Vul mutations as suppressors of a *lin-15* Muv mutation: *lin-15* mutations cause all six VPCs to become 1° or 2° (multivulva, or Muv, see Figure 4B) regardless of whether the inductive signal is present (FERGUSON and HORVITZ 1985; FERGUSON, STERNBERG and HORVITZ 1987; STERNBERG 1988; Sternberg and HORVITZ 1989). Mutations of genes whose products interact with *lin-15* gene product or of genes acting downstream of *lin-15* in the pathway might be expected to suppress the Muv phenotype of *lin-15*. We have isolated such suppressor mutations by mutagenizing *lin-15(n309)* animals with EMS and screening for phenotypically nonMuv revertants in the F_2 (Figure 3).

After screening approximately 100,000 EMS-mutagenized gametes, we isolated ten extragenic suppressors of the Muv phenotype of *lin-15(n309)*. Two of them are recessive, Vul mutations of *let-23* (R. V. AROIAN and P. W. STERNBERG, manuscript in preparation). Another suppressor also proved to be a recessive Vul mutation. This mutation, *sy96*, maps to the left of *unc-24* on chromosome *IV* and defines a new gene, *lin-45* (M. HAN and P. W. STERNBERG, unpublished results). The other seven mutations, which we analyze in this paper, have a dominant suppressor phenotype (Table 2) and all map to the same region on chromosome *IV*. They were located about 0.01 m.u. to the left of *dpy-20* by three-point mapping with a *lin-3* lethal allele and a *dpy-20* allele as markers (see MATERIALS AND METHODS, Table 1 and Figure 2). These seven mutations result in a dominant Vul phe-

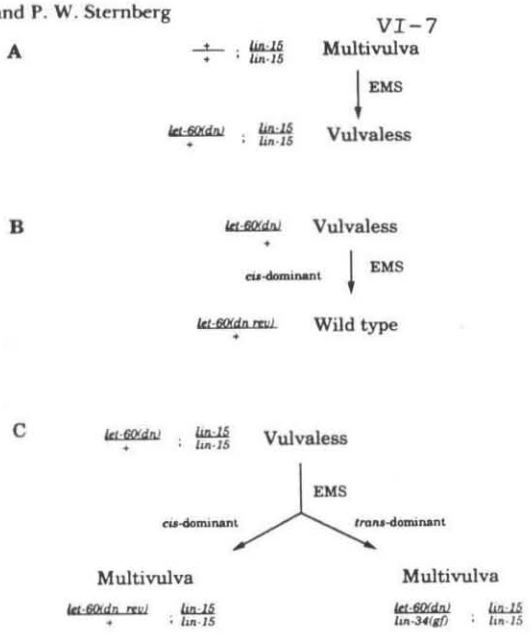

FIGURE 3.—Schematic illustration of the isolation of *let-60* and *lin-34* mutations as dominant suppressors. (A) Isolation of *let-60(dn)* mutations as dominant suppressors in strains with a *lin-15* mutation. Seven dominant *let-60* vulvaless mutations were isolated after screening about 100,000 EMS-mutagenized gametes for revertants of *lin-15* Muv phenotype. All these mutations were actually obtained in screening the F_2 progeny, although they were dominant and should be present in F_1. These mutations are referred to as *let-60(dn)* (*dn* for dominant negative, see text). (B) A loss-of-function allele of *let-60* was isolated as a *cis*-dominant suppressor of the vulvaless phenotype of *let-60(dn)/+*. The revertants have wild-type vulvae. (C) Isolation of dominant revertants of the dominant suppression phenotype of *let-60(dn)*. The suppressed *lin-15* Muv phenotype was re-isolated when the *let-60(dn)* dominant phenotype is suppressed *cis*-dominantly by a new mutation in *let-60* (putative loss-of-function (*lf*) allele, indicated by "rev" for revertant), or *trans*-dominantly by a new mutation in *lin-34*. Without *lin-15* in background, *let-60(dn rev)* has no dominant phenotype (see Figure 4H); *let-60(dn)/lin-34* has a weak Muv phenotype.

TABLE 2

Phenotypes of *let-60* and *lin-34* alleles

Class	Allele (*m*)	Hermaphrodite phenotype[a]				%Egl[c] (*m*/+)	Male mating[d] (*m*/+)	Mutant source[e]
		m/*m*	*m*/*m*; *n309*[b]	*m*/+	*m*/+; *n309*			
WT	+	WT	Muv	WT	Muv	0	+	
Deficiency	*sDf8*	Let	ND	WT	ND	0	+	(1)
Loss-of-function [*let-60*(*lf*)]	*sy101 sy163*	Let	Let	WT	Muv	0	+	This study
	syX sy127	Let	ND	WT	ND	0	+	This study
	s1124	Let	Let[f]	WT	Muv	0	+	(2)
	s1155	Let	ND	WT	ND	0	+	(2)
	s59	Let	ND	WT	ND	0	+	(3)
Dominant negative [*let-60*(*dn*)]	*sy93*	Vul	Vul	Vul	Vul	>99	−	This study
	sy99	Let	Let	Vul	Vul	97	−	This study
	sy101	Let	Let	Vul	Vul	97	−	This study
	sy94	Let	Let	Vul	Vul	93	−	This study
	sy100	Let	Let	Vul	Vul	89	−	This study
	sy95	Let	Let	Vul	Vul	59	−	This study
	sy92	Let	Let	Vul	Vul	42	−	This study
Gain-of-function [*lin-34gf*]	*n1046*	Muv	Muv	wMuv[g]	Muv	ND	+	(4)
	sy130	Muv	ND	wMuv	Muv	ND	+	This study

The mutations isolated and studied in this paper are divided into three different classes. The two gain-of-function mutations (*n1046* and *sy130*) are alleles of *lin-34* and the rest of the mutations are alleles of *let-60*.

[a] The phenotype of each *let-60* allele is described as "Vul" for vulvaless, "Muv" for multivulva, or "Let" for lethal. ND, not determined.

[b] Genotypes of *let-60*, *lin-34* and *lin-15*. "*m*" indicates the mutation in *let-60* or *lin-34*; "+" indicates the wild type allele. *n309* is an allele of *lin-15*. *m*/*m*; *n309* indicates the strain is homozygous for the *let-60* mutation on chromosome *IV* and homozygous for the *lin-15* mutation on chromosome *X*.

