The *Caenorhabditis elegans lin-15* locus

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This thesis is dedicated to my parents, Tsou-chiang and Jane,
who have always encouraged me to do my best.

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Abstract

In the nematode *Caenorhabditis elegans*, vulval development involves an inductive signalling event mediated by the LIN-3 growth factor and the LET-23 receptor tyrosine kinase. The LIN-3 growth factor is sent from the anchor cell, a cell in the somatic gonad, to the six multipotent vulval precursor cells (VPCs) in the ventral epidermis. Lack of the signal leads to none of the six VPCs adopting vulval fates. The *lin-15* locus is a negative regulator of *lin-3/let-23* mediated signalling; animals carrying loss-of-function mutations in *lin-15* have excessive vulval differentiation. Mosaic studies demonstrate this negative regulation to occur non-autonomously. Furthermore, the *lin-15* locus has two genetically defined functions, A and B. These A and B functions are shared by other loci with these functions, defining two distinct pathways for negative regulation. The A and B functions are redundant because animals defective in only one function display the wild-type phenotype; only animals defective in both A and B function display the excessive vulval differentiation phenotype.

This thesis describes the molecular and genetic analysis of the *lin-15* locus. The *lin-15* locus was cloned based on its genetic map position. The two functions of *lin-15* are due to two transcripts comprising the *lin-15* locus, one transcript corresponding to the A function, the other, the B function. These two transcripts are transcribed in the same direction, separated by 105 base pairs, and possibly transcribed polycistronically. The predicted proteins coded for by both transcripts are novel and hydrophilic. Antibodies raised against the LIN-15B protein show that LIN-15B is a 170 kD protein that is localized to the nucleus and broadly expressed. Genetic studies demonstrate

that *lin-15* acts upstream of the receptor and in parallel to the inductive signal. When a *lin-15* null allele is used to modulate signalling through weakly functioning LET-23 receptors, we see that the activity of the receptor can be correlated to the type of vulval fate specified.

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Chapter 1

A century of inductive studies

The current study of developmental biology has its roots in experimental embryology, which has been established as a science for a little over a century. The studies of early embryologists defined the phenomena of induction and competence, providing the groundwork for developmental biologists today. In the last decade, inductive processes have come to be viewed as cell-cell interactions involving a ligand and receptor interaction that mediates signal transduction; competence in these cases involves a cell's possession of the appropriate receptors, downstream signal transduction components and transcription factors for responding to the signal. In this chapter, I will outline the rise of experimental embryology and the early experiments that lay the foundation for studies on induction today. Next, I will review instances of signalling in *Saccharomyces cerevisiae*, *Caenhorhabditis elegans*, *Drosophila melanogaster*, *Xenopus laevis*, and mammalian development to demonstrate the similarities between these diverse group of organisms. Finally, I will discuss how the resolution with which modern techniques allows us to view inductive events brings up new questions regarding signalling specificity, signal regulation, the use of multiple signals in concert, the quality of the signal itself, and the use of a signal to generate different physiological outputs.

DEFINING THE PHENOMENOLOGY

The rise of experimental embryology

The emergence of classical descriptive embryology as a science began during the early 1800s, due to better preparatory techniques and improved

microscopes (Churchill, 1991). Embryology remained a predominantly descriptive study for several decades, involving careful recording of developmental events through observation and histology to explain the origin of more specialized structures as well as to explain evolutionary relationships between organisms. The change in the emphasis of embryology from descriptive studies to experimental studies in the late 1800s has been largely credited to Wilhelm Roux (Hamburger, 1988, Maienschein, 1991).

Roux was an experimental amphibian embryologist. His most famous experiment was the half-embryo experiment performed on frog eggs in 1888. In this experiment, Roux punctured a fertilized egg with a hot needle at the 2 cell stage to kill one of the blastomeres. He wished to investigate whether the blastomere was able to compensate for this death or whether the blastomere was "self-differentiating" and unable to compensate (Roux, 1888, reviewed in Hamburger, 1988). Roux found that the living blastomere would produce partial embryos, leading him to conclude that frog egg blastomeres were capable of self-differentiating and that the egg was likely composed of a mosaic of independent parts. [However, these experiments were later found to be flawed due to Roux's failure to remove the punctured blastomere (reviewed in Hamburger, 1988). Rotation of the frog egg by 180° after blastomere killing (Morgan, 1895) or the complete separation of blastomeres results in regulation (reviewed in Davidson, 1986, Hamburger, 1988), as seen by Driesch in sea urchins, described below.]

Although Roux's experiment did not uncover the regulation that can occur in the amphibian embryo, his major intellectual contribution to embryology was his urging for the application of the experimental approach for investigating development. From the 1880s, Roux advocated a more

experimental approach to embryology, eventually leading to his founding a journal for experimental embryology in 1895, *Wilhelm Roux's Archiv für Entwickelungsmechanik der Organismen* (also known as *Roux's Archives for Developmental Biology*). In this journal, he published his manifesto for experimental embryology, pronouncing "manipulative controlled experimentation" (carefully controlling experimental conditions and comparing what happens under altered conditions to that which happens under normal conditions) as the method for studying embryonic development (Roux, 1894). He believed that experimentation was better than cytology because working with tissues that have not been killed allows the direct observation of what actually happens. His insistence on proper experimental work and his editorial guidance were a great influence in determining the direction of embryological studies in the following decades (Maienschein, 1991).

Regulation

Following Roux's 1888 half-embryo experiments, Hans Driesch began a series of experiments to study whether or not blastomeres were capable of self-differentiation; these studies were published in 1892 (Driesch, 1892; reviewed in Davidson, 1986). Driesch studied this question in sea urchin eggs, which he considered more durable, easily obtainable, and easily observed than the frog eggs that Roux worked with. Instead of puncturing the blastomere with a hot needle as Roux, Driesch used the method devised by Oscar and Richard Herwig of shaking sea urchin eggs to separate blastomeres. Contrary to his expectations and Roux's results on frog eggs, Driesch found that when he separated the blastomeres after the first division,

he was able to obtain half-sized normally formed embryos; a small percentage of these developed into pluteus larvae. From these results, he concluded that the separated blastomeres had some regulative ability to respond to abnormal conditions (the experimental separation). These experiments defined the phenomenon known as regulation; the ability of a cell to regulate implies the ability of different parts of the embryo to interact with each other.

Lens Induction

Hans Spemann was very much influenced by the ideas of Roux and Driesch. His Habilitations Lecture (a requirement for admission to the faculty) at the University of Würzbug dealt with the half-embryo experiments of Roux and the blastomere separation experiments of Driesch in a seminar entitled "Critical consideration of experiments on the effects on development of removal or killing of single blastomeres" (Saha, 1991). This interest eventually led Spemann to study eye lens formation in *Rana fusca*, the European frog.

By the time Spemann began studying lens formation in 1901, a description of eye and lens development was already available (Hamburger, 1988, Spemann, 1938). The optic vesicles formed from evaginations from the anterior of the neural tube, with lenses forming at the point where the optic vesicle migrated to and contacted the overlying epidermis. The optic vesicle infolds and forms an optic cup; the cells that form the lens pinch off from the epidermis and fit inside the cup. Spemann was interested in investigating the role of the optic vesicle in lens formation.

To do so, he performed two experiments. First, he injured the eye rudiment such that the optic cup could not reach the epidermis. In this case,

he observed optic cups without lenses. Next, he used a hot needle to destroy the presumptive eye rudiment. Whenever the eye rudiment was destroyed, no lenses developed. From these experiments, he concluded that the lens is not essential in the development of the optic cup and that the optic vesicle was necessary for lens formation.

Independently of Spemann, Warren Lewis was studying eye development in *Rana palustris*, the American frog (Lewis, 1904). He also demonstrated that the epithelial cells that normally form lens do not do so when the optic vesicle does not establish contact. Furthermore, when he transplanted the optic vesicle such that it was in contact with trunk epidermis, he observed lenses developing from the trunk epidermis. This experiment demonstrated the sufficiency of the optic vesicle for lens formation.

However, subsequent studies by several investigators revealed the ability of lenses to self-differentiate and form without optic vesicle contact. Furthermore, it was also found that not all epidermis would respond to the optic vesicle stimuli (reviewed in Saha, 1991, Hamburger, 1988). Eventually, these differences were found to be species-dependent and a consensus was reached that different species had different needs for contact with the optic vesicle for lens development. Lens induction became the paradigm for embryonic induction and induction came to be defined as "an activity which determined the cytological fate of the reacting cells" (as in Hamburger, 1988).

Mangold and Spemann's Organizer Experiment

The most famous case of induction involves the formation of a second body axis in the amphibian embryo (reviewed in Hamburger, 1988, Spemann,

1938). These experiments, performed by Mangold and Spemann in 1924, involved the transplantation of a piece of the upper blastoporal lip of a gastrula *Triturus cristatus* (which have unpigmented eggs) to the ventral side of a similarly staged *Triturus taeniatus* (which have pigmented eggs). The transplant was found to cause the formation of a secondary embryo originating from the transplant site, composed of cells from both the host and the donor embryo. The ability of the transplant to both recruit and instruct cells from the host embryo to form a second embryo led Spemann to call the upper blastoporal lip the "organizer." This demonstration of the dramatic ability of the dorsal lip grafts to induce a complete secondary axis led to a heightened interest in the study of inductive events in development (Hamburger, 1988, Saha, 1991) and resulted in a Nobel Prize for Spemann.

Competence

The evidence for competence existed when it was found that various tissues in different species of frogs had varying abilities to form lens in response to the optic vesicle. However, the idea of competence was not formulated until 1936 by Conrad Hal Waddington (Gilbert, 1991, Gilbert, 1994). Waddington was interested in isolating the molecule responsible for the inductive event in the organizer. He discovered that many artificial and natural compounds had the ability to mimic the organizer's inductive effect (Waddington, et al., 1936). Because of this, his interest switched from the inductive signal, which appeared to be non-specific, to the receiving cells. He proposed that in order for the cells to be induced, they must be "competent" to respond. Furthermore, he considered the acquisition of this competence to result from an active process in the cells; he believed that the inductive signal

merely pushed these cells to a fate they had already progressed toward (Waddington, 1940).

A MOLECULAR FRAMEWORK FOR INDUCTIVE PROCESSES

In the late twentieth century, work on many systems has converged to provide a unified view of how cells can communicate with each other through receptor mediated signalling mechanisms. In this section, I will review the mating pheromone reception pathway in *S. cerevisiae*, vulval induction in *C. elegans*, photoreceptor R7 fate specification in *Drosophila*, and mesoderm induction in *Xenopus* (Figure 1). I will also discuss examples of signalling molecules that have been identified in mammalian systems.

Mating pheromone reception in *S. cerevisiae*

The budding yeast *S. cerevisiae* can exist in either haploid or diploid forms. Although unicellular, intercellular communication takes place between the haploid cell types for appropriate mating to occur (reviewed in Bardwell, et al., 1994). There are two haploid cell types, **a** and α, which mate with each other to form the diploid **a**/α cell. The **a** cell produces **a**-factor, which is received by the α cell through the **a**-factor receptor, STE3. Conversely, α-factor produced by the α cell is received by the **a** cell through the *STE2* encoded α-factor receptor. Upon reception of pheromone from the opposite cell type, the haploid cells undergo physiological changes including cell cycle arrest, new gene induction, and cell morphology changes to prepare the haploid cell to mate and fuse with the other haploid cell to form the diploid cell.

These physiological changes stem from the transduction of signal beginning upon pheromone reception. The ease of genetic, molecular, and biochemical manipulations in *S. cerevisiae* have led to an almost complete elucidation of the transduction events that occur from signal reception to gene transcription. STE2 and STE3, the pheromone receptors, are serpentine receptors coupled to a heterotrimeric G-protein, comprising GPA1, STE4, and STE18. Activation of the receptor causes dissociation of GPA1, the G_α subunit, from STE4 and STE18, the G_β and G_γ subunits, respectively. The $G_{\beta\gamma}$ complex then activates a protein kinase cascade through an unknown mechanism that requires STE20 and STE5: the STE11 MEK kinase phosphorylates the STE7 MEK, which then phosphorylates the MAP kinases FUS3 and KSS1 (Neiman and Herskowitz, 1994, Gartner, et al., 1992, Errede, et al., 1993). STE5 appears to be associated with all members of this kinase cascade independently and has been proposed to act as a scaffold for these molecules (Marcus, et al., 1994, Choi, et al., 1994, Printen and Sprague, 1994). Furthermore, this protein kinase cascade, also known as the MAPK module, is considered a functional unit that links signal input with cellular outputs, such as changes in gene expression (reviewed in Herskowitz, 1995). In the mating pheromone reception pathway, the activated MAP kinases phosphorylate many targets, including the STE12 transcription factor (Elion, et al., 1993), which, in association with the general transcription factor MCM1, leads to transcription of genes needed in preparation for the mating event.

Vulval induction in *C. elegans*

The inductive event in *C. elegans* vulval differentiation occurs between

the anchor cell, a cell in the gonad, and six multipotent cells in the ventral epidermis, P3.p through P8.p, which are also known as the vulval precursor cells (VPCs) (Sulston and White, 1980, Sternberg and Horvitz, 1986). The anchor cell sends a signal to the VPCs, causing three of the six cells to adopt vulval fates (Sulston and White, 1980, Kimble, 1981, Thomas, et al., 1990). The non-induced cells adopt non-specialized epidermal fates which involve at most one more round of division. There are two different vulval fates, 1° and 2°; these fates can be distinguished by the pattern of subsequent divisions in the induced cells and by the morphology adopted by the progeny of the VPCs (Sternberg and Horvitz, 1986, Katz, et al., 1995). In a wild-type animal, P6.p, the VPC closest to the anchor cell, adopts a 1° fate while P5.p and P7.p adopt 2° fates (Sulston and Horvitz, 1977). To produce this invariant pattern of cell fates, a lateral signal from P6.p to its neighboring VPCs, P5.p and P7.p is used (Greenwald, et al., 1983, Sternberg, 1988, Sternberg and Horvitz, 1989, Simske and Kim, 1995, Koga and Oshima, submitted), as well as a negative signal from the surrounding epidermis (Ferguson and Horvitz, 1985, Ferguson, et al., 1987, Herman and Hedgecock, 1990, Huang, et al., 1994, Clark, et al., 1994). The lateral signal and the negative signal are discussed later; here, I discuss the inductive signal and its transduction.

Molecular and genetic analyses of loss-of-function mutants that cause all six VPCs to adopt non-specialized epidermal fates have defined a growth factor/receptor tyrosine kinase signalling pathway involved in this inductive event. Because the lack of these gene functions produces a phenotype in the VPCs similar to that seen upon artificial removal of the signalling cell, the products of the wild-type genes were candidates for components that function in signalling between the anchor cell and the VPCs. By genetic epistasis

analysis (Ferguson, et al., 1987 and references below) and by analogy with other systems, these genes appear to act in the following order: *lin-3*, which encodes a growth factor of the TGFα family (Hill and Sternberg, 1992, Katz, et al., 1995) presumably binds to and activates *let-23*, which encodes a tyrosine kinase receptor (Aroian, et al., 1990). *sem-5*, which encodes a SH2-SH3 adaptor protein (Clark, et al., 1992) then binds activated *let-23*, which, through an unknown mechanism, activates *let-60*, which encodes a *ras* protein (Han and Sternberg, 1990). Activated *let-60* then interacts with *lin-45*, which encodes a *raf* protein (Han, et al., 1993); *raf* proteins are considered MEK kinases (reviewed in Herskowitz, 1995). *lin-45* then presumably phosphorylates *mek-1* (Wu, et al., 1995, Kornfeld, et al., 1995) which then phosphorylates *sur-1/mpk-1*, a MAP kinase (Wu and Han, 1994, Lackner, et al., 1994). The activation of this protein kinase cascade, by analogy to the *S. cerevisiae* example discussed above, presumably leads to changes in gene expression, causing the induced cells to adopt vulval fates.

A possible target for *sur-1/mpk-1* is the product encoded by *lin-1*. In animals carrying *lin-1* loss-of-function mutations, all VPCs to adopt vulval fates. Therefore, *lin-1* is a negative regulator of vulval development. *lin-1* can suppress the lack of vulval differentiation seen in *lin-3*, *let-23*, *sem-5*, *let-60*, *lin-45*, and *sur-1/mpk-1* (Ferguson, et al., 1987, Han, et al., 1990, Clark, et al., 1992), suggesting it acts downstream of the transduction cascade. *lin-1* encodes a protein containing an ETS-type DNA-binding domain (G. Beitel and H.R. Horvitz, personal communication), suggesting a role in gene regulation.

lin-25 is another possible target for the *sur-1/mpk-1* MAP kinase (Tuck and Greenwald, 1995). Genetic analysis demonstrates that *lin-25* is required

for vulval differentiation downstream of *let-60* and *lin-1*, suggesting that it acts downstream of the MAP kinase cascade. *lin-25* encodes a novel protein that has a putative MAP kinase phosphorylation consensus site and a possible nuclear localization sequence. Further analysis is needed to determine whether *lin-25* is a direct target of the MAP kinase cascade.

Photoreceptor cell R7 fate specification in *Drosophila*

In *Drosophila*, the specification of R7 photoreceptor cell fate by the R8 photoreceptor uses a signalling cascade very similar to the one used in *C. elegans* vulval induction. R7 fate specification has been extensively studied because R7 is the last photoreceptor of the 8 photoreceptors to begin development; thus, its absence has no effect on the other 7 photoreceptors (reviewed in Simon, 1994). An adult eye is composed of approximately 800 ommatidia; each ommatidium comprises 8 photoreceptor cells, 4 cone cells which secrete the lens of the eye, and 8 accessory cells. Although there are no fixed lineages within the eye, the cells in an ommatidium always develop in the same order, with R8 developing first, followed by R2 and R5, then R3 and R4, then R1 and R5, and finally, R7 (Tomlinson and Ready, 1987). The lack of a fixed lineage led to the proposal that signalling between the developing cells ensures the proper order of development.

Screens for animals lacking the R7 photoreceptor identified the *bride of sevenless (boss)* and *sevenless (sev)* genes. Mosaic analysis suggests that *boss* is required in the R8 cell for appropriate R7 development while *sev* is required in R7. Molecular and biochemical analyses of the *boss* and *sev* gene products indicate that *boss* encodes the ligand for *sev*, a receptor tyrosine kinase (Hart, et al., 1990, Krämer, et al., 1991, Cagan, et al., 1992, Hart, et

al., 1993, Hafen, et al., 1987, Basler and Hafen, 1988, Bowtell, et al., 1988, Tomlinson and Ready, 1987, Banerjee, et al., 1987).