[c] The percentage of hermaphrodites that fail to lay eggs (only tested for *m*/+ strains). To score %Egl for the *let-60*(*dn*) alleles, strains with genotype *unc-24* + *let-60*+/+*let-65* + *unc-22* were used except *sy93*, which was not linked to *unc-24* in the test. For each of the *let-60*(*dn*) alleles, more than 200 F_1 progeny of Egl parents were scored. Fewer than 1% of the hermaphrodites were sterile.

[d] Male mating indicates the capability of males of *m*/+ genotype to mate with hermaphrodites. More than 30 L4 or young adult males were used in tests for each *let-60*(*dn*) allele. "−" indicates no cross-progeny were found in a mating test. Defects in male spicules were found in *let-60*(*dn*)/+ animals for all the *let-60*(*dn*) alleles.

[e] The references for previously isolated alleles: (1) MOERMAN and BAILLIE (1981); (2) CLARK *et al.* (1988); (3) ROGALSKI, MOERMAN and BAILLIE (1982); (4) FERGUSON and HORVITZ (1985).

[f] Animals that escape the early larval lethality are Vul, and die as young adults.

[g] "wMuv" indicates a weakly penetrant Muv phenotype. For *lin-34*(*n1046*) and *lin-34*(*sy130*), about 10–20% of the heterozygous animals are Muv, compared to greater than 90% Muv among homozygotes.

notype with or without *lin-15*(*n309*) in the background (Figures 3E and 4C). All of these mutations have cell lineage defects: fewer than three VPCs adopt the vulval cell fates (1° or 2°). Although all seven alleles suppress completely the Muv phenotype of *lin-15*(*n309*), they differ with respect to the penetrance of the Vul phenotype (Table 2). Males heterozygous for these mutations (*m*/+) have defective copulatory spicules and fail to mate (Table 2). Six of the seven dominant alleles are recessive lethal, arresting at larval developmental stages (L1-L2). Animals homozygous for the seventh allele, *sy93*, are viable and are Vul with or without *lin-15* in the background (Table 2).

The similar map locations and similar phenotypes of these seven mutations suggested that they define a single locus. Complementation tests for the recessive lethal phenotype indicate that these mutations are indeed alleles of one locus. We found *sy92*, *sy95* and *sy94* fail to complement *sy100* for viability (see MATERIALS AND METHODS).

Deficiencies of the region spanning this locus (Figure 2) do not have a dominant Vul phenotype (Table 2). Therefore, these suppressors are not loss-of-function mutations. As described below, we have determined that these dominant Vul mutations are dominant negative (designated *dn*) alleles of the gene *let-60*.

Isolation of intragenic revertants and determinants of the dominant Vul mutations as *let-60* alleles: To ascertain the wild-type function of the gene identified by the dominant Vul mutations, it was necessary to obtain and characterize intragenic revertants. A *lf*/+ (*lf* for loss-of-function) heterozygote is expected to exhibit a phenotype similar to that of a *deficiency*/+ animal and should not exhibit any dominant phenotype. Adding a loss-of-function mutation in *cis* to a dominant *let-60* allele should thus suppress the dominant phenotype caused by the allele.

To revert the dominant phenotype of the *let-60*(*dn*) alleles, we carried out two different screens. In one screen, we sought to isolate revertants of the Vul phenotype of *let-60*(*dn*) (Figure 3B). One tightly

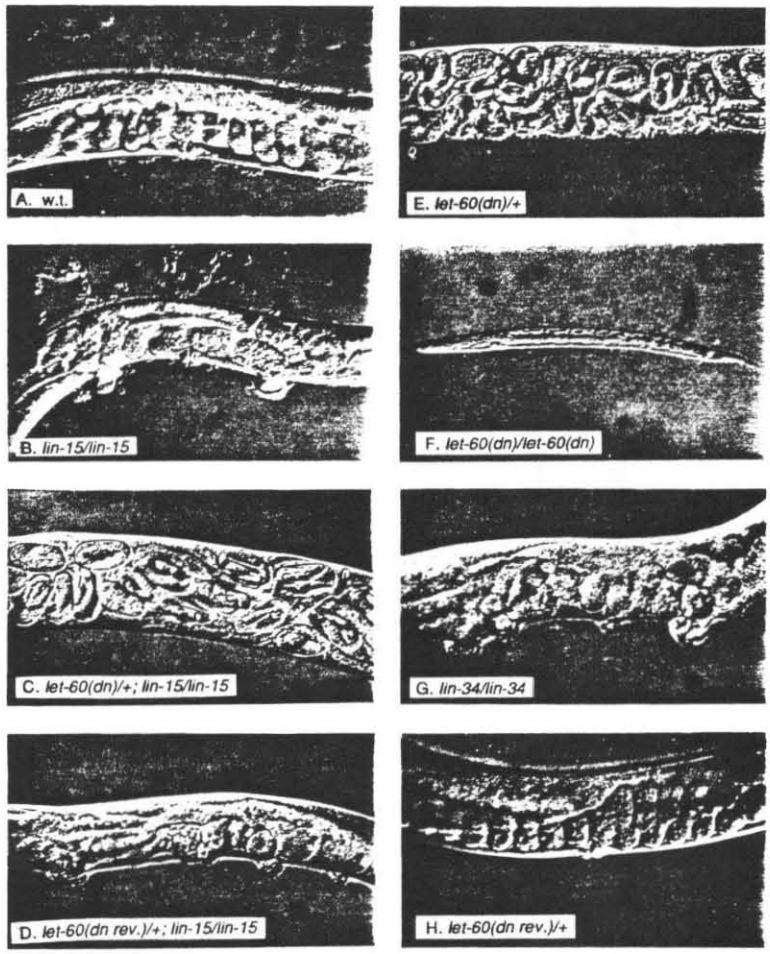

FIGURE 4.—Phenotypes in vulval development of hermaphrodites with mutations in *lin-15*, *let-60* and *lin-34*. Photomicrographs were taken with Nomarski optics (Plan Neofluor 40X dry lens with Kodak 2415 film). All animals are positioned with ventral side to the bottom of the micrograph. (A) and (H) show hermaphrodites with wild-type vulva. (B), (D) and (G) show multivulva (Muv) hermaphrodites. Additional vulval cells were induced in these Muv animals and gave rise two to three pseudo-vulval structures in the ventral hypodermis. (C) and (E) show vulvaless (Vul) hermaphrodites. The eggs hatch inside these Vul animals due to the defect in egg-laying. (F) shows a dead early larval hermaphrodite. The complete genotype for each of the animals is: (A), N2; (B), *lin-15(n309)*; (C), *unc-24 let-60(sy94dn) +/+lin-3 + dpy-20; lin-15*; (D), *unc-24 let-60(sy127) ++/ ++ dpy-20 unc-22; lin-15*; (E), *+let-60(sy100dn) dpy-20+/let-65 ++ unc-22*; (F), *let-60(sy100dn) dpy-20/let-60(sy100dn) dpy-20*; (G), *lin-34(sy130gf) dpy-20*; (H), *+unc-24 let-60(sy127) + /unc-8 ++ dpy-20*. Scale bar is 20 μm.

linked dominant suppressor of *let-60(sy101dn)*, *sy163* (Table 2), was isolated after screening approximately 9000 EMS-mutagenized gametes. *sy163* suppresses the *let-60(dn)* dominant phenotypes completely, and the double mutant alleles (*sy101dn sy163*) remain recessive lethal at a young larval stage. *sy101dn sy163/+* hermaphrodites have a wild-type level of vulval induction.

In another screen, we sought to restore the Muv phenotype to a *lin-15(n309)* strain which is dominantly suppressed by a *let-60(dn)* and hence Vul (Figure 3C). From a screen of approximately 35,000 mutagenized gametes, we isolated two new mutations that suppress the suppressor phenotype of *let-60(dn)*. In both isolates, the Muv phenotype of *lin-15(n309)* reappears [being no longer suppressed by the *let-60(dn)* mutation]. The dominant Vul phenotype of *let-60(dn)* is also completely suppressed by two new alleles in the absence of the *lin-15* mutation. In one of the revertants, *syXdn sy127* (*syX* is either *sy94dn* or *sy101dn*, see MATERIALS AND METHODS for explanation), the new mutation is also tightly linked to the dominant negative allele. *syXdn sy127* is also recessive lethal at an early larval stage (L1-L2) and fails to complement both *sy100dn* and a deficiency for its lethal phenotype. The *syXdn sy127/+* heterozygous strain has a wild-type level of vulval induction (see Figure 4, D and H), and males of this genotype mate normally.

The two linked revertants, *sy101dn sy163* and *syXdn sy127*, are most likely intragenic revertants and loss-of-function mutations of the *let-60* gene (also see Table 2 for comparison). Both revertants behave like deficiencies uncovering the region and the recessive lethal *let-60* alleles [loss-of-function mutations (*lf*); discussed below] isolated independently of *let-60(dn)*

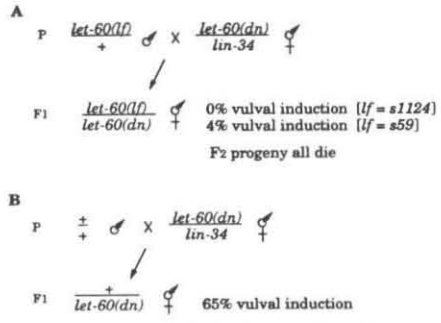

FIGURE 5.—Genetic interactions between *let-60(dn)* and *let-60(lf)*. The complete genotypes of the parent strains are described in MATERIALS AND METHODS. *dn*, dominant negative; *lf*, loss-of-function; *gf*, gain-of-function. The allele for *let-60(dn)* is *sy100*, and the allele for *lin-34(gf)* is *sy130*. *s1124* and *s59* were used as loss-of-function mutations. See Figure 6 for the maternal effect of *lin-34(gf)*. Only hermaphrodite F_1 and F_2 progeny were analyzed. Compared to 65% vulval induction in *+/let-60(dn)* animals (B), the 0% and 4% vulval induction phenotype of *let-60(lf)/let-60(dn)* animals (A) indicates the *let-60(lf)* alleles fail to provide function in vulval induction.

(CLARK *et al.* 1988). It is unlikely that our "*cis*" revertants (*rev*) and the dominant Vul alleles (*dn*) are in different, nearby genes: the phenotype of *let-60(dn rev)/+* is wild type and thus is distinct from the lethal phenotype of *let-60(dn)/let-60(lf)*.

Previously, three *let-60* larval lethal mutations were isolated by screening recessive lethal alleles in the region on chromosome *IV* (ROGALSKI, MOERMAN and BAILLIE 1982; CLARK *et al.* 1988) (Table 2). As described above, we carried out complementation tests between these previously isolated alleles and our dominant Vul recessive lethal mutations, and found they fail to complement for viability (see MATERIALS AND METHODS, also Figure 5). The three previously isolated lethal alleles as well as our two *cis* revertant alleles, *sy101dn sy163* and *syXdn sy127*, are recessive and behave in complementation tests similar to a deficiency uncovering the *let-60* locus. The three alleles were isolated in relatively high frequency (3 out of an equivalent of 6500 EMS-mutagenized gametes, ROGALSKI, MOERMAN and BAILLIE 1982; CLARK *et al.* 1988) [see MATERIALS AND METHODS for discussion of relative frequencies of obtaining *let-60(lf)*]. Based on all these results, we believe that the five recessive lethal alleles, including the two tightly linked *let-60(dn)* revertants and the three previously isolated alleles, represent loss-of-function mutations of *let-60*.

***let-60* function in vulval development and the nature of the dominant Vul mutations:** To determine the function of the *let-60* gene in vulval development, it is critical to know the phenotype of a loss-of-function mutation. We have already discussed above that the loss-of-function mutations are recessive lethal prior to the L3 stage and have no dominant phenotype. It is thus difficult to study the phenotype of loss-of-function mutations in vulval development, which occurs during the L3 stage. However, some of the *s1124lf/s1124lf* progeny from a *s1124lf/+* heterozygote can surpass the larval lethal stage to survive to the early adult stage. We have examined the vulval induction in such "survivor" animals of genotype *let-60(s1124lf)/let-60(s1124lf)* from a *let-60(s1124lf)/lin-34(sy130gf)* mother [see below for analysis of *lin-34(sy130gf)*]. Under Nomarski optics, we found that these survivors have 0% VPC induction (14 animals examined). Failure of vulval induction in these animals is not due to the fact that the animals are sick or dying, since the Vul phenotype of the surviving *let-60(s1124lf)* animals can be completely suppressed by *lin-1* (see below). This result indicates that Vul is also a loss-of-function phenotype.