Identification of downstream components demonstrated that the transduction machinery used in R7 cell fate development and *C. elegans* vulval development were very similar. In R7, *drk*, a *sem-5* homolog (Simon, et al., 1993, Olivier, et al., 1993), acts downstream of *sev* to link *sev* and *sos*. *Sos* is a guanine nucleotide exchange factor (GNEF) (Rogge, et al., 1991, Simon, et al., 1991) that stimulates the formation of GTP-bound *Ras1*, the *Drosophila ras* homolog.

Not all components of the MAPK module have been identified in this system. *Raf* likely acts in R7 development because animals containing a constitutively activated *raf* protein, when expressed under the control of the *sev* promoter had the same phenotype as animals in where *Ras1* is activated (cone cells are transformed to R7-like photoreceptors) (Dickson, et al., 1992). Downstream of *raf* may be *Dsor1*, the *Drosophila* MEK that has been shown to act in embryonic termini development, involved in signalling through the torso receptor tyrosine kinase (Tsuda, et al., 1993). A MAP kinase involved in R7 fate specification has been identified, encoded by *rolled* (Biggs and Zipursky, 1992, Biggs, et al., 1994, Brunner, et al., 1994).

Several nuclear factors that apparently act downstream of the MAP kinase cascade in R7 development have been identified (reviewed in Dickson, 1995). These include *sina*, (Carthew and Rubin, 1990); *pointed*, an ETS domain protein (Brunner, et al., 1994, O'Neill, et al., 1994); *yan*, another ETS domain protein that appears to have a role in inhibiting R7 fate specification much like the *C. elegans lin-1* locus discussed above (Lai and Rubin, 1992, Tei, et al., 1992); *D-jun*, the *Drosophila c-jun* homolog (Bohmann, et al., 1994)

and *phyllopod* (Dickson, et al., 1995, Chang, et al., 1995). The Yan and Pointed proteins are phosphorylated by MAP kinase *in vitro* (Brunner, et al., 1994), making them strong candidates for downstream targets of *rolled*.

Mesoderm induction in *Xenopus*

The *Xenopus* egg has an inherent polarity because the animal-vegetal axis is established during oogenesis. Upon fertilization, the dorsal-ventral axis is established, with the dorsal side being the side opposite the sperm entry point. Dorsalization likely involves secreted factors including *Wnt*-like molecules (Smith and Harland, 1991, Sokol, et al., 1991, Ku and Melton, 1993) and *noggin* (Smith and Harland, 1992), as well as the transcription factors *gsc* (Cho, et al., 1991) and *siamosis* (Lemaire, et al., 1995). During cleavage stage, a signal from the vegetal hemisphere is sent to the equatorial region, inducing mesoderm. Mesoderm induction is likely influenced by the dorsalization process (Kimelman, et al., 1992); this interaction will be discussed later in this chapter.

The interaction between the vegetal region and the animal region to induce mesoderm has been intensely studied since its discovery by Nieuwkoop in 1969 (reviewed in Kimelman, et al., 1992, Slack, 1993, Kessler and Melton, 1994). Mesoderm is normally formed from the equatorial region of the early embryo. In isolation, these cells form epidermis (which is of ectodermal origin), consistent with the requirement of a signal to induce mesoderm. The vegetal region below the equatorial region is the source of the inductive signal and normally forms endoderm; the animal region forms ectoderm. The induced mesoderm is not a uniform tissue, as the mesodermal region produces cells that eventually adopt very different fates such as blood,

lateral plate, muscle, and notochord; these fates are partitioned along the dorsal-ventral axis. These mesodermal specializations may arise in part from another inductive interaction originating from the organizer region. Mesoderm induction involves a field of cells (the vegetal region) inducing another field of cells (the equatorial region), instead of a single cell inducing a small number of defined cells, as in the examples described above. The experimental evidence suggests that there may be more than one inducing substance from the vegetal region (reviewed below), and, that the predetermined dorsal-ventral difference causes differences in mesodermal fate along that axis. Because of this complexity, the molecular mechanisms underlying mesoderm induction event are not well understood. However, many similarities still exist between this signalling event and those discussed above.

Candidate molecules involved in mesoderm induction have been identified, although precise *in vivo* roles have not yet been assigned. Some of these factors were isolated using an assay based on Nieuwkoop's animal-vegetal hemisphere recombinants, looking for molecules that could substitute for the vegetal hemisphere and induce animal caps to form mesoderm. These putative inducing molecules include activin, which can induce dorsal mesoderm (Smith, et al., 1990, Thomsen, et al., 1990); members of the FGF family, especially basic FGF (bFGF), which can induce ventral mesoderm (Kimelman and Kirschner, 1987, Slack, et al., 1987, Slack, et al., 1988) and can also form some dorsal mesoderm upon animal cap injection with bFGF RNA (Kimelman and Maas, 1992); Vg1, a maternal factor initially isolated for its localization in the vegetal hemisphere (Weeks and Melton, 1987) that, in its processed form, induces dorsal mesoderm (Thomsen and Melton, 1993);

and the bone morphogenetic protein BMP4, which can induce ventral mesoderm (Dale, et al., 1992, Jones, et al., 1992). None of these factors can clearly induce all forms of mesoderm, consistent with there being more than one signal from the vegetal region. Of these factors, bFGF, aFGF, XeFGF, Vg1, and BMP4 are maternally expressed; maternal activin transcripts have not been detected, although an activin-like activity exists in the early embryo (Asashima, et al., 1991).

The assay for receptors affecting mesoderm induction involves the construction of dominant negative receptor molecules and their reintroduction into the embryo, looking for inhibition of mesoderm induction. This was first done for the FGF receptor (Amaya, et al., 1991, Amaya, et al., 1993) and has also subsequently been demonstrated using a BMP receptor (Graff, et al., 1994) and an activin receptor (Hemmati-Brivanlou and Melton, 1992, Hemmati-Brivanlou and Melton, 1994). These studies are consistent with FGF, BMP, and activin as having roles in mesoderm induction, although the specificity of the interference of the dominant negative receptors is always a caveat in these types of experiments.

The use of components downstream of the receptor are less well defined. *Ras* may be involved in transducing the bFGF and activin signals (Whitman and Melton, 1992). Injection of dominant negative *ras* RNA blocks the mesodermal inducing ability of bFGF and activin as well as the ability of animal caps to receive the vegetally derived signal, while injection of activated *ras* induces some mesodermal tissues. Similar experiments with *raf* demonstrate its ability to block bFGF mediated induction of mesoderm but not activin mediated induction (MacNicol, et al., 1993). Biochemical studies have demonstrated a MAP kinase in embryos that is activated rapidly

by bFGF and activated upon prolonged exposure to activin (Hartley, et al., 1994). These results suggest that bFGF signalling probably occurs via a *ras*/*raf*/MAPK pathway; activin signalling may do so as well. However, since none of these studies are done looking at the response in isolated cells, it is difficult to determine if the *ras*/*raf*/MAPK pathway is acting downstream of bFGF signalling or in parallel, acting in neighboring cells and influencing bFGF signalling.

Molecules involved in mammalian development

Many components of the growth factor/receptor signalling systems used in *S. cerevisiae*, *C. elegans*, *Drosophila*, and *Xenopus* described above were initially defined for their oncogenic properties when mutated in mammalian cell culture studies (reviewed in Lodish, et al., 1995); the cellular (non-mutated) forms of these proteins are now known to mediate normal processes such as growth and differentiation. Studies of molecules defective in mutant mice strains and mice created which contain a targeted disruption in a gene have further demonstrated the importance of growth factor/receptor signalling systems. For example, activin-defective (Matzuk, et al., 1995), activin receptor-defective (Matzuk, et al., 1995), and FGF receptor-defective (Yamaguchi, et al., 1994, Deng, et al., 1994) mice demonstrate developmental defects, indicating a role in normal development. Similarly, studies of mutations in mice demonstrated that the coat color mutations *Dominant white spotting* and *Steel* encode a tyrosine kinase receptor protein of the Kit family and a ligand of the Kit receptor, respectively (Chabot, et al., 1988, Geissler, et al., 1988, Huang, et al., 1990, Copeland, et al., 1990, Flanagan and Leder, 1990, Zesebo, et al., 1990). Studies of both targeted gene

disruption and *waved-1* mutant mice indicated that the TGFα growth factor is involved in various developmental processes as well (Luetteke, et al., 1993, Mann, et al., 1993). These are only a few examples of the large body of work in mammalian systems indicating similarities in developmental mechanisms at the molecular level between organisms as diverse as yeast and mice.

NEW COMPLEXITIES REGARDING INDUCTION

From the work on many systems, it is now generally accepted that inductive signalling often involves cell-cell communication mediated by ligand/receptor interactions, leading to a protein kinase cascade. This knowledge now poses new mechanistic questions about how signal specificity is achieved, how the signalling event is regulated, how many signals work together to specify cell fate, what kind of informational content is in the signal, and how a single signal can cause different physiological responses within a cell.

Signalling specificity

As demonstrated in the previous section, many different organisms utilize cell-cell communication processes involving a ligand that signals through a receptor, transduced through a MAP kinase module. How this general pathway specifies specialized fates as different as a photoreceptor cell and a mesodermal cell could be argued to be due to the condition of the induced cells; that is, the induced cell contains materials to either produce a photoreceptor cell or a mesodermal cell and the signal causes the activation of the non-default fate that the cell is capable of executing. However, this is

likely an oversimplification, as I will discuss two cases where different signalling events use at least parts of the same transduction machinery to generate different outputs in the same receiving cell.

Studies in PC12 cells, a cell line derived from a rat pheochromocytoma, are beginning to elucidate the mechanism of how two different signals can cause two different outputs from a single cell type. PC12 cells proliferate in response to treatment with EGF and differentiate by growing neurites and ceasing cell division in response to treatment with either FGF or NGF (reviewed in Chao, 1992). The proliferation response and the differentiation response appear to share many of the same signal transduction components such as *ras* and MAP kinase; it is the level of activation of these components that appears to impart specificity. For example, NGF stimulation results in an sustained elevation of GTP-bound *ras* (the activated form of *ras*) and MAP kinase activity while EGF stimulation causes only a transient elevation of activated *ras* and MAP kinase (reviewed in Marshall, 1995).

One model for how MAP kinase activation is sustained involves the number of active receptors on the cell surface. The EGF receptor has been demonstrated to be more rapidly downregulated through internalization and phosphoyrlation than the TrkA-NGF receptors (Countaway, et al., 1992), consistent with the idea that ligand activation of EGF receptors would lead to a decrease in the length of time that a signal is transduced. Experiments that overexpress the EGF receptor on PC12 cells lead to prolonged MAP kinase activation and cell differentiation (Traverse, et al., 1994). Conversely, a mutant PC12 cell line that proliferates in response to NGF has a reduced number of TrkA-NGF receptor molecules per cell and shows only transient MAP kinase activation (Schlessinger and Bar-Sagi, 1995). These results

suggest that the quantity of activated receptors over time on a cell surface is reflected in the level of MAP kinase activation; different levels of MAP kinase activation lead to different responses. However, the simplest model that all cells proliferate upon transient MAP kinase activation and cease proliferation and differentiate upon prolonged MAP kinase activation is likely not true, as sustained activation of MAP kinase in fibroblasts leads to proliferation (reviewed in Marshall, 1995).

The difference in responses due to MAP kinase level has been proposed to be due to differences in new gene transcription. In PC12 cells, prolonged MAP kinase activation leads to translocation of MAP kinase to the nucleus; transient activation does not (Traverse, et al., 1992, Nguyen, et al., 1993, Dikic, et al., 1994, Traverse, et al., 1994). Studies of gene activation in stimulated PC12 cells has demonstrated transcriptional differences between NGF activation and EGF activation (reviewed in Ziff, 1993). Both EGF and NGF induce expression of the *c-fos* gene and the tyrosine hydroxylase (TH) gene while only NGF induces expression of the peripherin gene, a neuron-specific intermediate filament protein. *c-fos* expression is induced within 10 to 15 minutes of NGF or EGF addition, TH expression is induced maximally 1 hour after stimulation, and peripherin is induced 18 to 24 hours after NGF stimulation, consistent with prolonged MAP kinase activation under NGF stimulation.

The need for signal specificity is not an artifactual consequence of cells that grow in culture. In *S. cerevisiae*, many MAP kinase modules are known to exist within the same cell and are used for different cellular responses (reviewed in Herskowitz, 1995). Sometimes, the same transduction components are also used for different signalling events within the same cell.

For example, some components of the mating pheromone transduction cascade discussed in the previous section are also used during invasive growth (Roberts and Fink, 1994). Haploid *S. cerevisiae* can exhibit an invasive growth behavior resulting in penetration of the agar plate surface that they are being grown on. Agar penetration appears to require *STE20*, *STE11* (MEKK), *STE7* (MEK), and *STE12* (a transcription factor) but does not require *STE5* (scaffold), the pheromone receptors, the G protein, or *FUS3* (MAPK). *KSS1* (MAPK) also appears to be used in invasive growth, possibly in concert with an unidentified MAPK. Interestingly, although *STE12* is a target of both the invasive growth and mating pheromone response pathways, the mating specific gene *FUS1* is not induced during invasive growth. The signal for invasive growth is currently unknown; its identification as well as the identification of other components used during invasive growth will be informative in understanding how *STE20*, *STE11*, *STE7*, *KSS1*, and *STE12* can be used in the same cell to specify two distinct developmental processes.

Signal regulation

When the receptors on the receiving cell bind the signal, the signal transduction cascade is initiated, leading to new gene expression. The actual signalling event is likely more complicated than this description; the transduction cascade does not behave as a simple wire to relay information from the signal. It has become increasingly apparent that many components of this cascade are subject to regulation. The PC12 cell example discussed above demonstrates how receptor downregulation possibly affects the different outputs of the signal. Molecular genetic studies on vulval development in *C. elegans* has demonstrated both how the inductive process

occurs (discussed in the previous section) and the many ways this transduction cascade is negatively regulated.

Five distinct negative regulatory pathways involved in vulval development have been defined (reviewed in Sternberg, et al., 1994), represented by *lin-15*A, *lin-15*B, *unc-101*, *sli-1*, and *rok-1*. Loss-of-function mutations in any one of these genes show no vulval defect although many combinations of mutations in the different negative regulatory pathways exhibit excessive vulval proliferation, indicating considerable redundancy in the regulation of the *let-23* mediated signalling pathway.

The *lin-15*A and the *lin-15*B pathways were originally defined by a fortuitous event where an animal carrying two mutations, one in the A pathway and the other in the B pathway (*lin-8* and *lin-9*, respectively), was identified in a screen for animals with excessive vulval differentiation (Ferguson and Horvitz, 1985, Ferguson and Horvitz, 1989). The *lin-15* locus has both A and B activity; it encodes two novel gene products corresponding to the *lin-15*A and *lin-15*B activities (Huang, et al., 1994, Clark, et al., 1994). The *lin-15*A and *lin-15*B pathways appear to act together to regulate the basal level of *let-23* signalling for two reasons. Animals defective in *lin-15* exhibit the excessive vulval differentiation phenotype even in the absence of inductive signal (Ferguson, et al., 1987, Sternberg and Horvitz, 1989), indicating that the *lin-15* phenotype is signal independent. Secondly, *let-23; lin-15* double mutant animals display the *let-23* phenotype, indicating that *let-23* is required for the expression of the *lin-15* phenotype (Ferguson, et al., 1987, Huang, et al., 1994). In addition, genetic mosaic results indicate that *lin-15* function cell non-autonomously, possibly from the surrounding epidermis (Herman and Hedgecock, 1990). Thus, the negative regulatory

pathway mediated by *lin-15* likely comprises its own cell-cell signalling system.

sli-1 was isolated as a suppressor of a hypomorphic allele of *let-23* (Jongeward, et al., 1995). *sli-1* is thought to regulate stimulated activity of the *let-23* pathway because the *sli-1* phenotype appears to be partially inductive signal dependent. *sli-1* loss-of-function mutations will not suppress null mutations of *let-23*, indicating that mutations in *sli-1* do not allow bypass of the LET-23 pathway. The *sli-1* gene encodes a homolog of *cbl* (Yoon, et al., submitted), a gene initially identified for its transforming ability in cell culture (Blake, et al., 1991).

unc-101 was also isolated as a suppressor of a hypomorphic allele of *let-23*; it encodes a clathrin associated protein (Lee, et al., 1994). The ability of apparently null *unc-101* mutations to strongly suppress certain *let-23* alleles but weakly suppress alleles of *lin-3* and *lin-45* led to the proposal that *unc-101* acts to regulate the *let-23* pathway at the LET-23 step and not upstream or downstream.

rok-1 was isolated as a synthetic enhancer of *sli-1* (Lee, 1994). Because the *unc-101; rok-1; sli-1* triple mutant has more excessive vulval differentiation than any of the three double mutant combinations using those three genes, *rok-1* is thought to define the a negative regulatory activity separate from that of *unc-101* and *sli-1*. *rok-1* activity appears to be inductive signal dependent; thus *rok-1* likely regulates stimulated activity of *let-23* mediated signalling pathway.

These five negative regulatory pathways likely regulate different aspects of LET-23 mediated signalling. The fact that these pathways appear redundant may be a reflection of some threshold level of signalling that must

be crossed before a vulval defect can be observed, i.e., lack of one negative regulatory pathway increases LET-23 mediated signalling to a level that is not high enough to cause excessive vulval differentiation and thus we are unable to assay for this increase. The existence of five negative pathways, two of which are so far represented by only one gene, suggests that signalling events are very closely regulated processes.

Use of multiple signals

The use of a single signal for appropriate cell fate specification during normal development is likely an oversimplification. In fact, multiple signals are often used to specify a cell's fate. Examples of this include *C. elegans* vulval development, *C. elegans* male tail **pp** fate specification, and *Xenopus* mesoderm induction.

C. elegans vulval differentiation involves an inductive signal (discussed in the previous section), a lateral signal, and a negative signal mediated by *lin-15*(Herman and Hedgecock, 1990 as well as details above). The inductive signal may be sent in a graded fashion (Sternberg and Horvitz, 1986) to the VPCs, which can process the differing level of *lin-3* signal received (Katz, et al., 1995). The cell receiving the most inductive signal adopts a 1° fate while its neighbors on either side receive some signal and adopt 2° fates (Sternberg and Horvitz, 1986, Katz, et al., 1995).

Although the inductive signal appears to be sufficient for specification of a vulval fate (Sternberg and Horvitz, 1986, Katz, et al., 1995), two other signals are used during normal development to ensure the invariant pattern of three induced cells, the outside ones adopting a 2° fate and the middle one adopting a 1° fate. Interactions between the induced cells through a lateral

signal mediated by *lin-12* (Greenwald, et al., 1983, Sternberg, 1988, Simske and Kim, 1995, Koga and Oshima, submitted) enforces these fates; the 1° cell signals its 2° cell neighbors and inhibits them from adopting a 1° fate. The negative signal serves to modulate this signalling by preventing inappropriate receptor activity; without this signal, more than three cells adopt vulval fates (Ferguson and Horvitz, 1985, Herman and Hedgecock, 1990).

Multiple signals are also used during cell fate specification of the *C. elegans* male tail (Chamberlin and Sternberg, 1993). In the male tail, the specification of the **pp** cell fate requires four distinct signals, three which are also used in vulval fate specification: the inductive signal mediated by *lin-3*, a lateral signal mediated by *lin-12*, a negative signal mediated by *lin-15*, and a second negative signal from the Y.p cell that is distinct from the *lin-15* signal (Chamberlin and Sternberg, 1994).

Xenopus mesoderm induction (discussed in previous section) is another example of multiple signals used for appropriate fate specification, although whether these signals are used in a temporally distinct fashion is currently not understood (reviewed in Kimelman, et al., 1992). The dorsal-ventral axis is established through the cortical rotation of the egg; this happens at fertilization and thus precedes mesoderm induction. This rotation has been proposed to cause the localization of dorsal determinants at the new dorsal region (Vincent and Gerhart, 1987, Lemaire and Gurdon, 1994).

Candidates for molecules involved in this dorsalization include the members of the *wnt* family (Smith and Harland, 1991, Sokol, et al, 1991, Ku and Melton, 1993) and *noggin*, a secreted protein (Smith and Harland, 1992). Neither *wnt* nor *noggin* can induce mesoderm alone, although they may able

to modify the action of mesodermal inducers (Christian, et al., 1992, Smith, et al., 1993). Although different concentrations of activin, a mesodermal inducer, can elicit at least five different responses (Green, et al., 1992, Green, et al., 1994), there exists no evidence for an activin gradient *in vivo* (reviewed in Kessler and Melton, 1994). Furthermore, miniature embryos with a complete body axis can be formed from blastula stage explants cultured at a homogeneous activin concentration (Sokol and Melton, 1991), suggesting that an activin gradient is not necessary. Thus, it has been proposed that perhaps activin activity is uniform dorsal-ventrally while either *wnt* or *noggin*, which act as dorsal determinants, synergize with activin to cause the specification of different fates (Kimelman, et al., 1992, Lemaire and Gurdon, 1994, Green, et al., 1994).

Although it is clear that multiple signals are involved in specifying cell fates, it is unclear how these inputs are integrated for appropriate fate specification. For example, in *C. elegans* vulval development, one model of vulval development involves a VPC adopting a 2° fate due to two reasons: the reception of a low level of inductive signal from the anchor cell and the reception of the lateral signal from its 1° fate adopting cell neighbor. It is unknown whether the reception of the lateral and inductive signal combine to cause the new expression of genes used in 2° fate specification, whether reception of the lateral signal results in some kind of down-regulation of the transduction components that were activated as a result of LIN-3 signalling, or both. Similarly, in *Xenopus* mesoderm induction, the mechanisms by which either *wnt* or *noggin* modulate responses to activin is unknown. *wnt* and/or *noggin* could act early to predispose dorsally located cells to reception of activin signal by upregulating the activin responsive signalling machinery,

or *wnt* and/or *noggin* could modulate activin response by acting in concert with activin.

Signal content: two different types of inductive signals?

As more is discovered about inductive processes, questions have been raised about how much informational content the signal contains. Some would argue that, in the extreme, two different types of inductive events exist. One type would require the signal to carry very little information and merely tip the receiving cell toward a fate it was already predisposed to. The second type would require the signal to encode the information required to specify the receiving cell's fate. Here, I will discuss the two types of inductive signals and suggest how these two types may not be so different.

The inductive signal in *C. elegans* vulval development would be a signal that falls in the latter category. This signal not only instructs the receiving cells to adopt vulval fates, the signal can elicit different responses from the receiving cell depending on the level of signal received (Katz, et al., 1995). Lack of this signal causes the receiving cells to adopt a default fate of a non-specialized epidermal cell (Kimble, 1981, Sternberg and Horvitz, 1986).

The *boss* signal used in R7 cell fate specification has been proposed to carry much less informational content. Lack of *boss* leads to lack of R7 development (Reinke and Zipursky, 1988) while ubiquitous expression of boss will transform the cone cells to adopt an R7 cell fate (Van Vactor, et al., 1991). Similarly, consitutively activating sev in all cells that normally express sev results in both the mystery cells and the cone cells adopting R7 cell fate (Basler, et al., 1991). When an activated sev is produced through a fusion of the intracellular kinase domain of sev with an extracellular portion

of an activated Torso mutant protein and expressed under the control of the sev enhancer, cone cells adopt R7 fate as expected. However, when the sev-Torso fusion is expressed along with the *rough* gene (which is normally expresed in R2 and R5), the cone cells still adopt a photoreceptor cell fate although not an R7 fate (Dickson, et al., 1992). These results are consistent with experiments which demonstrate ectopic expression of rough in an R7 cell inhibits its development (Kimmel, et al., 1990, Basler, et al., 1990). The conclusion drawn from these experiments was that the R7 cell is predisposed to respond to *boss* by differentiating as R7 and the information from *boss* merely tells it to develop as a photoreceptor cell (Dickson, et al., 1992).

It could be argued that the signals encoded by *lin-3* and *boss* are different because of their different properties. *lin-3* appears to act in a graded fashion with the ability to specify two different vulval fates, one at a high dose and the other at a low dose (Sternberg and Horvitz, 1986, Katz, et al., 1995). On the other hand, *boss* is involved in a binary switch. Reception of *boss* by the presumptive R7 cell causes it to adopt an R7 fate; lack of *boss* leads to no R7 cell differentiation.

However, it could also be argued that the difference between the activities of *lin-3* and *boss* are due to differences in the abilities of the receiving cell to process this information. All responding cells are not naive (not even the original blastomere of the fertilized egg); for one, their developmental history endows them with the transduction machinery necessary to respond to the signal. The expression of *rough* in the cone cells in the experiments described above essentially transforms their developmental history to that of a different photoreceptor cell, causing its transformation into a non-R7 photoreceptor upon receptor activation.

Presumably, if one could alter the developmental history of a VPC, it might be possible to cause it to adopt a fate other than that of a vulval cell. Thus, the difference between *lin-3* and *boss* activities are not necessarily due to differences in the quality of the signals themselves *per se*. The informational content of the signal as well as the competence to respond to the signal (as proposed by Waddington in 1936; see first section) are both likely to be important during inductive signalling.

Signal content: are all inductive signalling events promoting cell fates?

Another question regarding the content of information encoded by the signal involves how an inductive signal promotes cell fate. The examples described above in *S. cerevisiae*, *C. elegans*, *Drosophila*, and *Xenopus* involve an inductive signal actively promoting the induced fate. However, an inductive signal may promote a cell fate by indirectly inhibiting a cell from adopting a particular fate. Here, I discuss an example from *Xenopus* neural induction where the inductive signal may carry an inhibitory content and act as an antagonist for a positive signalling event.

The action of follistatin during *Xenopus* neural induction illustrates another potential action of an "inducer" molecule. *Xenopus* neural induction occurs during gastrulation and involves an inductive event from the dorsal mesoderm to the dorsal ectoderm (reviewed in Kessler and Melton, 1994); Spemann's "organizer" (discussed in the first section of this chapter) resides in this dorsal mesoderm and is a potent neural inducer of isolated animal caps (Kintner and Dodd, 1991). Follistatin has been proposed to act as a neural inducer because its RNA is localized to the organizer region and

because injection of follistatin RNA to animal pole explants results in the induction of neuronal marker genes (Hemmati-Brivanlou, et al., 1994). However, follistatin does not appear to be a ligand that binds a receptor; follistatin is an activin anatagonist that directly binds and inhibits activin (Nakamura, et al., 1990, Kogawa, et al., 1991).

Consistent with these results is the observation that a dominant negative form of the activin receptor, when expressed in either animal cap explants or in whole embryos, can neuralize embryonic cells (Hemmati-Brivanlou and Melton, 1994). This suggests that inhibition of activin signalling results in neural development, which further suggests that neural development is the default state for these cells. Evidence that cultured dissociated embryonic cells can display neural differentiation is consistent with neural development being a default fate (Grunz and Tacke, 1989, Godsave and Slack, 1991, Sato and Sargent, 1989). Therefore, an antagonist inhibits activin signalling to promote a neuronal fate.

However, neuronal induction is likely more complex and involves multiple pathways (reminiscent of examples from the previous section). *Noggin* is a neuronal inducer (Lamb, et al., 1993) which does not inhibit activin (Smith and Harland, 1992). Furthermore, *noggin* transcription does not appear to be regulated by follistatin and *noggin* does not regulate follistatin transcription (Hemmati-Brivanlou, et al., 1994), suggesting that noggin and follistatin do not function in the same pathway. Thus, the neural induction by noggin and follistatin likely define two redundant and independent pathways. The understanding of how noggin induces neuronal fates should give insight into how the follistatin antagonism of activin works in concert with noggin *in vivo*.

Signals that result in several outputs

In general, the output of inductive signals have been looked upon simplistically. The LIN-3 signal induces vulval fates, mating pheromone causes haploid cells to mate, and *boss* induces development of the R7 photoreceptor. However, these induced cells are very specialized cells and the execution of these fates likely involves the coordination of several physiological events.

The coordination of these events is best understood in *S. cervisiae* (described above) where the process of mating has been observed to include new gene transcription, cell cycle arrest, and morphological changes that allow for conjugation and cell fusion (Figure 2). Transcription of genes required for mating occurs through the phosphorylation and activation of the STE12 transcription factor upon mating pheromone reception (Elion, et al., 1993, reviewed above). On the other hand, cell cycle arrest does not involve STE12 and occurs through the phosphorylation of FAR1 by activated FUS3 (MAPK) (Peter, et al., 1993, Elion, et al., 1993, Tyers and Futcher, 1993). Activated FAR1 then binds CDC28-CLN1 and CDC28-CLN2 to inhibit their activity (Peter and Herskowitz, 1994). Thus, inhibition of the cell cycle occurs at a branch of the transduction pathway at the MAPK. How the morphological changes that occur to cause cell polarization and mating projection formation are linked to the signal transduction cascade are currently unknown. These events are hypothesized to occur through a much earlier branch from the transduction cascade, possibly at the G-protein or the receptor (reviewed in Chenevert, 1994).

Branching in other signal transduction pathways has not been as well defined as in yeast. However, studies on the *torso* receptor tyrosine kinase

used during embryonic terminal development in *Drosophila* have defined the existence of an upstream branch in the signal transduction cascade. Torso signalling occurs through essentially the same downstream components as those used in R7 cell fate specification (reviewed in Duffy and Perrimon, 1994, R7 cell fate specification discussed above). Activation of Torso normally results in the localized expression of *tailless* (Pignoni, et al., 1990, Pignoni, et al., 1992) and *huckebein* (Weigel, et al., 1990, Brönner, et al., 1994) at the termini of the embryo; conversely, lack of *torso* greatly diminished *tailless* and *huckebein* expression in the termini. Expression of *tailless* and *huckebein* in embryos lacking *D-raf* is essentially the same as in embryos lacking *torso*. However, embryos lacking *Ras1* have greater expression of *tailless* and *huckebein* than embryos lacking *torso*, suggesting that Torso mediated signalling is not completely blocked by lack of *Ras1* activity. Embryos lacking *drk* (adaptor) and embryos lacking *Sos* (GNEF) demonstrate even more *tailless* and *huckebein* expression than *Ras1*-lacking embryos, with *drk*-lacking embryos having the greatest expression of the target genes (Hou, et al., 1995). These results suggest the existence of a branch in the pathway at the receptor, allowing for *ras* independent activation of *raf*. Also, because *drk*-, *Sos*-, and *Ras1*- lacking embryos do not have the same phenotype, it is possible that other branches in this signalling pathway also exist.

The function of any of these proposed branches is currently unknown. In fact, many more pathways involved in inductive signalling in development probably have branches that cause different physiological changes required for the appropriate specification of fate. It is clear, for example, that the execution of R7 fate involves both cell cycle arrest, new gene transcription (i.e. the *Rh3* and *Rh4* R7 specific opsins), and morphological changes such as

sending out an axon and elaborating a rhabdomere (the light gathering structure); some of these changes may involve direct branches from the *boss/sev* mediated signal transduction cascade as seen in *S. cerevisiae*. These studies may await a new resolution of phenotypic visualization for a true understanding of the physiological changes required for appropriate fate specification.

CONCLUSION

A century's worth of experimentation has brought us from the initial observation of inductive phenomena to an understanding of the mechanisms involved in cell-cell signalling during inductive processes. This understanding has raised many more questions regarding the detailed mechanisms of inductive signalling, which another century's worth of work should elucidate.

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Figure 1. Examples of inductive signalling in development. See text for further description. A "?" indicates a step in the transduction pathway that likely exists but has not been yet defined. "? X ¿" indicates a molecule, X, that may act at that particular step.

From left to right: Mating pheromone signalling in *S. cerevisiae*. A pheromone is sent from a haploid cell to a different haploid cell of the opposite cell type.

Inductive signalling in vulval differentiation in *C. elegans*. An inductive signal is sent from the anchor cell (AC) to the multipotent vulval precursor cells (VPCs) below.

R7 fate specification in *Drosophila* eye development. The R8 photoreceptor cell sends an inductive signal to the developing R7 cell.

Mesoderm induction in *Xenopus*. The vegetal hemisphere is the source of the inductive signal to the animal hemisphere, inducing mesodermal development in the equatorial region.

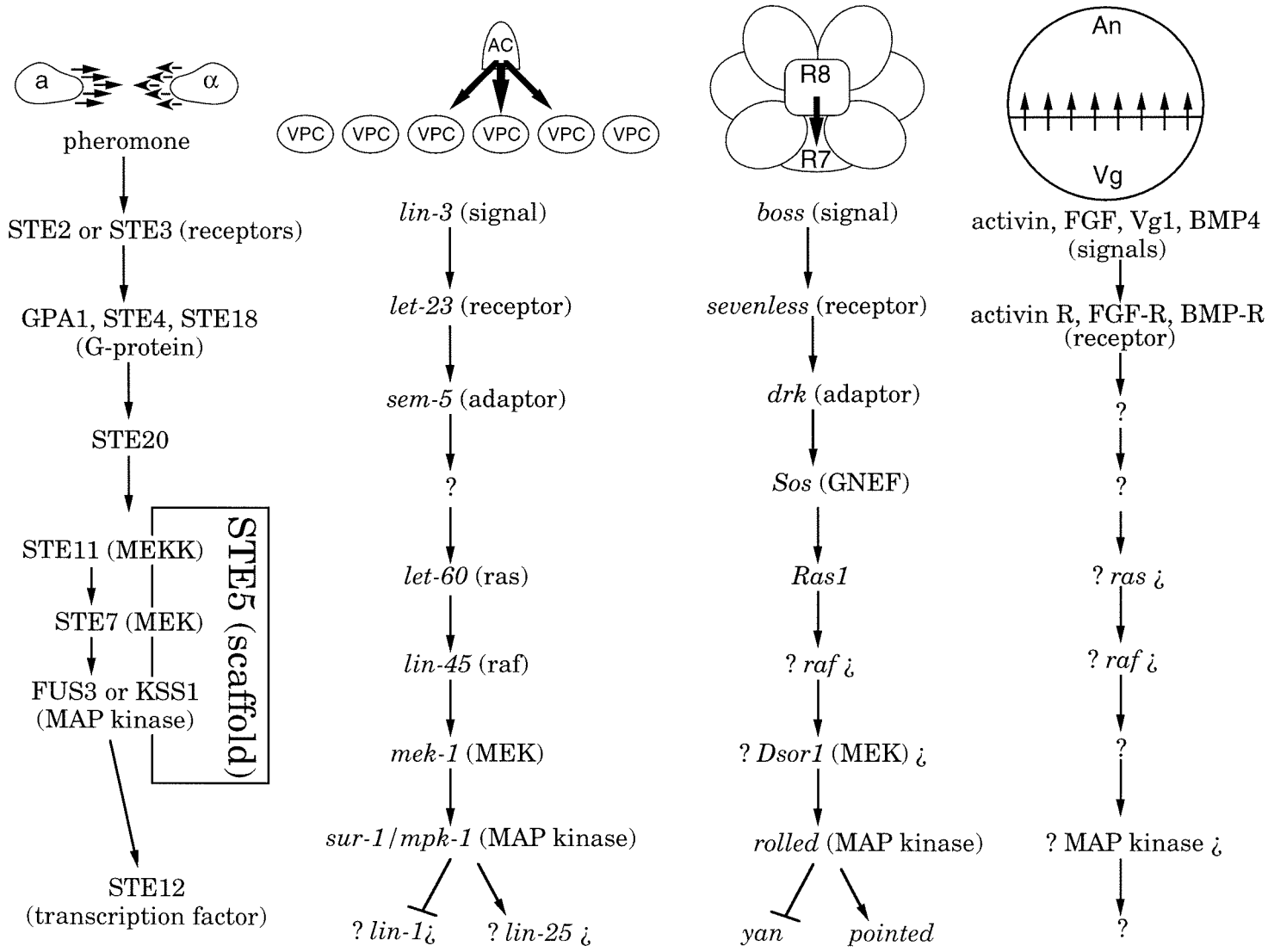
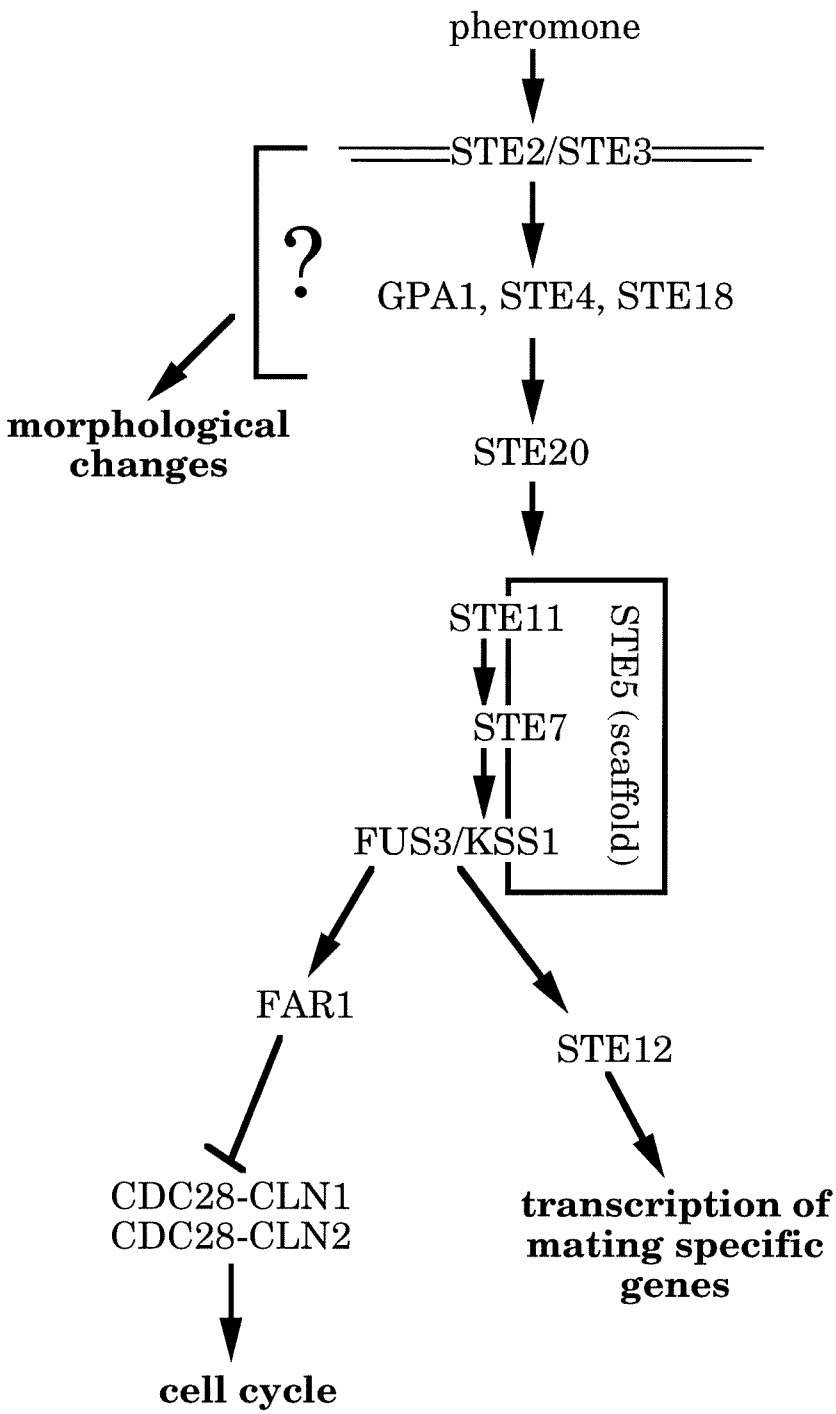