We also performed a genetic interaction analysis to overcome the recessive lethal problem of *let-60(lf)* and determine the phenotype of loss-of-function alleles in vulval induction. Two previously isolated recessive lethal alleles, *s1124* (CLARK *et al.* 1988) and *s59* (ROGALSKI, MOERMAN and BAILLIE 1982), were placed in *trans* to our dominant Vul, recessive lethal allele *let-60(sy100dn)*. A *let-60(sy100dn)* homozygote from a *lin-34(gf)/let-60(dn)* mother is viable for one generation (it normally would be larval lethal from a *let-60(dn)/+* mother) (see below and Figure 6). We took advantage of this maternal effect of *lin-34(gf)* Muv mutations to examine interactions between *let-60(sy100dn)* and *let-60(lf)*. A *let-60(dn)/let-60(lf)* heterozygote from a *let-60(dn)/lin-34(gf)* mother is also expected to live for one generation and hence allows us to examine the phenotype in vulval induction (Figure 5). We crossed *let-60(lf)/+* males with *let-60(sy100dn)/ lin-34(sy130gf)* hermaphrodites, we found that *let-60(lf)* fails to provide any function in vulval induction when lethality is rescued. Examined with Normarski opitics, *let-60(lf)/let-60(sy100dn)* animals from a *let-60(sy100dn)/lin-34(sy130gf)* mother have nearly no vulval induction (0% VPC induction among 16 *s1124lf/sy100dn* heterozygous hermaphrodites and 4% VPC induction among eight *S591f/sy100dn* heterozygous hermaphrodites.) By contrast, *sy100dn/+* animals (18 examined) from a cross between wild-type (N2) males and hermaphrodites *let-60(sy100dn)/lin-34(sy130gf)* display about 65% VPC induction (Figure 5). This result confirms that loss-of-function results in a vulvaless phenotype. We thus conclude that *let-60* is necessary for vulval development.

Since the *let-60(dn)* mutations act in the same phenotypic direction (Vul and Let) as *let-60(lf)*, these dominant Vul mutations of *let-60* are "dominant negative" (*dn*) mutations ("antimorphic mutations"). In a

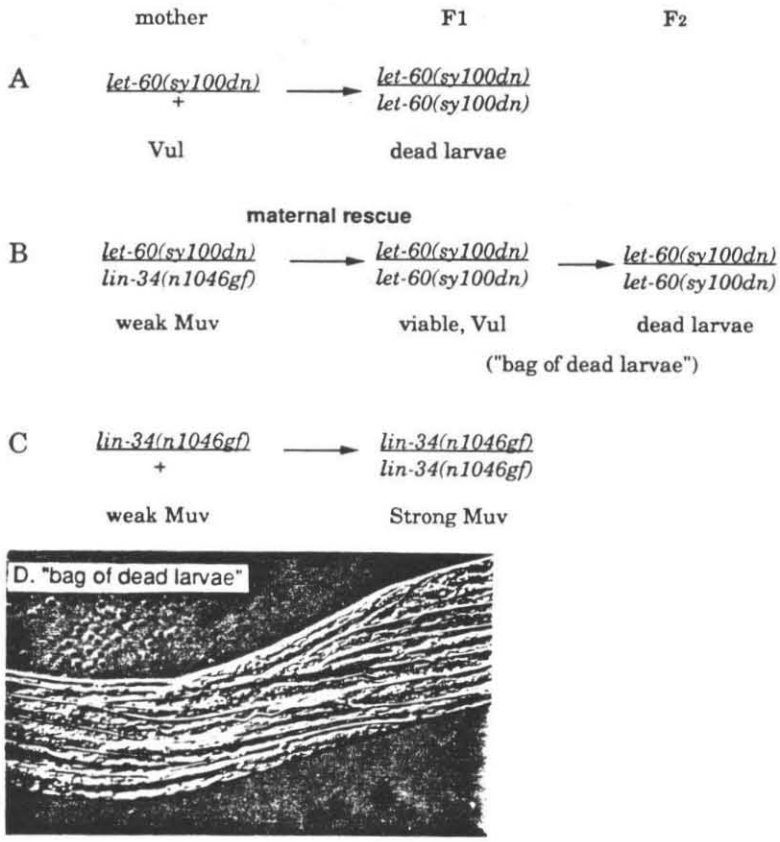

FIGURE 6.—Dominant suppression of *let-60(dn)* by semidominant Muv mutations of *lin-34gf*. (A) *let-60(sy100dn)* is dominantly vulvaless and recessively lethal at an early (L1-L2) larval stage (Table 2). (B) A *lin-34(gf)* allele (*n1046* or *sy130*), in *trans* to *let-60(sy100dn)*, completely suppresses the Vul phenotype of *let-60(sy100dn)*. The lethality of *sy100/sy100* is also suppressed through the dominant maternal effect of the *lin-34* mutation. The F_1 *sy100/sy100* progeny are viable and completely Vul, and their progeny (F_2) are all lethal at larval stages. Most of these F_2 larvae die in their mother's body so that a "bag of larvae" phenotype results. (C) *lin-34* mutations show a strong Muv phenotype (above 95% penetrant) as homozygotes and a weak Muv phenotype (less than 40%) as heterozygotes. (D) A maternally rescued hermaphrodite ("bag of dead larvae") described in (B). The genotype of the hermaphrodite is *let-60(sy100dn) dpy-20/let-60(sy100dn) dpy-20* and the genotype of its parent is *let-60(sy100dn)dpy-20 +/lin-34(n1046gf) + unc-22*. The photomicrograph was taken under Nomarski optics as in Figure 4 (same scale as in Figure 4).

let-60(dn)/+ heterozygote, there is less wild-type gene activity than that in a *let-60(lf)*/+ heterogyzgote. The dominant Vul phenotype of *let-60(dn)* is the result of this reduction of gene activity.