a
α
pheromone
STE2 or STE3 (receptors)
GPA1, STE4, STE18
(G-protein)
STE20
STE11 (MEKK)
STE7 (MEK)
FUS3 or KSS1
(MAP kinase)
STE5 (scaffold)
STE12
(transcription factor)
AC
VPC
VPC
VPC
VPC
VPC
VPC
lin-3 (signal)
let-23 (receptor)
sem-5 (adaptor)
?
let-60 (ras)
lin-45 (raf)
mek-1 (MEK)
sur-1/mpk-1 (MAP kinase)
? lin-1¿
? lin-25 ¿
R8
R7
boss (signal)
sevenless (receptor)
drk (adaptor)
Sos (GNEF)
Ras1
? raf ¿
? Dsor1 (MEK) ¿
rolled (MAP kinase)
yan
pointed
An
Vg
activin, FGF, Vg1, BMP4
(signals)
activin R, FGF-R, BMP-R
(receptor)
?
?
? ras ¿
? raf ¿
?
? MAP kinase ¿
?

Figure 2. Branching of the transduction pathway. Upon pheromone reception, the *S. cerevisiae* haploid cell undergoes several physiological changes resulting in new gene transcription, cell cycle arrest, and cell polarization toward the pheromone source. See details in text.


pheromone
STE2/STE3
?
GPA1, STE4, STE18
morphological changes
STE20
STE11
STE7
FUS3/KSS1
STE5 (scaffold)
FAR1
STE12
CDC28-CLN1
CDC28-CLN2
transcription of mating specific genes
cell cycle

Chapter 2

The *lin-15* locus encodes two negative regulators of *Caenorhabditis elegans* vulval development

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The *lin-15* Locus Encodes Two Negative Regulators of *Caenorhabditis elegans* Vulval Development

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During *Caenorhabditis elegans* vulval development, an inductive signal from the anchor cell stimulates three of the six vulval precursor cells (VPCs) to adopt vulval rather than nonvulval epidermal fates. Genes necessary for this induction include the *lin-3* growth factor, the *let-23* receptor tyrosine kinase, and *let-60 ras*. *lin-15* is a negative regulator of this inductive pathway. In *lin-15* mutant animals, all six VPCs adopt vulval fates, even in the absence of inductive signal. Previous genetic studies suggested that *lin-15* is a complex locus with two independently mutable activities, A and B. We have cloned the *lin-15* locus by germline transformation and find that it encodes two nonoverlapping transcripts that are transcribed in the same direction. The downstream transcript encodes the *lin-15*A function; the upstream transcript encodes the *lin-15*B function. The predicted *lin-15*A and *lin-15*B proteins are novel and hydrophilic. We have identified a molecular null allele of *lin-15* and have used it to analyze the role of *lin-15* in the signaling pathway. We find that *lin-15* acts upstream of *let-23* and in parallel to the inductive signal.

INTRODUCTION

Intercellular signaling is a common mechanism used by multicellular organisms to specify cell fate during development, and receptor tyrosine kinases (RTKs)[1] (reviewed by Fantl *et al.*, 1993) represent one important class of signaling molecules. In general, the extracellular domain of an RTK binds an intercellular signal and regulates an intracellular tyrosine kinase activity. Upon ligand stimulation, RTKs associate with substrates and other signal transducers. RTKs involved in development include the insulin family RTK *sevenless* required for *Drosophila* photoreceptor development (Hafen *et al.*, 1987), the platelet-derived growth factor (PDGF) family RTK *torso* involved in *Drosophila* terminal development (Sprenger *et al.*, 1989), the *Drosophila* epidermal growth factor (EGF) receptor homologue DER involved in *Drosophila* dorsal/ventral patterning, ommatidial patterning in eye imaginal discs, and neuronal fate (Price *et al.*, 1989; Schejter and Shilo, 1989), the *c-kit* RTK encoded by the W-locus utilized in mouse hematopoiesis and spermatogenesis (Chabot *et al.*, 1988), and the fibroblast growth factor receptor used in *Xenopus* mesoderm induction (Amaya *et al.*, 1991).

RTK activity is normally modulated by reception of ligand (Massagué and Pandielle, 1993). Thus, both ligand expression and localization are important for regulating RTK activity. Control of the expression of the RTKs themselves is another regulatory mechanism; overexpression of the EGF-RTK is seen in human solid tumors as well as tumor cell lines, although it is unclear whether this is a cause or an effect (Cowley *et al.*, 1984; Lin *et al.*, 1984; Merlino *et al.*, 1984; Ullrich *et al.*, 1984; Derynck *et al.*, 1987). Furthermore, downregulation of RTK-mediated signaling through phosphorylation of defined sites of the receptor C-terminus, the activities of phosphatases, and the activity of protein kinase C also occurs (reviewed by Carpenter and Wahl, 1990). Negative regulation of the EGF-RTK has been proposed to occur through Müllerian Inhibitory Substance (Cigarro *et al.*, 1989), a transforming growth factor-β homologue (Cate *et al.*, 1986), although this inhibition may be indirect. Other events, such as ligand-mediated internalization and receptor dimerization, also define steps where potential regulation of RTK activity could occur.

[1] Abbreviations used: AC, anchor cell; Berg, Bergerac; Bris, Bristol; EMS, ethylmethonesulfonate; Muv, multivulva; PCR, polymerase chain reaction; Rol, rolling; RTK, receptor tyrosine kinase; VPC, vulval precursor cell; Vul, vulvaless.

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During *Caenorhabditis elegans* vulval development, a signal is sent from the anchor cell (AC) of the hermaphrodite gonad to the vulval precursor cells (VPCs), which are P3.p, P4.p, P5.p, P6.p, P7.p, and P8.p, in the ventral epidermis (Kimble, 1981). This inductive signal causes three of the six VPCs to undergo vulval development, whereas the other three cells undergo nonvulval epidermal development. The three VPCs adopting vulval fates do so in a stereotyped pattern. P6.p, the VPC closest to the anchor cell adopts a 1° fate; the neighboring P5.p and P7.p adopt 2° fates. Removal of the inductive signal causes all VPCs to adopt nonvulval epidermal fates (Kimble, 1981; Sulston and White, 1980). Cells that adopt vulval fates undergo three rounds of cell divisions, characterized by stereotyped cell division patterns and morphogenetic movements; cells that adopt nonvulval epidermal fates divide at most only once (Sulston and Horvitz, 1977). The direct observation of cell division patterns provides a straightforward assay of VPC fate.

The inductive signal provided by the AC is an EGF-like growth factor encoded by *lin-3* (Hill and Sternberg, 1992; Hill, Katz, and Sternberg, unpublished data). *let-23*, the candidate receptor for the signal, encodes an EGF-like RTK (Aroian *et al.*, 1990). Several molecules downstream of *let-23* have been identified, including *sem-5*, a SH2/SH3 adaptor protein (Clark *et al.*, 1992); *let-60*, a *ras* homologue (Han and Sternberg, 1990); and *lin-45*, a *raf* homologue (Han *et al.*, 1993) (see Figure 1). Reduction of function mutations in any of these genes result in a vulvaless (Vul) phenotype, where all six VPCs adopt nonvulval epidermal fates.

Loss of function mutations in the *lin-15* locus result in an opposite phenotype. All six VPCs adopt vulval fates, leading to a multivulva (Muv) phenotype (Ferguson and Horvitz, 1985; Ferguson *et al.*, 1987). Thus, *lin-15* is a negative regulator of vulval differentiation. Genetic mosaic analysis indicates that *lin-15* acts in cells other than the AC and the VPCs (i.e., nonautonomously) (Herman and Hedgecock, 1990). *lin-15* is not the only locus involved in this aspect of negative regulation. *lin-15* is a member of a set of negative regulators; appropriate combinations of mutations in these loci cause a Muv phenotype (Ferguson and Horvitz, 1989). This group of negative regulators was first defined by the genes *lin-8* and *lin-9* and now includes many other loci. *lin-8* and *lin-9* define class A and class B genes, respectively. Animals carrying a single mutation in either A or B genes are wild-type; only animals homozygous mutant for both an A and a B gene are Muv. *lin-15* is a complex locus; it is the only genetically identified locus in this pathway to have three kinds of alleles. The A and B alleles of *lin-15* have a wild-type vulval phenotype. There are also alleles that confer a Muv phenotype; we refer to these as AB alleles. We cloned the *lin-15* locus to study the basis for the two functions and to elucidate the role of *lin-15* in vulval differentiation.

MATERIALS AND METHODS

Strains and Culture Conditions

Nematodes were cultured as described (Brenner, 1974; Sulston and Hodgkin, 1988). All experiments were performed at 20°C unless otherwise noted. The following mutations and strains were used in this study: linkage group X: the *lin-15* alleles *n309*, *e1763*, *n1139*, *n765* (Ferguson and Horvitz, 1985), *n744*, *n767* (Ferguson and Horvitz, 1989), *n744sy212*, *n744sy211* (this study), *n767sy222* (Hill, personal communication), *sy197* (Lee, personal communication), *sup-10(n983)* (Greenwald and Horvitz, 1982), *mec-5(e1340)* (Chalfie and Sulston, 1981), *unc-3(e151)* (Brenner, 1974), *lin-8(n111)* (Ferguson and Horvitz, 1985), and *mnDp1(X;V)*; *mnDf4 (X)* (Meneely and Herman, 1979); linkage group III: *lin-36(n766)* (Ferguson and Horvitz, 1989); linkage group II: *let-23(sy97)/mnC1(dpy-10 unc-52)* (Herman, 1978; Aroian and Sternberg, 1991); the wild-type *C. elegans* strain N2 (Brenner, 1974), and the Bergerac (Berg) BO *C. elegans* strain (Emmons *et al.*, 1979). Genetic nomenclature follows Horvitz *et al.* (1979), except we designate the *lin-15* allele *n765* as "*lin-15AB*^{n}*(n765)*" because animals carrying this mutation display a Muv phenotype at 20°C, although the B function is restored at 15°C (Ferguson and Horvitz, 1985).

Physical Mapping of the lin-15 Locus

We mapped the *lin-15* locus by constructing recombinants between the *C. elegans* Bristol (Bris) strain and the Berg strain, a *C. elegans* strain that contains many polymorphisms with respect to the Bris strain as described (Rose *et al.*, 1982). Because *lin-15* is X-linked and *C. elegans* males are XO, we allowed Bris N2 males to mate with Berg BO hermaphrodites. *Berg*/0 males from this cross were then allowed to mate with *lin-15AB*^{n}*(n765 Bris) sup-10(n983 Bris)* hermaphrodites, and their heterozygous cross-progeny hermaphrodites (*lin-15AB*^{n}*(n765 Bris) sup-10(n983 Bris)/lin-15(+ Berg) sup-10 (+ Berg)*) were selected and grown at 25°C. Their progeny were screened for animals with the Sup-10(n983) phenotype but not the Muv phenotype; these were presumed to be of the genotype *lin-15AB*^{n}*(n765 Bris) sup-10(n983 Bris)/lin-15(+ Berg) sup-10(n983 Bris)*. These animals were allowed to self-fertilize, and their Sup-10 progeny that did not segregate Muv animals in the next generation were kept. Twenty recombinants were found. DNA from these animals was prepared, digested with restriction enzymes, Southern blotted, and probed with TU#W1723, a clone that identified a polymorphism from the *sup-10* contig (Driscoll and Chalfie, personal communication). Of the genomic DNA from the 20 recombinants, 13 displayed a Bris pattern, five displayed a Berg pattern, and two were ambiguous.

Germline-mediated Transformation by Microinjection

Microinjection was performed as described (Mello *et al.*, 1991), modified from Fire (1986). For all injection experiments, the plasmid pRF4, containing the *rol-6(su1006)* mutant gene (Mello *et al.*, 1991), was used as a transformation marker at a concentration of 50 ng/μl. Injected animals were placed on new plates at least once per day, and their progeny were examined one to four times per day. F1 rolling (Rol) animals were placed on individual plates as soon as they were identified and scored under a dissecting microscope for rescue of the Muv phenotype upon reaching adulthood. Their progeny were examined for the presence of Rol animals to determine whether the line was stable, and if stable, were checked for their Muv phenotype. All injected animals, F1 transformants, and transgenic lines were maintained at 20°C. Animals of the genotype *lin-15AB*^{n}*(n765)*, *lin-8A(n111)*; *lin-15B(n744)*, and *lin-36B(n766)*; *lin-15A(n767)* were injected, as described in Figure 2, Figure 8, and Table 1.

RNA Analysis

Mixed stage RNA was prepared from N2 animals using guanidinium thiocyanate and pelleted using CsTFA (Pharmacia, Piscataway, NJ).

Poly A$^+$ selection was performed using a poly dT column (Pharmacia). Twice selected poly A$^+$ RNA (7.5 μg) was separated on a 1% denaturing formaldehyde agarose gel, blotted onto Hybond-N (Amersham, Arlington Heights, IL), and probed with random-primed DNA labeled with ^{32}P, all according to standard methods (Sambrook *et al.*, 1989; Ausubel *et al.*, 1992). Exposures were done for 2 1/2 wk at −70°C with an intensifying screen on Kodak (Rochester, NY) XAR film.

Subclones and Southern blots

All subcloning, Southern blotting, and DNA manipulations were performed according to standard methods (Sambrook *et al.*, 1989; Ausubel *et al.*, 1992). *C. elegans* genomic DNA was prepared according to the methods of Andy Fire as described by Sulston and Hodgkin (1988).

cDNA Analysis

Two cDNA libraries were screened with the 6.8-kilobase (kb) *Bam*HI fragment of plasmid p68B3 (see Figure 1 for location) using standard methods (Sambrook *et al.*, 1989; Ausubel *et al.*, 1992) because p68B3 hybridizes with both transcripts detected on a Northern blot (see Figure 2). One cDNA library was in λgt10 (kindly provided by S. Kim, Stanford University); the other was in λSHLX2 (kindly provided by C. Martin, Columbia University). Two cDNAs were obtained from the Kim library and six from the Martin library. Two noncross-hybridizing classes of cDNAs were detected, and both classes were represented in both libraries. These cDNAs were mapped to genomic DNA through Southern hybridization using probe synthesized from the cDNA clones against genomic clones digested with various restriction enzyme combinations and blotted on nylon membranes.

The composite cDNA for the upstream transcript appeared not to include the 5′ end of the gene based on the apparent size of the transcript as detected on a Northern Blot (see Figure 3). Therefore, a third cDNA library in λZAP (kindly provided by B. Barstead, Oklahoma Medical Research Foundation) was probed with pbLH10, which contains a 1.3 kb-*Eco*RI fragment from base 3045 to base 4396, upstream of the most 5′ cDNA sequence. Six cDNAs were obtained; three were identical clones (cF1, cD1, cF2). The four unique cDNAs were *lin-15* clones, as demonstrated by DNA sequencing. However, two of the four cDNAs (cF1, cD3) were fused to cDNAs from other genes, as evidenced by the presence of an *Eco*RI linker fused to a poly A$^+$ tail in the middle of the clone. A third cDNA (cD2) ended at the 3′ end of the *lin-15* upstream transcript but had a highly rearranged 5′ end in previously sequenced DNA. The fourth cDNA (cL1) was an intact cDNA that started 848 basepair (bp) 5′ of the genomic 3′ end of the upstream transcript. Sequence from cL1 gave 286 bp more cDNA sequence from the previous 5′ end. Sequence from the *lin-15* portion of cF1 gave 1110 bp more cDNA sequence upstream of that obtained from cL1. However, the cDNA sequence from cF1 was separated from the cDNA sequence inferred from cL1 by a 35 bp genomic gap that was later found to be transcribed, as described below.

Exon Predictions

Because the most 5′ cDNA of the upstream transcript was not full length, we predicted potential exons by examining the genomic sequence. Because *C. elegans* introns have, on average, a 68% AT content (Fields, personal communication), the genomic region was plotted for its relative AT versus GC content. AT-rich regions were considered potential introns. The genomic DNA sequence was scanned by eye for potential splice donor and acceptor sites (Emmons, 1988) that would maintain the open reading frame, on the basis of our known cDNA sequence. Thus, the exons predicted had to fulfill three criteria. 1) The exon had to have an open reading frame that would splice in-frame to the next exon. 2) The exon had to have splice donor and acceptor sites of reasonable consensus. 3) The exon had to have a GC content of >35%.

To confirm exon predictions, we used 4.5 μg of once-selected poly A$^+$ RNA to synthesize cDNA using primer m1sq (CGG GTA ACG TAG TTG TAG), primer w2sq (GGA AAC GAT ATC CCA TTG AG), primer 18BJ7B (GCG GTT TAG ATG ATG TGC TG), and primer 20J7A (GCG ATA AAT ACA GCC CAA C). The polymerase chain reaction (PCR) was performed on one-tenth of the synthesized cDNA using primer bF (ACA GCA CGA CTT ACA TCA AAC CCG GCA TCA), primer wF (GGC CGT TGC CAC CGA GAC CAG CCA ATG TGC C), primer mR (CGT TTG ACA ACT GCA CTC TCT TCA ACC AGC), primer wR (GTT GCG AAT TGA CAT GAT GGC TGG CAC AAC), and primer xR (CGG TTG CAA GCT CTG CGC TTC TGG AAT TCC) to confirm exon predictions. PCR was performed using Pfu DNA Polymerase (Stratagene, La Jolla, CA) using the following conditions: 95°C, 1 min; 60°C, 1 min; 72°C, 1 min 30 sec, for 26 cycles. Band of predicted sizes were obtained using combinations of primer bF with primer mR, primer bF with primer wR, and primer wF with primer xR. The bands obtained were cloned and sequenced according to standard methods.

Identifying Trans-splicing to SL2

cDNA was made from 30 μg of N2 RNA using primers A4-RA6 (GAG ATA CTC GAT GAT CCA GC) and D32R (CGG TTT GAC AGT TGG GAG). PCR was performed using primers SL2 (GGT TTT AAC CCA GTT ACT CAA G) and A4-VR6 (GAT GAG TTG AAT GAG TTC TTG CTT ATC AGC), using one-fifth of the cDNA synthesized. Primer SL2 is the exact sequence of the SL2 trans-splice leader (Huang and Hirsh, 1989). One-tenth of the PCR reaction was separated on a 0.8% Tris-Borate-EDTA (TBE) gel and blotted onto Hybond-N (Amersham). The blot was probed with random primed B11c, a cDNA of the downstream transcript obtained from the Martin library, to establish that the 1.2-kb band visualized with ethidium bromide staining was indeed a *lin-15* clone. Three different thermostable polymerases, Taq (Cetus, Berkeley, CA), Vent (New England Biolabs, Beverly, MA), and Pfu (Stratagene) were used in three independent PCR reactions to minimize polymerase artifacts. The 1.2-kb bands from the three different reactions were isolated from a low melting point TBE agarose gel, cloned, and sequenced to confirm their identity as *lin-15* clones.

Sequencing

Double strand sequencing of all genomic and cDNA clones was performed using a combination of primer walking, subcloning, and γδ transposon sequencing as described (Sambrook *et al.*, 1989; Strathmann *et al.*, 1991; Ausubel *et al.*, 1992). Sequencing reactions were performed using Sequenase version 2.0 and other reagents from United States Biochemical (Cleveland, OH). Sequence was compiled using the International Biotechnologies program, MacVector 3.5 (New Haven, CT). The predicted proteins were subjected to database searches against both Genbank and EMBL (Heidelberg, Germany) using BLAST (Altschul *et al.*, 1990), TFASTA (Pearson and Lipman, 1988), and BLOCKS (Henikoff and Henikoff, 1991). The BLAST search was performed at the National Center for Biotechnology Information using the BLAST network service. The protein motif finding programs MacPattern and Motifs were used on both predicted proteins. Motifs is within the GCG Package, Version 7, from the Genetics Computer Group, University of Wisconsin (1991). DNA Strider was used to calculate the predicted molecular weight of the proteins and to determine the amino acid composition. MacVector 3.5 was used to plot hydrophilicity, using the Kyte Doolittle hydrophilicity scale.

Screen for New lin-15AB Alleles Starting with a lin-15B Allele

Hermaphrodites homozygous for *lin-15B(n744)* were mutagenized at 20°C using ethylmethonesulfonate (EMS) as previously described (Brenner, 1974; Sulston and Hodgkin, 1988). P0 animals were allowed to self-fertilize for two generations, and F2 progeny were screened for Muv animals. Of 6900 mutagenized gametes screened, two new *lin-15*AB alleles, *sy212n744* and *sy211n744*, were recovered.

Noncomplementation Screen Starting with lin-15AB^{ts}(n765)

mec-5(e1340) males were mutagenized at 20°C with EMS as above and allowed to mate with *unc-3(e151) lin-15AB^{ts}(n765)* animals. After EMS treatment, all animals and their progeny were grown at 15°C. The F1 generation was screened for Muv animals. Of 35 000 mutagenized gametes screened, no candidates were found. Dominant mutations of other genetic loci were seen, indicating that the mutagen was effective.

unc-3(e151) lin-15AB^{ts}(n765)/mnDf4 animals were constructed by mating N2 males with hermaphrodites of the genotype *mnDp1/+; mnDf4*. *mnDf4* is a deficiency of the X chromosome that removes *lin-15* along with other nearby genes (Meneely and Herman, 1979); this deficiency is covered by the duplication *mnDp1*, which is attached to chromosome V (Herman *et al.*, 1976). This cross was performed at 20°C; the rest of this experiment was performed at 15°C. *mnDp1/+; mnDf4/0* males from this cross were allowed to mate with *dpy-17(e164); unc-3(e151) lin-15AB^{ts}(n765)* hermaphrodites. In the next generation, nonDumpy Uncoordinated hermaphrodites were scored for their Muv phenotype. The nonDumpy Uncoordinated animals were presumed to be of the genotype *dpy-17(e164)/+; unc-3(e151) lin-15AB^{ts}(n765)/mnDf4*. Of the 40 nonDumpy Uncoordinated animals scored, 31 of them (77%) displayed protrusions on their ventral surface that could be easily scored under a dissecting scope.

lin-15A(n767) Deletion Localization

DNA from N2 and *lin-15A(n767)* animals was digested with *Eco*RI, *Bam*HI, and *Hin*dIII, singly and in combination, separated by electrophoresis on agarose gels, and blotted onto Hybond-N according to standard methods (Sambrook *et al.*, 1989; Ausubel *et al.*, 1992). These Southern blots were then probed with pgLH2, a 2.8-kb *Sal* I/*Bam*HI fragment containing DNA from base 6886 to 9659 (see Figure 5). The *lin-15A(n767)* deletion was localized to within a *Eco*RI/*Hin*dIII 336-bp fragment. Primers were designed to allow for PCR of an ~300-bp fragment from *lin-15A(n767)* DNA. PCR on 50 ng of *lin-15A(n767)* and N2 DNA using both Taq (Cetus) and Pfu (Stratagene) DNA polymerases was performed using primers n767F (GGC GAC CTG CAC AAA GGG AAG GTT ACG TCG) and n767R (CGC TTT CTG CGT TCT ACA GTG TTC TGC ATC). One-tenth of the PCR reaction was separated on a 1% TBE agarose gel, and the PCR product was directly sequenced using primers SQ7F (TTA CGT CGA ACA CTC CGC) and SQ6R (CAG TGT TCT GCA TCT CAC). As primer n767F was only 43 bp away and primer SQ7F was only 33 bp away from the 5' end of the deletion breakpoint, it was difficult to read the sequence on the minus strand all the way to the 5' breakpoint. Therefore, we confirmed the location of both the 5' and 3' breakpoint and the structure of the *lin-15A(n767)* mutation by repeating this procedure using primers n767FZ (TGC CAC CGG ACA GAT TGA TGC GTG GCC ATG) and n767RZ (GGG AAT ATA CGG TTC ACT AGG TGC ATC TTC) for PCR and primers 7SQFZ (GGC CAT GAT ATC TAT AGA GC) and 6SQRZ (GCA TCT TCA GTG TGA TCT CG) for sequencing. Both primer sets revealed the same rearrangement.

lin-15AB(e1763) Deletion Localization

Because of repetitive DNA, we were unable to determine the 3' deletion breakpoint of *lin-15AB(e1763)* by mapping with genomic Southern blots followed by PCR as performed for *lin-15A(n767)*. Therefore, the *lin-15AB(e1763)* deletion was localized using a variation of ligation-mediated PCR (Mueller *et al.*, 1992). Six micrograms DNA from *lin-15AB(e1763)* animals was ethanol precipitated and resuspended in 18 μl ddH_2O. The DNA was modified by adding 54 μl 88% formic acid (Mallinckrodt, Paris, KY), vortexing for 25 sec, and incubating for 1 min 30 sec. One hundred sixty-four microliters stop solution (0.36 M NaOAc pH 7.0, 0.14 mM EDTA pH 8.0, 0.5 μg/μl tRNA) was added before ethanol precipitation. The modified DNA was then cleaved by resuspension in 200 μl 1:10 diluted piperidine (Aldrich, Milwaukee, WI) at room temperature and heating to 90°C for 30 min. Piperidine-cleaved samples were then recovered as described (Mueller *et al.*, 1992). First strand synthesis was carried out on 200 ng cleaved DNA using a denaturing time of 5 min at 95°C, annealing time of 30 min at 50°C, and extension time of 10 min at 76°C. The remainder of the first strand synthesis and ligation were performed as described (Mueller *et al.*, 1992) using primer LMPCR.1 (GCG GTG ACC CGG GAG ATC TGA ATT C), primer LMPCR.2 (GAA TTC AGA TC), and the *lin-15* specific primer, e1763.1 (TCC TTT ATC CAC AAT GGT CA).

The first round of PCR was performed on one-half of the ligated DNA in a mixture consisting of 10 μl DNA, 20.5 μl ddH_2O, 2.5 μl 20 μM primer LMPCR.1, 2.5 μl 20 μM primer e1763.2 (TGT CTA GAA CAC TAT CTC CTC TCC AT), 5 μl Pfu buffer (Stratagene), 8 μl 1.25 mM dNTPs, 1.5 μl Pfu (3.75u, Stratagene), and 50 μl mineral oil, using PCR conditions as follows: 95°, 1 min; 60°C, 1 min; 72°C, 3 min, 20 cycles total. One-half of the PCR reaction was separated on a 0.8% low melting point TBE agarose gel. DNA migrating between 600 bp and 1.2 kb was isolated from the gel, and one-half of the recovered DNA was subjected to a second round of PCR in a mixture consisting of 10 μl DNA, 23.5 μl ddH_2O, 1 μl 50 μM kinased primer LMPCR.1, 1 μl 50 μM kinased primer e1763.3 (CTA GAA CAC TAT CTC CTC TCC ATT GTC TA), 5 μl Pfu buffer, 8 μl 1.25 mM dNTPs, 1.5 μl Pfu (3.75 u, Stratagene), and 50 μl mineral oil, using PCR conditions as follows: 95°C, 1 min; 61°C, 1 min; 72°C, 3 min for 25 cycles. One-half of the PCR reaction was separated on a 0.8% low melting point TBE agarose gel, and DNA running between 600 bp and 1.2 kb was recovered from the gel. The recovered DNA was cloned into Bluescript SK$^+$ (Stratagene), and four independent isolates were sequenced to determine the 3' breakpoint of *lin-15AB(e1763)*. This result was confirmed by performing PCR on 50 ng of *lin-15AB(e1763)* genomic DNA using primers e1763.chF (GCT GCC ATA AAA TCT AAT AAT CCG CTG CAC) and e1763.chR (TTC TAA CCT GAA AAA TTC CGT GTG ACC GA). The ~350-bp band obtained from this PCR reaction was sequenced to confirm its identity as a *lin-15AB(e1763)* clone.

Construction of let-23(sy97); lin-15AB(e1763)

let-23(sy97)/mnC1 (dpy-10 unc-52) males were allowed to mate with *lin-15AB(e1763)* hermaphrodites. L4 hermaphrodite cross-progeny were placed onto individual plates and allowed to self-fertilize. The genotype of *let-23(sy97)/+; lin-15AB(e1763)/+* was determined upon scoring of both Muvs and Vuls in the next generation. Muv and Vul progeny from *let-23(sy97)/+; lin-15AB(e1763)/+* were selected. No Muvs were found segregating from Vul hermaphrodites, whereas Vuls were found segregating from Muv hermaphrodites. The Vuls segregating from the Muv hermaphrodites were the desired genotype *let-23(sy97); lin-15AB(e1763)*.

Gonad Ablations, Vulval Differentiation, Vulval Lineages, and Photography

Animals were mounted on 5% noble agar pads containing ~3 mM sodium azide as an anesthetic, and gonad precursor cells were killed by laser ablation in L1 animals as previously described (Sulston and White, 1980; Avery and Horvitz, 1987; Sternberg, 1988). Ablations were verified 4–20 h after surgery, before the time of vulval differentiation. Vulval differentiation was scored during or after the L3 molt.

To score vulval differentiation, living animals were mounted on 5% noble agar pads and examined under Nomarski microscopy as previously described (Sulston and Horvitz, 1977; Sternberg and Horvitz, 1981). The number of cells undergoing vulval differentiation was tabulated by scoring for the presence of vulval cells versus large nonvulval epidermal cells after the L3 molt, the time after vulval differentiation occurs in intact animals. The percentage of cells undergoing vulval differentiation was determined as previously described (Aroian and Sternberg, 1991). In some cases, vulval cell lineages were followed by watching cell divisions under Nomarski microscopy. Photomicrographs were taken on Kodak technical pan film on a Zeiss Axioskop (Thornwood, NY) under Nomarski optics with a 40× objective.

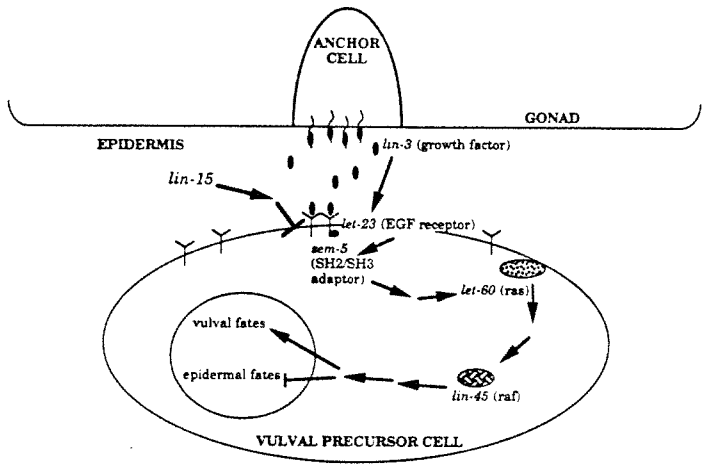

Figure 1. Model for vulval induction. This pathway summarizes the work of many people (see Sternberg, 1993, for review).

RESULTS

Identification of the lin-15 Genomic Region

We correlated the genetic and physical map in the *lin-15 sup-10* region of the X chromosome by making recombinants between the highly polymorphic *C. elegans* Berg BO strain and the *C. elegans* Bris strain as described in MATERIALS AND METHODS. By probing the recombinant genomic DNA with radiolabeled probe synthesized from the polymorphism-containing clone, TU#W1723 (M. Driscoll and M. Chalfie, personal communication), we determined that TU#W1723 was approximately one-third of the distance from *sup-10* to *lin-15* (see MATERIALS AND METHODS for details). The cloning of *sdc-1* (Nonet and Meyer, 1991), which genetically maps between *sup-10* and *lin-15* (Villeneuve and Meyer, 1990), confirmed our map data and strongly suggested that *lin-15* was near the end of the then existing *sup-10* contig, an overlapping set of genomic clones for the left end of the X chromosome. Thus, we injected PS#74B3, a cosmid mapping to the end of the *sup-10* contig, overlapping cosmids F14H4 and C29B12 (Mendel and Coulson, personal communication). Microinjection-mediated germline transformation using PS#74B3 rescued the *lin-15* Muv phenotype (Figure 2). To further define the *lin-15* containing region, we injected smaller subclones from the cosmid and found that the pJM23 plasmid would rescue *lin-15*.

Genomic Analysis of the Rescuing Region

Genomic subclones from the region contained in the pJM23 15-kb rescuing fragment were used to probe a Northern blot containing RNA from wild-type animals. Two transcripts were detected (Figure 3). The larger transcript was recognized by both probes (p18B3 and p68B3); the smaller transcript was only recognized by one probe (p68B3). cDNA libraries were probed using a subclone that recognized both transcripts (see MATERIALS AND METHODS). Two noncross-hybridizing classes of cDNAs were found. The clones were mapped to the genomic DNA. As both classes of cDNAs had members that contained poly A^+ tails, we could infer

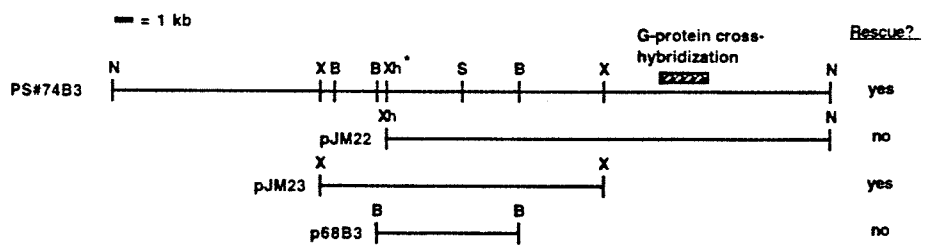

Figure 2. Rescuing ability of cosmid PS#74B3 and various subclones. PS#74B3 was originally isolated as a G-protein cross-hybridizing cosmid (Mendel, personal communication). The G-protein cross-hybridizing region is shown with a hatched box and is distinct from the pJM23-rescuing plasmid. Only relevant restriction sites are shown. Abbreviations: N, *Not* I; X, Xba I; B, *Bam*HI; Xh, *Xho* I; S, Sal I. *, two *Xho* I sites are here, separated by 330 bp. Rescue was demonstrated by microinjection-mediated germline transformation as described in MATERIALS AND METHODS. All injection mixes contained 50 ng/μl of the genomic subclone. We injected animals bearing the *lin-15*AB^{ts} allele, *n765*.