***lin-34* Muv mutations, tightly linked to *let-60*, suppress *let-60(dn)* phenotypes:** *lin-34* was previously defined by the semidominant Muv mutation *n1046* (FERGUSON and HORVITZ 1985). This mutation confers a "strong Muv" phenotype (defined here as greater than 80% penetrance) in homozygotes and a "weak Muv" phenotype (defined here as less than 30% penetrance) in heterozygotes. Additional semidominant Muv alleles of *lin-34* have been isolated as suppressors of mutations in *lin-10* (D. PARRY, S. KIM and R. HORVITZ, personal communication), *let-23* (G. JONGEWARD and P. W. STERNBERG, UNPUBLISHED RESULTS) AND *let-341* (S. CLARK and R. HORVITZ, personal communication). We have also isolated a semidominant Muv allele (*sy130*), as a dominant suppressor of the dominant suppressor phenotype of *let-60(dn)* (see above and Figure 3C). *sy130/sy130* animals are Muv (about 95%) (Figure 4G and Table 2). *sy130*/+ animals have a weak Muv phenotype (about 10%). *sy130* interacts in *trans* with *lin-34(n1046)* to produce a highly penetrant (strong) Muv phenotype (>95% *sy130/n1046* heterozygotes are Muv, see MATERIALS AND METHODS). Based on this result and similar mapping data for *sy130* and *lin-34(n1046)* (MATERIAL AND METHODS; G. BEITEL and R. HORVITZ, personal communication), we suggest that *sy130* is also an allele of *lin-34*. As *Df*/+ animals do not have a semidominant Muv phenotype, *sy130* is a gain-of-function (*gf*) mutation in *lin-34*. *sy130gf* also maps between *dpy-20* and *let-65*, very close to the *dpy-20* gene, as do all the *let-60* alleles (see MATERIALS AND METHODS and Figure 2). The fact that both *lin-34* and *let-60* are located in the same small chromosome interval suggests that the *lin-34* and *let-60* mutations might be different alleles of the same gene. This possibility is consistent with our observations of the genetic interactions between *let-60* and *lin-34*.

As described above, *lin-34(sy130gf)* was isolated as

a *trans*-dominant suppressor of *let-60(dn)*, indicating a close relationship between these two classes of mutations. We have further examined the interactions of *let-60* alleles with other *lin-34* Muv alleles. Three types of results demonstrate that the *lin-34* Muv mutations strongly suppress the *let-60* mutations (Figure 6). (1) The *lin-34* mutations dominantly suppress the dominant Vul phenotype of *let-60(dn)*: *let-60(dn)/lin-34* animals show the weakly penetrant Muv phenotype of *lin-34/+* rather than the Vul phenotype of *let-60(dn)/+*. Specifically, between 5% and 20% of animals of genotypes *lin-34(n1046)* or *lin-34(sy130)* in *trans* to each of six *let-60(dn)* alleles are Muv (the remaining 80–95% are wild type, data not shown). The suppression of the Vul phenotype of *let-60(dn)* by *lin-34* mutations is complete, even though the majority (80–90%) of *lin-34/+* animals are not Muv. (2) *lin-34* mutations suppress maternally the lethality of some *let-60(dn)* alleles (*sy100*, *sy92* and *sy95*). This maternal effect is also dominant. For example, homozygous *let-60(sy100dn)* F_1 progeny from a *let-60(sy100dn)/+* mother are normally lethal at a larval stage. However, the *let-60(sy100dn)/let-60(sy100dn)* F_1 progeny, from a *let-60(sy100dn)/lin-34(n1046)* parent are viable for one more generation; the F_2 progeny are all dead larvae (Figure 6). *lin-34* mutations do not rescue maternally the defect in vulval induction in *let-60(dn)/let-60(dn)* animals. The *sy100dn* homozygotes rescued by the *lin-34* maternal effect have 0% VPC induction (none of 10 animals examined under Nomarski optics had any VPCs induced to vulval cell types). (3) *lin-34* Muv mutations can partially overcome the male mating defect of some *let-60(dn)/+* animals; *sy92dn*, *sy95dn*, *sy100dn* and *sy94dn* males can mate at low efficiency if placed in *trans* to *lin-34(n1046)* (see MATERIALS AND METHODS).

A *lin-34* mutation in *trans* to a deficiency (*e.g.*, *lin-34(n1046)/sDf8*) displays a weak Muv phenotype (about 8% of animals are Muv), similar to *lin-34/+* (about 10–20%, see MATERIAL AND METHODS; also see FERGUSON and HORVITZ 1985). This observation suggests that the *lin-34* mutations are not loss-of-function mutations, because otherwise, the *lin-34/Df* hemizygotes should display a Muv phenotype of equal or greater penetrance than *lin-34/lin-34* homozygotes (above 90% Muv). Moreover, *lin-34(gf)*, which are most likely alleles of *let-60*, have a phenotype (Muv) opposite to that of *let-60(lf)* (Vul). Therefore, all the *lin-34* Muv alleles are likely to be gain-of-function mutations. A simple explanation for our results is that the activity of *let-60* is elevated by the presence of a *lin-34(gf)* mutation, either because *lin-34(gf)* mutations are gain-of-function alleles of *let-60*, or that *lin-34(gf)* mutations are gain-of-function alleles of another gene that acts positively in the same signaling pathway as *let-60*.

TABLE 3

Mutual suppression of *let-60(sy100dn)* and *lin-15(n309)*

Genotype		Phenotype			
let-60	*lin-15*	%Egl[b]	%Muv	%Induction[c]	%Induction without signal[d]
+/+;	+/+	<1	<1	100	0
+/+;	*n309/n309*	<1	100	200	200
sy100/+;	+/+[a]	87	<1	57	ND
sy100/+;	*n309/n309*	21	<1	88	0

[a] The complete genotype on chromosome *IV* is + + *let-60(sy100dn)* + */unc-24 mec-3* + *dpy-20*.

[b] Egl stands for egg-laying defective, which, in this case, results from an animal being vulvaless. More than 200 animals were scored.

[c] Percentage VPCs induced to vulval cells relative to wild type, scored with Nomarski optics (see MATERIALS AND METHODS).

[d] The signal is eliminated by ablation of gonad cells during the first larval stage (MATERIALS AND METHODS). Data for wild type are from SULSTON and WHITE (1980), and for *lin-15(n309)* from STERNBERG and HORVITZ (1989) and STERNBERG (1988).

Genetic interactions of *let-60* with other genes in the vulval induction pathway: To understand the role of *let-60* in the genetic pathway specifying the VPC fates, we constructed and analyzed several double mutant strains carrying a *let-60(dn)* mutation and Muv mutations in *lin-1*, *lin-12* and *lin-15*. In addition, we examined the interaction between *let-23* and *lin-34(gf)*. Our results suggest that *let-60* acts downstream of *let-23* and *lin-15* but upstream of *lin-1* and *lin-12* in the pathway specifying the VPC fates.

lin-15 acts upstream of let-60: The seven dominant negative *let-60* Vul mutations were isolated as suppressors of *lin-15(n309)*. This suppression is not specific to the *n309* allele because another *lin-15* allele, *n765*, can also be dominantly suppressed by *let-60(dn)* mutations. We have also examined the interaction between *lin-15(n309)* and a loss-of-function mutation of *let-60*, *s1124*. This analysis was possible, because as mentioned above, a small percentage of animals homozygous for the recessive lethal allele *let-60(s1124lf)* can grow to an early adult stage. While the Muv phenotype of *lin-15(n309)* is fully displayed in a *let-60(s1124lf)/+* background, the Muv phenotype is changed to a completely Vul phenotype in "survivors" of genotype *let-60(s1124lf)*; *lin-15(n309)*. This suppression itself suggests that the *let-60* gene acts downstream of *lin-15* in the genetic pathway that specifies VPC types. Furthermore, we have observed that the Vul and Muv phenotypes are mutually suppressed in a *let-60(sy100dn)/+*; *lin-15(n309)* double mutant; not only is the Muv phenotype of *lin-15* suppressed by *let-60(sy100dn)*, but the Vul phenotype of *let-60(sy100dn)/+* is also partially suppressed by the presence of the *lin-15* mutation. The level of VPC induction is close to wild type in a *sy100/+*; *n309/n309* double mutant (88% VPC induction; 21% Egl) in contrast to 57% VPC induction (87% Egl) in the strain with *sy100dn/+* only, Table 3). More impor-

tantly, although the Muv phenotype of *lin-15(n309)* is independent of the inductive signal from the gonad anchor cell (FERGUSON, STERNBERG and HORVITZ 1987; STERNBERG 1988), the induction of VPCs depends absolutely on the inductive signal in the mutually suppressed *let-60(sy100dn)/+*; *lin-15(n309)* double mutant. We have ablated all the gonad cells and hence the signal-producing anchor cell of ten *let-60(sy100dn)/+*; *lin-15(n309)* double mutants at the L1 larval stage; all VPCs generated hypodermal cells in these animals (Table 3). These results suggest that *let-60* and *lin-15* may function antagonistically in the pathway specifying VPC fates, and that the *let-60(sy100dn)* mutation can compensate to some degree for the *lin-15(n309)* defect and restore the relative normal output of the signal response pathway. One possibility is that *lin-15* is a negative regulator of *let-60* activity. Reduction of *lin-15* activity could then result in a higher level of *let-60*, which is no longer subject to the regulation by the upstream signal. This view is supported by the fact that the gain-of-function *lin-34* mutations also display a signal-independent Muv phenotype. Specifically, an average of 120% VPC induction was found among five *lin-34(gf)* animals whose gonad primordia were ablated at an early larval stage (100% is wild type, 200% is maximal for Muv; see MATERIALS AND METHODS). *lin-34(gf)* animals with intact gonads display an average of 165% induction (13 animals).

lin-1 acts after let-60: lin-1 is another Muv gene that acts in the genetic pathway specifying VPC fates (HORVITZ and SULSTON 1980; SULSTON and HORVITZ 1981; FERGUSON and HORVITZ 1985; FERGUSON, STERNBERG and HORVITZ 1987). The Muv phenotype of *lin-1* is epistatic to the Vul phenotype of many Vul genes in the pathway (FERGUSON, STERNBERG and HORVITZ 1987), and the *lin-1* phenotype is coexpressed with *lin-12* phenotypes in double mutants (P. W. STERNBERG, unpublished observation). These results lead to a hypothesis that *lin-1* acts downstream of Vul genes (*e.g.*, *let-23*) and other Muv genes (*e.g.*, *lin-15*) and, as a negative regulator of 1°- and 2°-specific functions. To further characterize the position of *let-60* in the pathway, we constructed double mutants with *lin-1(e1275)* and the loss-of-function mutation of *let-60*, *s1124*. We found that *lin-1(e1275)* does not rescue the lethality of *let-60(s1124lf)/let-60(s1124lf)*: the typical double homozygous animals are larval lethal, but a small percentage of them survive to reach adulthood stage and are sick and sterile. However, those small number of surviving adult animals are all Muv, indicating the Vul phenotype of the *s1124* mutation is suppressed by the *lin-1* mutation. We have also found that the Vul phenotype of *let-60(sy100dn)* is suppressed by the *lin-1* Muv mutation. The *lin-1* Muv phenotype is fully expressed even in a double homozygote. We could observe this phenotype because the lethality of *let-60(sy100dn)/let-60(sy100dn)* is suppressed by *lin-1(e1275)*. The homozygous double mutant is viable and can be continuously propagated. These results suggest that *lin-1* acts downstream of *let-60* in the vulval induction pathway, and that *lin-1* interacts with *let-60* in a pathway required for larval growth.

lin-12 acts after let-60 in 2° fate specification: One of many *lin-12* functions is to distinguish between 2° and non-2° (1° or 3°) VPC types during vulval induction (GREENWALD, STERNBERG and HORVITZ 1983; STERNBERG and HORVITZ 1989). *lin-12* dominant mutations (*lin-12(d)*) cause all six VPCs to be 2°, while *lin-12* loss-of-function mutations cause all six VPCs to be non-2°. It has been proposed that *lin-12* is involved in the lateral signaling which prevents the neighbors of a presumptive 1° from also becoming 1°, and that *lin-12* acts downstream of most Muv and Vul genes whose function is to specify the choice between 3° and non-3° cell fates (STERNBERG and HORVITZ 1989). For example, a *let-23* Vul mutation causes all six VPCs to adopt the 3° cell type. In a *lin-12(d)*; *let-23* double mutant, all six VPCs are 2°. To order the action of *let-60* with respect to *lin-12*, we constructed and examined a double mutant with a *lin-12(d)* allele *n137* and each of four *let-60* alleles (dominant negative alleles *sy100dn*, *sy99dn*, *sy94dn* and a loss-of-function allele *s1124*) (see MATERIALS AND METHODS). We found that the *lin-12(d)* phenotype (five ventral protrusions and egg-laying defective) is fully expressed in all *lin-12(d)/+*; *let-60(dn/+)* strains, and in survivors of genotype *lin-12(d)/+*; *let-60(s1124)/let-60(s1124)*. In other words, all six VPCs are 2° in the double mutants. Therefore, *lin-12* hyperactivity bypasses the need for *let-60* function for promoting 2° fate, suggesting that *lin-12* acts after *let-60* in 2° fate specification.