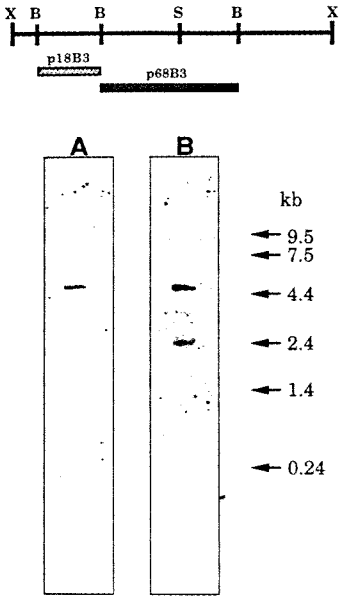

Figure 3. RNA analysis. poly A$^+$ RNA from the wild-type nematode strain N2 was electrophoresed and transferred to a nylon membrane. A was probed with p18B3, a 1.8-kb *Bam*HI fragment. B was probed with p68B3, a 6.8-kb *Bam*HI fragment. p18B3 and p68B3 are both subclones of cosmid PS#74B3 (see Figure 2). Abbreviations: X, *Xba* I; B, *Bam*HI; S, *Sal* I.

that both cDNAs are transcribed in the same direction (Figure 4).

The cDNAs and the corresponding genomic regions were sequenced (Figure 5). Exons are shown in capital letters, and the predicted amino acids are shown below. Only 105 bp of genomic sequence separates the 3′ end of the upstream transcript from the 5′ end of the downstream transcript.

Analysis of the Downstream Transcript

One cDNA of the downstream transcript, B11c, is likely to be near full length as it contains 3 bp of the *trans*-spliced leader SL2 (Huang and Hirsh, 1989). SL2 is a 22 nucleotide *trans*-spliced leader that is spliced to the 5′ end of certain *C. elegans* transcripts (for a review of *trans*-splicing, see Blumenthal and Thomas, 1988). We confirmed that the downstream transcript is indeed *trans*-spliced to SL2 by performing PCR using reverse transcribed cDNA. We were able to amplify a band using the *lin-15* specific primer A4-VR6 and an SL2 primer. This band hybridized to probe made from the B11c cDNA. DNA sequencing confirmed that it was a *lin-15* clone and that *trans*-splicing of SL2 occurs as predicted from the cDNA.

Analysis of the Upstream Transcript

Three cDNA libraries were screened, and a composite cDNA of 3.5 kb was compiled (see MATERIALS AND METHODS and Figure 4). Because this composite cDNA was not complete, introns and exons were predicted from the genomic sequence (see MATERIALS AND METHODS). Predictions were confirmed by performing PCR on reverse-transcribed RNA. From the predicted exons, primers for reverse transcription as well as for PCR were designed. All PCR primers were required to span a predicted intron to allow discrimination of potential genomic contaminants (none were seen). These bands were cloned and sequenced, and the corresponding exons are shown in Figure 4. The first 21 nucleotides of the 5′-most exon, including the initiating methionine, is predicted from the genomic sequence.

Both Transcripts Constitute lin-15 Function

Southern blots containing DNA from the *lin-15*AB alleles *n1139*, *n377*, *e1763*, and *n309* were probed with radiolabeled subclones of the 15-kb *lin-15* rescuing ge-

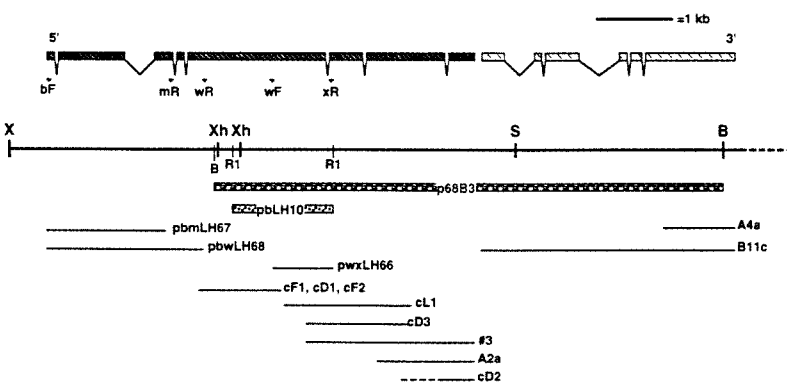

Figure 4. cDNA analysis. Primers used for RT-PCR are shown below the exon intron map with an arrowhead indicating their orientation. Only relevant restriction sites are shown. Probe p68B3 was used to isolate cDNAs #3, A2a, A4a, and B11c. Probe pbLH10 was used to isolate cDNAs cF1, cD1, cF2, cL1, cD2, and cD3. Only cDNAs that were analyzed beyond sizing are shown on this map. pbmLH67 is an RT-PCR fragment generated using primers bF and mR, pbwLH68 is an RT-PCR fragment generated using primers bF and wR, and pwxLH66 is an RT-PCR fragment generated using primers wF and xR. Details about the isolation of the cDNAs and the RT-PCR fragments are given in MATERIALS AND METHODS. Abbreviations: X, *Xba* I; B, *Bam*HI; Xh, *Xho*I; R1, *Eco*RI; S, *Sal* I.

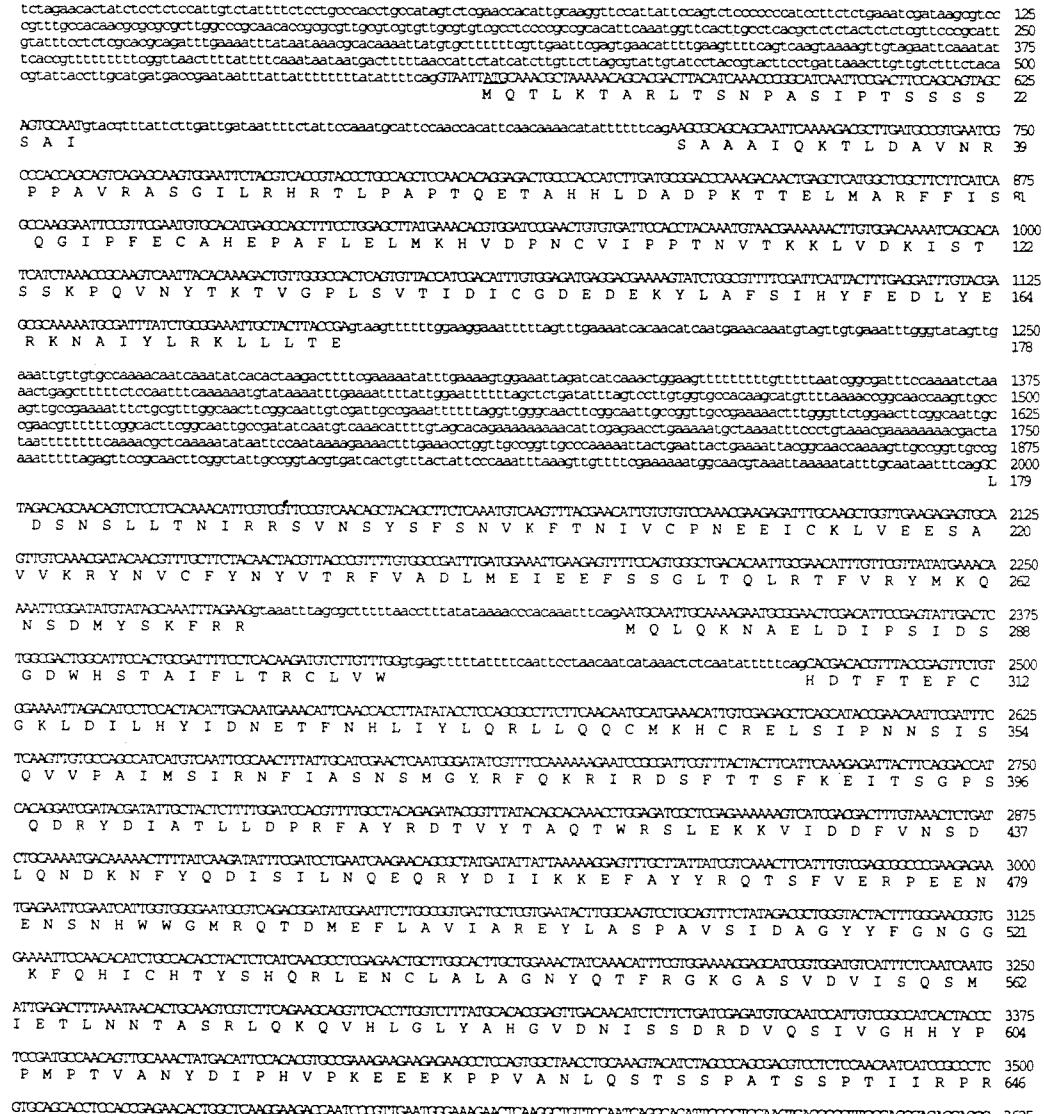

Figure 5. (Continued)

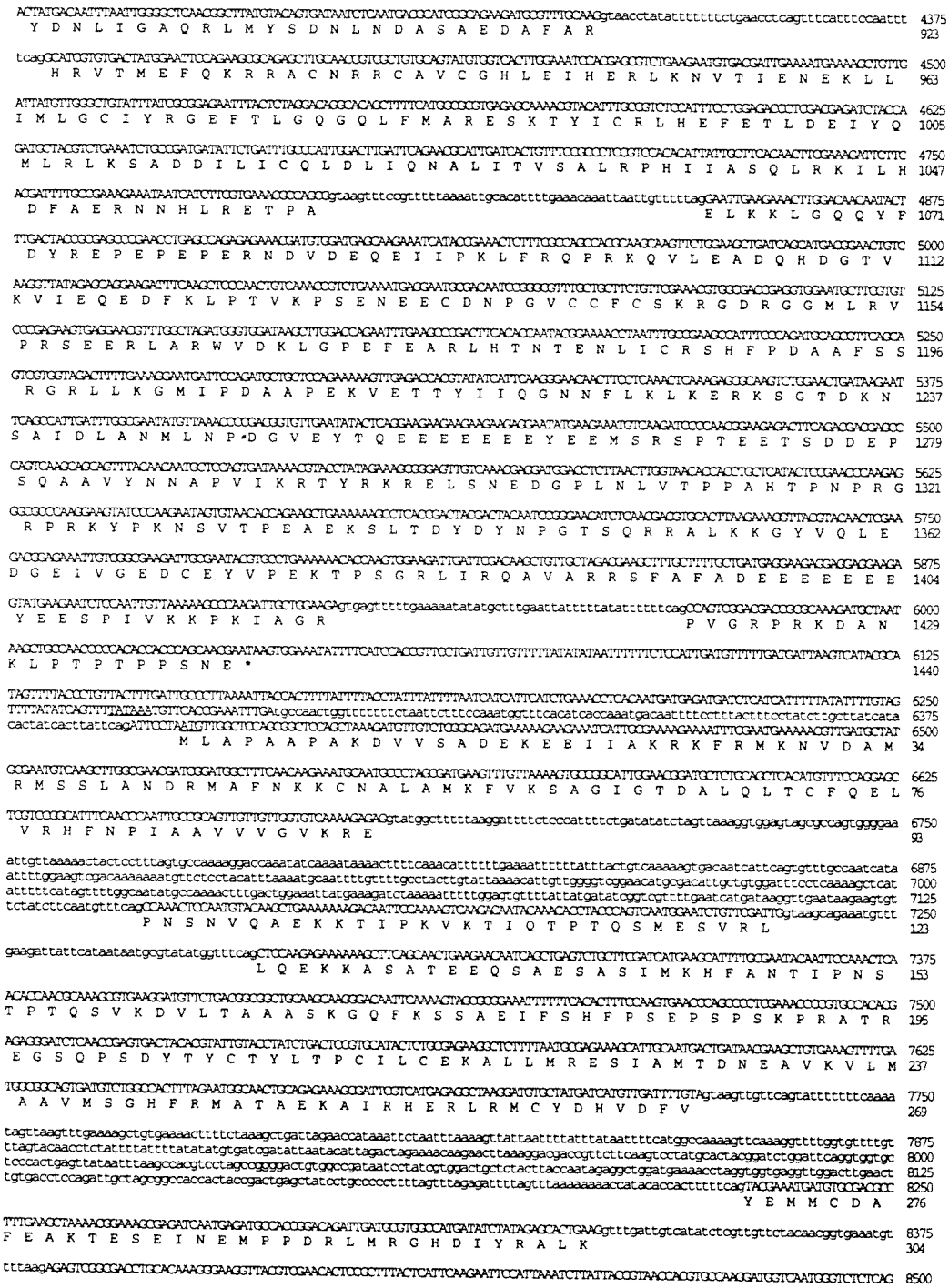

Figure 5. (Continued)

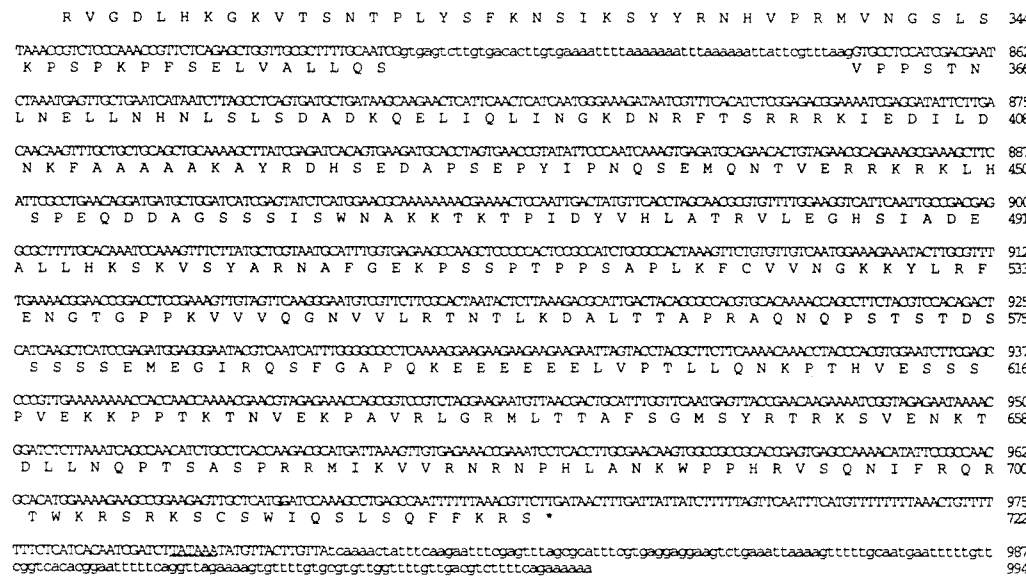

Figure 5. Nucleotide and predicted protein sequence of the *lin-15* locus. The first methionine codon and a potential polyadenylation signal are underlined for each transcript. Uppercase letters indicate exons. The predicted amino acids are shown below. The first exon, part of which is predicted, is shown as starting at a splice consensus 5′ to the predicted Methionine. Genomic sequence is shown with base 1 as the *Xba* I site at the 5′ end of the rescuing plasmid, pJM23, to 290 bp past the 3′ *Bam*HI site at base 9660. EMBL accession number is Z29967.

nomic DNA contained in pJM23. These alleles were all derived from EMS mutagenesis of N2 hermaphrodites (Ferguson and Horvitz, 1985); all exhibited polymorphisms of the *lin-15* rescuing region (Figure 6, lanes 2–5). These polymorphisms were determined to be deletions of the DNA from the *lin-15* rescuing region by mapping of the polymorphisms using *Bam*HI-, *Hin*dIII-, *Eco*RI-, and *Eco*RV-digested DNAs on Southern blots that were probed with subclones of the 15-kb *lin-15* rescuing genomic DNA contained in pJM23. These deletions appear to affect both transcribed regions.

However, not all Muv alleles of *lin-15* show detectable polymorphisms for the *lin-15* rescuing region. Screens for new *lin-15*AB alleles using EMS starting with the *lin-15*B allele *n744* yielded two new *lin-15*AB alleles, *sy211n744* and *sy212n744*. These alleles were recovered at a frequency of ~1/3500. We looked for polymorphisms using *Bam*HI-, *Hin*dIII-, *Eco*RI-, and *Eco*RV-digested DNA on Southern blots that were probed with subclones of the 15 kb *lin-15* rescuing genomic DNA contained in pJM23. No polymorphisms were detected (for example, see lane 13 and 14 of Figure 6). New polymorphisms were also not found for the *lin-15*AB allele *n767sy222*, which was obtained in a similar screen starting with *lin-15*A(*n767*) (Hill, personal communication) (lane 11).

We were unable to detect polymorphisms associated with the A and B alleles of *lin-15*, except for the *lin-15*A allele, *n767* (lanes 9, 10, and 12 of Figure 6). We determined the location of the *n767* polymorphism through restriction analysis followed by PCR. This 179-bp deletion lies in the downstream transcript and spans the final two exons (see Figure 7 for details). The *n767* mutation would lead to a truncated A protein, missing 347 amino acids from the C-terminus, as well as 40 amino acids from the middle of the predicted protein.

All these results together suggest that both transcripts constitute wild-type *lin-15* activity and both must be eliminated to produce a Muv phenotype. This hypothesis is consistent with results from a *lin-15* noncomplementation screen that could recover null alleles, as 77% of animals of the genotype *lin-15*AB^{ts}*(n765)/Df* are Muv at 15°C. We mutagenized *mec-5(e1340)* males, allowed them to mate with *unc-3(e151) lin-15*AB^{ts}*(n765)* hermaphrodites, and screened 35 000 F1 progeny raised at 15°C. Although standard mutagenesis protocols result in a *C. elegans* gene knockout frequency between 1/2000 to 1/3300 (Brenner, 1974; Greenwald and Horvitz, 1980; Park and Horvitz, 1986), we recovered no new *lin-15* mutations. However, EMS usually generates point mutations (Anderson and Brenner, 1984; Dibb *et al.*, 1985). Thus, if two independent point mutations or a deletion are required for recovery of *lin-15*AB alleles, mutations that fail to complement *lin-15*AB^{ts}*(n765)* would be rare. Our failure to recover additional *lin-15* alleles is consistent with *lin-15* being a complex locus.