lin-34 acts after let-23: let-23 is another essential gene with a function in vulval induction (FERGUSON and HORVITZ 1985; FERGUSON, STERNBERG and HORVITZ 1987). Some recessive mutations of *let-23* cause a Vul phenotype. However, loss-of-function of *let-23* results in a larval lethal phenotype (R. V. AROIAN and P. W. STERNBERG, manuscript in preparation). A *lin-34(gf)* Muv mutation has been isolated as a suppressor of the *let-23* Vul phenotype (G. JONGEWARD and P. STERNBERG, unpublished results), suggesting that *lin-34* acts downstream of *let-23* during vulval induction. We constructed a double mutant with *lin-34(n1046gf)* and a loss-of-function, recessive lethal mutation of *let-23*, *mn23* (HERMAN 1978). We found that *let-23(mn23)*; *lin-34(n1046gf)* hermaphrodites were sterile adults and showed a Muv phenotype (88% of the animals are Muv). Sterility is another phenotype associated with some *let-23* muta-

tions (R. V. AROIAN and P. W. STERNBERG, manuscript in preparation) and was not suppressed by *lin-34(n1046gf)*. However, the *let-23* lethal and Vul phenotypes were clearly suppressed by the *lin-34(gf)*. Therefore, we conclude that *lin-34* acts after *let-23* in the genetic pathways involved both in vulval induction and larval growth.

DISCUSSION

Dominant negative mutations of *let-60*: We have exploited the properties of dominant vulvaless (Vul) mutations in the *let-60* gene to analyze its role in vulval induction. Loss of *let-60* activity results in death at an early larval stage, prior to vulval induction. These dominant Vul mutations were isolated as extragenic suppressors of a *lin-15* multivulva mutation, in effect selecting for vulvalessness and viability. These mutations thus allowed us to conclude that *let-60* plays an important role in vulval induction. We used the dominant Vul mutations to obtain both recessive lethal loss-of-function alleles of *let-60* as well as semidominant multivulva *lin-34* mutations that behave as gain-of-function alleles of *let-60* (see below). Analysis of these mutations has allowed us to understand the role of *let-60* in the switch between vulval and nonvulval VPC fates during vulval induction, as detailed below.

We have found that these dominant Vul mutations are dominant negative, *i.e.*, they result in a *let-60* product that appears to compete with the wild-type product ("antimorphic," MULLER 1932). In *let-60(dn)*/+ heterozygotes, *let-60* activity is reduced more than in a heterozygote carrying one copy of a loss-of-function mutation (*lf*/+), indicating that its function in vulval induction is disrupted. There are many possible ways that a mutant gene product can compete with a wild-type gene product and cause the dominant negative effects (reviewed by HERSKOWITZ 1987). For example, a *let-60* gene product may normally form multimers, and the multimeric complex containing wild-type and mutant products could be defective in vulval induction.

A key component of a developmental switch: *let-60* has the properties of a component of a developmental switch because its activity determines which of two alternative fates the six VPCs have. We propose that, in wild-type animals, *let-60* activity is increased by the inductive signal. Mutations with opposite effects on *let-60* activity have opposite consequences for VPC fates (Table 4). Loss or significant reduction of *let-60* activity causes the VPCs to become the nonvulval cell type (3°) even in the presence of inductive signal. In contrast, in *lin-34* Muv mutants, all six VPCs become vulval cell types. Based on mapping results and genetic interactions between mutations of *let-60* and *lin-34*, *lin-34* Muv mutations appear to be either gain-of-function mutations of *let-60* or gain-of-function mutations of an intimately related gene that elevates *let-60* activity. In either case, *lin-34(gf)* mutations apparently result in elevation of *let-60* activity. Thus, an increase of *let-60* activity causes all six VPCs to become vulval cell types compared to the three in wild type, even in the absence of the inductive signal (Table 4). The site of *let-60* action is unknown; however, we hypothesize that *let-60* acts in the VPCs in the pathway of response to inductive signal because this is the simplest interpretation of existing data.

TABLE 4

Illustration of the function of *let-60* activity in controlling the fate of each VPC in response to inductive signal

let-60 genotype	+/− signal	*let-60* activity	VPC fate
Wild type	+	High	Vulval [1° or 2°]
	−	Low	Nonvulval [3°]
Mutants	+ or −	Always high	Vulval [1° or 2°]
	+ or −	Always low	Nonvulval [3°]

We propose that in each of the six VPCs, inductive signal indirectly regulates the *let-60* activity which controls VPC fates. In the column marked "+/− signal", "+" means the individual VPC receives the signal from anchor cell, "−" means the individual VPC does not receive the signal either due to the position of the cell or due to elimination of the signal source by ablation of gonad cells (MATERIALS AND METHODS). *let-60* activity levels are defined genetically: *lin-34(gf)* causes "high" activity (hyperactive), and *let-60(lf)* or *let-60(dn)* cause "low" activity.

If *let-60* and *lin-34* are the same gene, changes of the gene activity caused by dominant negative ("antimorphic") mutations *let-60(dn)* and gain-of-function ("hypermorphic") mutations *lin-34(gf)* may be the consequence of qualitatively different changes in protein structure. For example, the *let-60* product might contain a functional domain and a regulatory domain. The *let-60(dn)* Vul phenotype may result from defects in the functional (*e.g.*, catalytic) domain, while the *lin-34(gf)* Muv phenotype may be caused by defects in the regulatory domain. The regulatory domain could be a site for interacting with a negative regulator, which would keep *let-60* inactive until the VPC receives inductive signal.

let-60 appears to act in more than one aspect of *C. elegans* development. We have described that all the putative loss-of-function mutations and most of the dominant negative mutations are recessive lethal at an early larval stage. We have also described that the *let-60(dn)* mutations result in defects in male spicules and mating. The spicule defect of *let-60(dn)* males is due to at least one alteration in cell fate (H. CHAMBERLIN and P. W. STERNBERG, unpublished results). These observations suggest that *let-60* acts in multiple cells during development.

***let-60* function is regulated by *let-23* and *lin-15*:** Vulval induction is a complicated and multistep process. Along with other Muv and Vul genes, *let-60* functions in one of the key steps in distinguishing

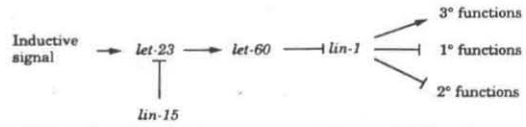

FIGURE 7.—Functional relationship between *let-60* and some other genes in the genetic pathway of vulval fate specification. Based on known genetic interactions (FERGUSON, STERNBERG and HORVITZ 1987; STERNBERG and HORVITZ 1989) we propose the functional relationships between *let-60* and some other genes in the pathway. The arrows indicate the positive regulation of one gene by another. "T" bars indicate the negative regulation of one gene by another. The arrows and bars do not necessarily indicate a direct interaction. We propose that *let-60* activity is positively regulated by inductive signal through *let-23* and negatively controlled by *lin-15* via *let-23*. *let-60* controls the 1°- and 2°-specific functions through inhibition of *lin-1*. *lin-12* could act either in combination with *lin-1* or downstream of *lin-1* to specify 2° functions. The interaction between *lin-12* pathway and *let-60* pathway might involve intercellular or autocrine signals (STERNBERG and HORVITZ 1989).

whether a VPC becomes a vulval cell types (1°, 2°) or a nonvulval cell type (3°) in response to an inductive signal. By studying genetic interactions between *let-60* and other Muv or Vul genes, we can start to elucidate the functional relationship between these genes. The relationship between *let-60* and other Vul and Muv genes is proposed as shown in Figure 7. Since the ordering of gene action is based on dominant mutations [*lin-12(d)*, *lin-34(gf)*, *let-60(dn)*] and possibly non-null recessive mutations (*lin-15*, *lin-1*), we regard these conclusions, which represent the simplest interpretations of our data, as tentative.

We propose that *let-60* activity is positively controlled by *let-23* activity. Again, this is based on our conclusion that *lin-34(gf)* are either gain-of-function alleles of *let-60* or gain-of-function mutations of an intimately related gene that activates *let-60*. Both the lethal and vulvaless phenotypes of *let-23* are suppressed by *lin-34(gf)* mutations (G. JONGEWARD and P. W. STERNBERG, unpublished results; this study), and *lin-34(gf)* mutations result in a signal-independent Muv phenotype. In other words, a *lin-34(gf)* mutation bypasses the need for either inductive signal or *let-23*.

lin-15 is proposed to be a negative regulator of the vulval induction pathway acting before *let-60*, since a decrease in *let-60* activity suppresses the Muv phenotype of *lin-15*. However, *lin-15* could exert its negative effect on *let-60* via *let-23*, since the *lin-15* Muv phenotype is also suppressed by *let-23* Vul mutations. If *lin-15* interacts with *let-60* via *let-23* as proposed in Figure 7, the mutual suppression between *lin-15(n309)* and *let-60(sy100dn)* (Table 3) could be explained by an increase in *let-23* activity in the *lin-15(n309)* background which compensates for the reduction in *let-60* activity. It is known that to some extent, there is also mutual suppression between particular *lin-15* and *let-23* mutations (STERNBERG and HORVITZ 1989). This mutual suppression could result from partial defects in the *lin-15* and *let-23* gene products, which either have antagonistic regulatory effects on *let-60* gene activity, or directly interact with each other. We do not believe that the controlling effect of the inductive signal on *let-60* is exerted via *lin-15*, because the dependence on inductive signal is not relieved by the *lin-15* mutation in a *lin-15(n309)*; *let-60(sy100dn)*/+ double mutant. Moreover, although a *lin-15* mutation alone causes a signal-independent Muv phenotype, the exact pattern of VPC fates in a *lin-15* mutant can be responsive to the inductive signal (STERNBERG 1988). Furthermore, *lin-15* most likely acts in cells other than the VPCs (R. HERMAN and E. HEDGECOCK, personal communication).

***let-60* controls VPC fates via *lin-1* and *lin-12*:** *lin-1* is proposed to act downstream of the *let-60* gene because *lin-1* mutations are epistatic to *let-60* mutations (Figure 7). *lin-1* mutations cause a Muv phenotype, and *lin-1* might act as a negative regulator of the expression of 1°- and 2°-specific functions. *lin-12* is proposed to act downstream of the *let-60* gene in promoting the 2°-specific functions because dominant *lin-12* mutations are epistatic to *let-60* mutations with respect to the 2° cell fate. *lin-12* is a component of a developmental switch specifying 2° *vs.* non-2° (1° or 3°) VPC fates (GREENWALD, STERNBERG and HORVITZ 1983; STERNBERG and HORVITZ 1989). In contrast, *let-60* is a component of a developmental switch specifying 3° *vs.* non-3° VPC fates (1° or 2°). The Vul/Muv pathway is likely to control, at least in part, the activity of *lin-12* (STERNBERG and HORVITZ 1989). It is not known whether the interaction of these pathways occurs within the same VPC or via intercellular signals. The precise pattern of VPC fates is established by the combined action of these two pathways. The activity states of *let-60* and *lin-12* define the action of each pathway.

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The Answer

"There is nothing without an answer," said a monk as he entered the teahouse where Mulla Nasrudin and his friends sat.

"Yet I have been challenged by a scholar with an unanswerable question," observed the Mulla.

"Would that I had been there! Tell it to me, and I shall answer it."

"Very well. He said: 'Why are you stealing into my house through a window by night?'"

From "The Pleasantries of the Incredible Mulla Nasrudin" by Idries Shah, E.P. Dutton & Co., U.S.A., 1971.