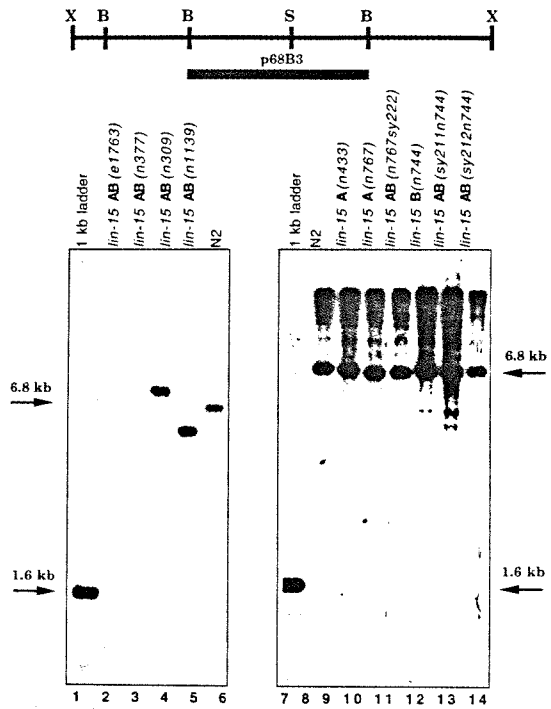

Figure 6. Southern analysis of the *lin-15* locus. One microgram genomic DNA for each strain was digested with *Bam*HI, separated on an agarose gel by electrophoresis, and transferred to a nylon membrane. These membranes were probed with p68B3, a 6.8-kb *Bam*HI genomic subclone. The *lin-15* Muv alleles *e1763*, *n377*, *n309*, and *n1139* are all derived from mutagenesis of the wild-type N2 strain (Ferguson and Horvitz, 1985). The *lin-15* Muv allele *n767sy222* is derived from EMS mutagenesis of *lin-15A(n767)*. The *lin-15* Muv alleles *sy210n744* and *sy211n744* are derived from EMS mutagenesis of *lin-15B(n744)*. Abbreviations: X, *Xba* I; B, *Bam*HI; S, *Sal* I.

The lin-15 Locus Can Be Divided into a lin-15A and a lin-15B Region

Because animals carrying either *lin-15A* alleles or *lin-15B* alleles are phenotypically wild-type, it is conceivable that rescued animals could be phenotypically wild-type while still genotypically A or B. To exclude this possibility, we tested for *lin-15A* and *lin-15B* function independently. To test for rescue of *lin-15B*, we injected animals of the genotype *lin-8A(n111); lin-15B(n744)*. To test for rescue of *lin-15A*, we injected animals of the genotype *lin-36B(n766); lin-15A(n767)* (Figure 8 and Table 1). The plasmid pJM24, which contains the genomic sequence that encodes the upstream transcript and the first exon of the downstream transcript, will rescue *lin-8A(n111); lin-15B(n744)* but not *lin-36B(n766); lin-15A(n767)*. Therefore, the upstream transcript is the *lin-15B* transcript.

Transgenes containing pJM23 rescue *lin-36B(n766); lin-15A(n767)* animals to a wild-type vulval phenotype. Therefore, the *lin-15A* region is contained within this plasmid. As pmLH4 does not rescue *lin-36B(n766); lin-15A(n767)* animals, a third transcript 3′ of the downstream transcript encoding *lin-15A* function is unlikely. A 12-kb plasmid (pmLH5) containing all of the downstream transcript plus ~3 kb upstream will not rescue *lin-15A*. Although this could indicate a requirement for 5′ sequences, we believe this is because transcription of the upstream transcript is required for transcription of the downstream transcript. Spieth *et al.* (1993) have demonstrated that polycistronic transcription exists in *C. elegans* and that polycistronic transcripts are *trans*-spliced to the SL2 leader. In these polycistronic messages, the downstream transcript is often separated by ~100 bp from the upstream transcript. Because the *lin-15A* transcript is *trans*-spliced to SL2 and is separated from the upstream *lin-15B* transcript by 105 bp, it is likely that *lin-15A* is being cotranscribed with *lin-15B* and then processed.

The construct pbLH51 is the same as pJM23 except that 330 bp is deleted from an exon in the *lin-15B* transcript. *lin-36B(n766); lin-15A(n767)* animals are rescued to a wild-type phenotype when they carry this construct as a transgene. Injection of pbLH51 into *lin-8A(n111); lin-15B(n744)* animals does not rescue the vulval phenotype. Thus, pbLH51 will rescue *lin-15A* but not *lin-15B*. Because pJM24 will not rescue *lin-36B(n766); lin-15A(n767)* animals, we believe the ability of pbLH51 to rescue *lin-36B(n766); lin-15A(n767)* animals results from the addition of important regulatory sequences for the *lin-15A* transcript, rather than some function of the *lin-15B* gene product.

lin-15A and lin-15B Protein Products

The sequences of the predicted protein products from the two transcripts (Figure 5) are different and appear to show no similarity to any currently known proteins. Each transcript contains one large open reading frame that spans all exons. Because the other potential reading frames contain many stop codons and do not code for continuous proteins, we presume the large open reading frames code for the *lin-15A* and *lin-15B* proteins. The methionines at the start of the proteins were chosen because they were the ones closest to the 5′ end.

Both predicted proteins are hydrophilic. The 1440 amino acid *lin-15B* protein has a predicted molecular weight of 163 kDa and contains 8.4% acidic and 15.3% basic residues. The 722 amino acid *lin-15A* protein has a predicted molecular weight of 81 kDa and contains 10.7% acidic and 17.8% basic residues. Neither protein contains known protein motifs except for potential N-glycosylation, phosphorylation, and myristoylation sites in both, and potential amidation sites in the *lin-15A* protein. However, the presence of these sites is not suf-

N2 ..G AAC ACT CCG CTT TAC TCA TTC AAG AAT TCC ATT AAA TCT TAT TAC CGT AAC CAC GTG CCA
lin-15(n767) ..G AAC ACT CCG CTT TAC TCA TTC AAG AAT TCC ATT AAA TCT TAT TAC AAA AAA AAA AAA

N2 AGG ATG GTC AAT GGG TCT CTC AGT AAA CCG TCT CCC AAA CCG TTC TCA GAG CTG GTT GCG CTT
lin-15(n767)

N2 TTG CAA TCG gtgagtcttgtgacacttgtgaaaattttaaaaaaaatttaaaaaaattattcgtttaag GTG CCT CCA TCG
lin-15(n767)

N2 ACG AAT CTA AAT GAG TTG CTG AAT CAT AAT CTT AGC CTC AGT GAT GCT GAT AAG CAA GAA CTC
lin-15(n767) AAT CATAAAT CTT AGC CTC AGT GAT ACT GAT AAG CAA GAA CTC

N2 ATT CAA CTC ATC.....
lin-15(n767) ATT CAA CTC ATC.....

Figure 7. The *lin-15A(n767)* mutation. The *lin-15A(n767)* deletion detectable on a Southern blot (see Figure 6) was localized using PCR (see MATERIALS AND METHODS). The sequence in this figure represents the sequence of the PCR fragment obtained by using primer n767FZ and n767RZ as described in MATERIALS AND METHODS. The *lin-15A(n767)* mutation deletes 120 bp from the A transcript as well as a 59-bp intron, while inserting 12 As, thus fusing the final two exons of the *lin-15A* transcript in frame. An extra A is added six bases after the fusion, causing a frame shift resulting in a stop codon three codons later (TGA is boxed). In addition, 11 bp after the stop codon, a G has been changed to an A. The relevance of this G to A change is unknown and could have occurred upon maintenance of this strain, as there would be no pressure to maintain the fidelity of the bases after the new stop codon in *lin-15A(n767)*.

ficient to conclude that these sites are used, and we have no evidence that the presence of these sites is significant.

e1763 Is a Candidate Null Allele of lin-15

As Southern blot analysis demonstrated *lin-15AB(e1763)* was a deletion of a large part of the genomic DNA contained in pJM23, we localized the deletion breakpoints of *lin-15AB(e1763)* by ligation-mediated PCR. The extent of the deletion was confirmed by PCR directly from *lin-15AB(e1763)* genomic DNA. Our analysis demonstrates that the *e1763* allele deletes DNA from base 33 beyond the *Xba* I site to base 9588 in the 3′ UTR of the *lin-15A* transcript, effectively deleting all coding regions contained in the rescuing fragment, pJM23. Previously published experiments with *lin-15* have used the allele *n309*, which contains a smaller deletion than *e1763* (see Figure 8). Thus, we characterized animals carrying the *lin-15* molecular null, *e1763*, to determine if there were any differences that would affect interpretation of earlier studies.

Gonad Independence of lin-15AB(e1763) Vulval Differentiation

In *lin-15AB(e1763)* animals, essentially all VPCs undergo vulval differentiation. To determine if the Muv phenotype of *lin-15AB(e1763)* mutant animals is dependent on the inductive signal from the gonad, we performed gonad ablation experiments. *lin-15AB(e1763)* animals whose gonads were ablated in the first larval stage (L1)

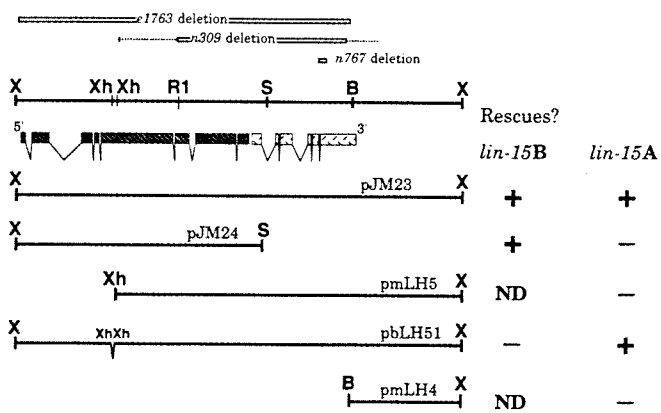

Figure 8. The *lin-15* locus can be separated into a *lin-15A* and a *lin-15B* region. The orientation of the two transcripts of the *lin-15* locus is shown on the top left with the intron/exon structure of the two transcripts below a partial restriction map of the 15-kb *Xba* I subclone, pJM23. The location of the deletions for the *lin-15AB* alleles, *e1763* and *n309*, and the *lin-15A* allele, *n767*, are shown above. Abbreviations: X, *Xba* I; Xh, *Xho* I; R1, *Eco*RI; S, *Sal* I. Rescuing ability was determined using germline transformation (see MATERIALS AND METHODS). The *e1763* deletion was localized as described in MATERIALS AND METHODS; the deletion is described in the RESULTS. The *n309* defect was localized using Southern blots probed with different regions of the *lin-15* genomic region. The left breakpoint lies somewhere between the second *Xho*I site and the *Eco*RI site shown. The right breakpoint is either close to the *Bam*HI site or to the right of it; we were unable to determine its location because of repetitive DNA to the left of the *Bam*HI site. The *n767* defect is described in Figure 7. To determine the ability of a genomic subclone to rescue *lin-15B*, animals of the genotype *lin-8A(n111); lin-15B(n744)* were injected. To determine the ability of a genomic subclone to rescue *lin-15A*, animals of the genotype *lin-36B(n766); lin-15A(n767)* were injected. See Table 1 for details.

Table 1. Rescue of *lin-15A* and *lin-15B*[a]

		Rescue of *lin-36B(n766); lin-15A(n767)*				Rescue of *lin-8A(n111); lin-15B(n744)*			
Plasmid injected	Concentration injected	Number F1 rescued	Total number F1s	Number stable lines rescued	Number of stable lines	Number F1 rescued	Total number F1s	Number stable lines rescued	Number of stable lines
pJM23	50 ng/μl	8[d]	8	4	4[e]	0	16	1	1[f]
pJM23	25 ng/μl	29	29	3	3	5	24	4	4[g]
pbLH98[b]	25 ng/μl			ND		33	50	11	11
pbLH99[c]	25 ng/μl			ND		26	42	8	8
pJM24	50 ng/μl	0	11	0	3	15	16	2	2
pmLH5	25 ng/μl	0	15	0	2			ND	
pbLH51	50 ng/μl	27	28	5	5	0	19	0	1
pbLH51	25 ng/μl			ND		0	26	0	2
pmLH4	25 ng/μl	0	27	0	5			ND	

[a] Stable rescue of *lin-15A* and *lin-15B* was determined by transmission of the Rol phenotype, unless otherwise noted. These Rol nonMuv animals typically have progeny rescued for *lin-15*, which are nonMuv Rol and nonMuv nonRol (which are rescue for *lin-15* but do not express the Rol marker), as well as nonrescued progeny, which are Muv nonRol. This is a typical range of phenotypes, as injected DNA exist in extrachromosomal arrays that are not transmitted to 100% of an animal's progeny.

[b] pbLH98 is an independently derived isolate of the 15-kb *lin-15*–rescuing fragment subcloned directly from cosmid PS#74B3, like pJM23. pbLH98 and pJM23 carry the insert in the *Xba* I site of Bluescript SK$^+$ (Stratagene) in the same orientation.

[c] pbLH99 is an independent isolate of the 15-kb *lin-15*–rescuing fragment subcloned directly from cosmid PS#74B3 into the *Xba* site of Bluescript SK$^+$ (Stratagene) in the opposite orientation of pJM23 and pbLH98. We cannot explain the incomplete F1 rescue of pJM23, pbLH98, and pbLH99 of *lin-15B*, especially given the rescuing ability of pJM24 of *lin-15B*. However, this incomplete rescue does not appear to be caused by a mutation in pJM23 or by the orientation of the insert relative to the vector sequences.

[d] Of the eight F1 rescued animals, only two of the eight were nonMuv Rol; the other six were nonMuv nonRol.

[e] Of the two Rol F1–rescued lines, both produced Muv and nonMuv progeny, although the Rol phenotype used as a selectable marker was lost. Of the six nonMuv nonRol F1 animals, two of the six produced both Muv and nonMuv progeny, although no Rol animals were seen. This suggests that the expression of the pRF4 Rol plasmid was not sufficient for cotransformation with pJM23. Thus, we lowered the concentration of pJM23 to 25 ng/μl and the results are given above.

[f] This stable line segregated nonMuv Rol, Muv Rol, nonRol Muv, and nonRol nonMuv animals.

[g] One stable line never segregated Rol animals but segregated animals that were nonMuv. When these nonMuv nonRol animals were cloned, they segregated Muv and nonMuv animals, but no Rol animals. The other stable lines behaved normally.

display vulval differentiation (see Table 2). This gonad independence is consistent with previous results using *lin-15AB(n309)* (Sternberg and Horvitz, 1989).

Epistasis of lin-15AB(e1763) and let-23(sy97)

To confirm the order of action of *lin-15* and *let-23*, the candidate receptor for the inductive signal, we performed double mutant analysis with *let-23(sy97)*, the most severe viable allele of *let-23* (Aroian and Sternberg, 1991). Wild-type animals are defined as having 100% vulval differentiation, corresponding to three of six VPCs generating vulval tissue (Figure 9 and Table 2). Animals homozygous for *let-23(sy97)* are Vul and display no (0%) vulval differentiation; animals homozygous for *lin-15AB(e1763)* are Muv and have essentially 200% vulval differentiation (all six VPCs assume vulval fates). The double mutant *let-23(sy97); lin-15AB(e1763)* displays the *let-23(sy97)* Vul phenotype and 14% vulval differentiation. Gonad ablations on *let-23(sy97); lin-15AB(e1763)* animals were performed to determine whether the residual activity is gonad dependent. No vulval differentiation was observed in gonad ablated *let-23(sy97); lin-15AB(e1763)* animals. Therefore, the residual differentiation seen in *let-23(sy97); lin-15AB(e1763)* animals is probably because of residual activity of the *sy97* allele (see below).

DISCUSSION

We have cloned the *C. elegans lin-15* locus by isolating a 15-kb genomic fragment, pJM23, that will rescue the *lin-15* Muv, A, and B phenotypes in transgenic animals. *lin-15AB* alleles *n309*, *n1139*, *n377*, and *e1763*, derived from EMS mutagenesis of a wild-type strain, all contain deletions corresponding to sequences within this rescuing fragment. Two transcripts are encoded by this region, transcribed in the same direction, and separated by 105 bp. The upstream transcript encodes the *lin-15B* activity; a subclone containing essentially only the upstream transcript will rescue *lin-15B* mutant animals but not *lin-15A* mutant animals. Two experiments suggest that the downstream transcript encodes the *lin-15A* activity. First, the *lin-15A* allele *n767* contains a deletion/insertion in the downstream transcript. Second, pbLH51, a derivative of pJM23 that deletes 330 residues from an

exon of the upstream B transcript, will rescue *lin-15*A but not *lin-15*B. Clark, Lu, and Horvitz (personal communication) have also cloned *lin-15* and have obtained similar results.

lin-15A and lin-15B Encode Novel Gene Products

Sequence analysis of the cDNAs and genomic sequence corresponding to the two *lin-15* transcripts indicates their predicted protein products are not similar to any currently identified proteins. Furthermore, motif analyses show no significant recognizable protein motifs. Because the *lin-15* locus behaves nonautonomously in genetic mosaic analysis, it has been proposed that *lin-15* is or controls the production of a negative signal produced by the epidermis that prevents vulval differentiation (Herman and Hedgecock, 1990). Because there are no clear consensus signal sequences, it is unlikely that either *lin-15*A or *lin-15*B is secreted. Thus, *lin-15* may regulate production of such a signal. However, the lack of a signal sequence does not formally rule out the possibility that either product of *lin-15* may be secreted. For instance, *Saccharomyces cerevisiae* a-factor is clearly exported although its sequence does not have a consensus signal sequence (Brake *et al.*, 1985).

It is not surprising that *lin-15*A and *lin-15*B are novel gene products, because they act in a pathway that nonautonomously regulates RTK activity or effect, originating from neither the signaling nor the receiving tissue but from a third tissue, the nonvulval epidermis. Such nonautonomous regulators of RTKs have not been previously identified because of the assays typically used to study RTKs. Cell culture systems usually contain homogeneous cell populations supplied with growth factors in the media; in vivo the receiving cell resides near the signaling tissue and is surrounded by other cell types. Therefore, a negative signal from a nonreceiving nonsignaling tissue might not have been detected by cell culture assays. A genetically identified locus in *Drosophila, argo/giant lens(gil),* may behave like *lin-15* in *Drosophila* photoreceptor development (Freeman *et al.*, 1992; Kretzschmar *et al.*, 1992); *argos/gil* acts nonautonomously to affect cell fate determination (Freeman *et al.*, 1992) and encodes a potentially secreted factor with an EGF-like motif that displays no sequence similarity to either *lin-15*A or *lin-15*B.

What Is the Nature of the Redundancy of lin-15A and lin-15B?

Two genetic activities, A and B, were previously defined for *lin-15* as well as for the other A and B genes, such as *lin-8*A, *lin-9*B, *lin-36*B, *lin-37*B, and *lin-38*A (Ferguson and Horvitz, 1989). Although *lin-15* was shown to have both A and B genetic activity, the mechanism of this dual function was unclear. For instance, the *lin-15* locus could have encoded a bifunctional protein that could be independently mutated to become defective in either A or B activity. This would have suggested that *lin-15* was an integrator of the A and B pathways. Instead, the *lin-15* locus contains two nonoverlapping transcripts; one encodes A function, the other encodes B function. In fact, the *lin-15* locus could be viewed as containing a class A and a class B gene. Their physical juxtaposition could have no more functional significance than, for example, if *lin-8*A and *lin-9*B were to be tightly linked on the same chromosome. However, the *lin-15* genomic structure fits the emerging consensus for polycistronic transcripts (Spieth *et al.*, 1993). Thus, it is possible that *lin-15*A and *lin-15*B together are important either in controlling or in being controlled by other A and B genes because of the potential for *lin-15*A and *lin-15*B to be cotranscribed and coordinately regulated.

Ferguson and Horvitz (1989) demonstrated that A and B activities are redundant, because animals homozygous mutant for either A or B are wild-type; animals display the Muv phenotype only when homozygous mutant for both A and B. However, the nature of the redundancy was unknown. Our transgenic studies demonstrate that the *lin-15*A product and the *lin-15*B product cannot substitute for each other, nor can they substitute for other class A and class B genes. If *lin-15*B could substitute for *lin-15*A, we would not have been able to distinguish the two transcripts by rescue experiments, as injection of a subclone containing the *lin-15*B transcript would have rescued *lin-15*A and vice versa. Furthermore, if *lin-15*B could substitute for *lin-36*B, injection of a subclone containing the *lin-15*B transcript would have rescued *lin-36*B. A similar argument can be used for *lin-15*A substituting for *lin-8*A. Finally, sequence comparison of the *lin-15*A protein to the *lin-15*B protein reveals no similarity. Therefore, A and B are genetically redundant but not molecularly redundant. They do not appear to encode related proteins and are not able to functionally substitute for each other in transgenic assays.

Role of lin-15 in Regulation of the let-23 Pathway

lin-15 acts as a negative regulator of the *let-23*–mediated signaling pathway (Ferguson *et al.*, 1987). Our genetic analysis demonstrates that *lin-15* acts upstream of *let-23;* a *let-23* Vul mutation is epistatic to a *lin-15* Muv mutation. This conclusion is supported by the fact that alleles of *let-23,* as well as alleles of the downstream genes *sem-5, let-60,* and *lin-45,* have been isolated as suppressors of *lin-15* (Beitel *et al.*, 1990; Han *et al.*, 1990, 1993; Clark *et al.*, 1992a,b). Furthermore, the patterning of vulval differentiation in *lin-15* mutant animals is dependent on the inductive signal. P6.p, the VPC closest to the AC (Sulston and Horvitz, 1977), always adopts a 1° fate in intact *lin-15* mutant animals, whereas in gonad-ablated *lin-15* mutant animals, P6.p can adopt either a 1° fate or a 2° fate (Sternberg, 1988). VPCs in *lin-15* mutant animals thus retain the ability to respond

Table 2. Double mutant analysis of *lin-15* and *let-23*

Genotype	Gonad	Percentage[a] differentiation	n[b]	# of VPCs differentiated[c] 0	1/2, 1	1 1/2, 2	2 1/2, 3	3 1/2–5	5 1/2, 6
N2	+	100	many[d]				all		
N2	−	0	many[e]	all					
lin-15(e1763)	+	199	28						28
lin-15(e1763)	−	200	13						13[f]
let-23(sy97)	+	0	19	19					
let-23(sy97)	−	0	3	3					
let-23(sy97);lin-15(e1763)	+	14	40	24	13	2	1		
let-23(sy97);lin-15(e1763)	−	0	15	15					

[a] Percentage differentiation reflects the amount of VPC differentiation observed of a genotype relative to wild-type animals.

[b] n is the number of animals examined.

[c] The numbers in this column represent the number of animals displaying a particular amount of VPCs differentiated.

[d] See Sulston and Horvitz, 1977; Sulston and White, 1980; Sternberg and Horvitz, 1986.

[e] See Sulston and White, 1980; Kimble, 1981.

[f] Occasionally, P cells were killed during gonad ablations of *lin-15AB(e1763)*, resulting in fewer than normal VPCs. However, all VPCs present differentiated in the *lin-15AB(e1763)* animals.

to inductive signal. Therefore, *lin-15* acts in parallel with the inductive signal to regulate *let-23*. Because we use vulval differentiation as the assay for activity of *let-23* and *lin-15*, we can only discuss the genetic activities of these loci; our analysis does not imply a direct biochemical interaction of *lin-15* with *let-23*. Formally, the *let-23* pathway could act positively on a downstream molecule, X, whereas the *lin-15* pathway acts negatively on X in parallel to the *let-23* pathway (where X has no activity completely dependent on ligand stimulated *let-23*). Our genetic analysis indicates that *lin-15* regulates *let-23*, as *lin-15* is required only in the presence of functional *let-23*.

It is clear that *lin-15* regulates *let-23* basal activity. Vulval differentiation occurs independently of the inductive signal in *lin-15AB(n309)* mutant animals (Ferguson *et al.*, 1987; Sternberg and Horvitz, 1989). Similarly, in *lin-15AB(e1763)*, a molecular null, all VPCs undergo the cell division patterns and morphogenetic movements characteristic of vulval fates even in the absence of the gonad. The VPC's ability to undergo inductive-signal independent differentiation implies *lin-*

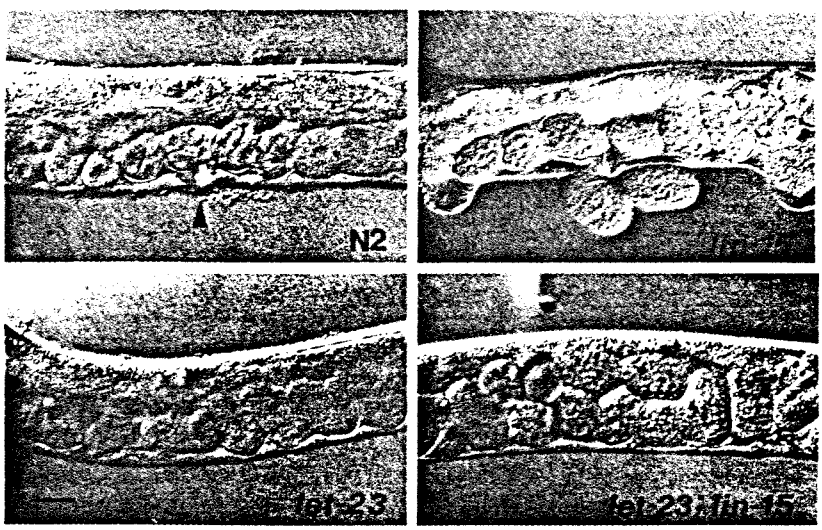

Figure 9. Double mutant analysis of *let-23(sy97); lin-15AB(e1763)*. All photographs are of the middle third of adult hermaphrodite animals. Top left: the wild-type N2 strain. Arrow points to vulva. Top right: *lin-15AB(e1763)*. Bottom left: *let-23(sy97)*. Note the lack of a functional vulva. Bottom right: *let-23(sy97); lin-15AB(e1763)*. Bar, 20 μm. See text.

15 regulates *let-23* basal activity, where basal activity is defined as the potential of *let-23* activity to cause vulval differentiation in the absence of its ligand.

We cannot conclude whether *lin-15* preferentially regulates basal or stimulated activity of *let-23*. Signal-dependent vulval differentiation is seen in the *let-23(sy97); lin-15*AB*(e1763)* double mutant, although the extent of vulval differentiation is not wild-type. Thus, removal of the negative regulation conferred by *lin-15* allows detection of the low level of residual-stimulated activity of the LET-23(sy97) mutant protein. The response of the LET-23(sy97) mutant protein to ligand activation is not surprising, as the *let-23(sy97)* allele harbors a point mutation in a 3′ splice acceptor, causing misspliced RNAs that, when translated, would result in a C-terminal truncation of the protein (Aroian *et al.*, 1994). Because this truncation does not disrupt the kinase domain and *let-23(sy97)* can function in at least one tissue (Aroian and Sternberg, 1991), the truncated molecule retains a low level of activity. The gonad dependent differentiation seen in *let-23(sy97); lin-15*AB*(e1763)* implies that *lin-15* has an effect on ligand bound receptors, assuming that all receptors present were occupied by ligand during the signaling event. However, if receptors are in excess on each cell, it would be unclear whether *lin-15* activity regulates both ligand unbound as well as ligand bound receptors.

Negative Regulation and Inductive Signaling

The signal transduction pathway functioning in vulval development uses *C. elegans* homologues of molecules involved in cell proliferation and determination throughout the animal kingdom. Besides *lin-3* and *let-23*, there is *sem-5*, SH2/SH3 adaptor protein, *let-60*, a *ras* protein, and *lin-45*, a *raf* protein. This particular signaling cascade is used in *Drosophila* R7 photoreceptor determination and in the proliferative response of mammalian cell culture systems. The high degree of conservation of these components throughout the animal kingdom strongly suggests that *lin-15* homologues may exist in these and other systems.

The emerging picture of signal transduction shows that, in general, receptors are expressed with less specificity than their ligands. For example, the RNA and protein products of the *Drosophila* PDGF-family RTK *torso* have no apparent localization and are expressed all over the embryo, even though *torso* acts to specify cell fate in the termini of the embryo (Sprenger and Nüsslein-Volhard, 1992). Similarly, the RTK *sevenless* required for *Drosophila* R7 photoreceptor development is expressed in cells other than R7 (Banerjee *et al.*, 1987; Tomlinson *et al.*, 1987). The localization of the *boss* ligand to the apical surface of R8, the signaling cell, is one reason why R7 is the only *sevenless* expressing cell that responds to the *boss* ligand (Van Vactor *et al.*, 1991).

During *C. elegans* vulval development all six VPCs are competent to undergo vulval differentiation upon receipt of the inductive signal (Sulston and White, 1980; Sternberg and Horvitz, 1986; Thomas *et al.*, 1990; Hill, Katz, and Sternberg, unpublished data). Presumed overexpression of the *lin-3* signal causes up to six VPCs to adopt vulval fates (Hill and Sternberg, 1992). Thus, *lin-3* must be precisely regulated during wild-type vulval development.

As Gurdon (1992) points out, the number of cells competent to respond to a particular signal exceeds the number that actually respond. Davidson (1993) has argued that competence could be viewed as a cell possessing the appropriate receptors, signal transduction molecules, and transcription factors, with the molecular response of a cell being limited by the expression of the signal. Receptor tyrosine kinase activation most likely involves precise temporal, spatial, and quantitative control of ligand expression. However, because receptor is more ubiquitously expressed, receptor activity must be precisely regulated to prevent incorrect development. Thus, the study of *lin-15* and how this pathway prevents inappropriate RTK activity should yield new insights on RTK regulation as well as how cells can respond appropriately to a localized ligand.

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Chapter 3

Expression of *C. elegans* LIN-15B, a negative regulator of vulval differentiation

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ABSTRACT

The decision to adopt a vulval fate by a *C. elegans* vulval precursor cell (VPC) involves an inductive signal from the anchor cell in the gonad to the VPC. Normally, three of the six multipotent VPCs adopt vulval fates while the other three adopt non-specialized epidermal fates. The inductive signal is encoded by *lin-3*, a growth factor of the TGF-α family. The LIN-3 signal is received by LET-23, a receptor tyrosine kinase. *lin-15* is a negative regulator of this inductive signalling. Previous genetic studies indicate that *lin-15* acts in parallel to the inductive signal and upstream of the receptor; mosaic analysis demonstrates that *lin-15* acts non-autonomously. We have raised antisera against LIN-15B, one of the proteins encoded by the *lin-15* locus. We demonstrate that LIN-15B is a nuclear protein that is expressed broadly. Our results indicate that LIN-15B is expressed in the appropriate tissues for non-autonomous regulation of vulval differentiation. These results also indicate that the negative regulation of LIN-15B on LET-23 is likely not due to a direct physical association.

INTRODUCTION

The many steps involved in growth factor signalling through tyrosine kinase receptors have been extensively defined through both biochemical and genetic studies (reviewed in van der Geer, et al., 1994). The current (and oversimplified; see Chapter 1) model deduced from these studies involves presentation of a growth factor to a receptor, resulting in dimerization and autophosphorylation of the receptor molecule. Once the receptor is activated,

an SH2/SH3 domain containing protein binds the receptor and potentially activates a guanine nucleotide exchange factor, which proceeds to activate the *ras* protein. *Ras* then binds *raf*, activating a kinase cascade involving MEK and MAP-kinase. MAP-kinase has then likely enters the nucleus to phosphorylate various transcription factors of the ETS-domain class, resulting in transcriptional differences in cells that receive signal.

Each step during this transduction event is potentially a target for negative regulation; many negative regulators of this cascade have been identified. Some negative regulators are pharmacological agents that have been developed to inhibit this cascade (Levitzki and Gazit, 1995). A number of endogenous negative regulators have also been identified. These include Müllerian Inhibitory Substance (Cigarro, et al., 1989), which inhibits EGF receptor tyrosine kinase signalling, and phosphatases that may act to dephosphorylate molecules phosphorylated upon receptor activation (reviewed in Sun and Tonks, 1994) such as SH-PTP1, a negative regulator of the erythropoietin receptor that binds JAK2 and downregulates the receptor signal (Klingmüller, et al., 1995).

Studies on vulval differentiation in *C. elegans* have allowed genetic identification of at least five negative regulatory pathways that act on the *let-23* receptor tyrosine kinase mediated signalling that occurs during the inductive event that leads to vulval precursor cells (VPCs) adopting vulval fates. During normal development, the anchor cell in the gonad sends the *lin-3* signal, causing three of the six multipotent VPCs to adopt vulval fates (reviewed in Sternberg, 1993). Animals carrying loss-of-function mutations in the genes involved in the *lin-3*/*let-23* signalling cascade are Vulvaless; too few VPCs adopt vulval fates. On the other hand, animals with loss-of-

function mutations in any one of the five negative regulatory pathways appear wild-type. These negative regulatory pathways are redundant; only certain combinations of mutations in the negative regulatory pathways cause inappropriate vulval differentiation (reviewed in Sternberg, et al., 1994). Three of these five negative regulatory pathways are represented by *unc-101*, which encodes a clathrin associated protein recovered for its ability to suppress a hypomorphic *let-23* allele *sy1* (Lee, et al., 1994); *sli-1* , a *c-cbl* homolog that supresses *let-23* (Jongeward, et al., 1995, Yoon, et al., submitted); and *rok-1*, isolated as a synthetic enhancer of *sli-1* in vulval differentiation (Lee, 1994). The negative regulation mediated by *unc-101*, *sli-1*, and *rok-1* are likely to involve regulation of activated *let-23* (Sternberg, et al., 1994).

The other two negative regulatory pathways are represented by the two activities of *lin-15*, namely, *lin-15*A and *lin-15*B. *lin-15* mediated negative regulation has been demonstrated genetically to occur in parallel to the *lin-3* growth factor signalling and upstream of *let-23*, acting on the basal level of *let-23* signalling (Ferguson, et al., 1987, Huang, et al., 1994). Mosaic analysis of *lin-15* has shown that *lin-15* acts non-autonomously, i.e., *lin-15* activity is needed in cells other than the VPCs for proper negative regulation to occur (Herman, 1989). Furthermore, *lin-15*A and *lin-15*B are not the only identified members of their associated negative regulatory pathways. Genetic screens have identified many loci that act in these pathways; these loci act as enhancers of *lin-15*A or *lin-15*B loss-of-function activity (Ferguson and Horvitz, 1989, Huang, et al., 1994, J. Thomas and H.R. Horvitz, personal communication). These loci are called either class A or class B "syn Muv" (synthetic Multivulva) genes (Ferguson and Horvitz, 1989). Animals bearing

mutations in both a class A syn Muv and a class B syn Muv have inappropriate vulval differentiation; too many VPCs adopt a vulval fate, causing ectopic pseudovulvae, resulting in the Multivulva (Muv) phenotype.

The cloning of *lin-15* demonstrated that two distinct novel hydrophilic gene products were responsible for the genetically defined *lin-15*A and *lin-15*B activities (Huang, et al., 1994, Clark, et al., 1994). Because *lin-15* mediated negative regulation was demonstrated to occur non-autonomously, we wanted to know where *lin-15* was expressed to determine from which tissue it could potentially act, as well as where in the cell it was expressed. Here, we describe antisera against the LIN-15B protein and the use of these antisera to determine the expression of LIN-15B.

MATERIALS AND METHODS

Strains and culture conditions

Nematodes were cultured as previously described (Brenner, 1974, Sulston and Hodgkin, 1988). All strains were grown at 20°C unless otherwise noted. The following mutations were used in this study: the wild-type N2 (Brenner, 1974) and *lin-15(e1763)* (Ferguson and Horvitz, 1985), which is on linkage group X.

Expression of *lin-15* fusion proteins

The RT-PCR fragment pbm67 and the Xho1-EcoR1 genomic fragment pbLH11 have been previously described (Huang, et al., 1994). The pbm67 insert was excised from Bluescript SK+ using Xho1 and Pst1 and cloned into

the Xho1 and Pst1 site of pRSETA (Invitrogen), creating prLH101. pRSETA is an expression vector that uses the T7 promoter to drive the expression of a recombinant protein containing six histidine residues in tandem followed by the enterokinase cleavage site followed by a multiple cloning site for the insertion of DNA fragment of choice. The pbLH11 insert was excised from Bluescript SK^+ using Xho1 and EcoR1 and cloned into the Xho1 and EcoR1 site of pRSETA, creating prLH100. prLH100 and prLH101 were first grown in the *E. coli* strain DH5α to check for proper plasmid construction. Once plasmid construction was verified, the plasmids were transferred into the *E. coli* strain BL21 (Studier, et al., 1990) by electroporation for protein expression.

All BL21 strains were grown in M9ZB media (Studier, et al., 1990). Cultures for protein induction were grown at 37°C. 100 ml M9ZB containing chloramphenicol and carbenicillin was inoculated with 1 ml of a saturated culture of the *E. coli* strain containing either prLH100 or prLH101, depending on the protein desired. The diluted culture was grown until the OD at A_{600} was between 0.2-0.4 (usually 2 to 2 1/2 hours). At this point, IPTG was added to 1 mM and grown for 4 more hours. Cells were harvested (Sorval GSA rotor, 8 min. at 4000 rpm), resuspended in 5 ml ice cold PBS supplemented with 50 µl 100 mM Pefabloc®SC (Boehringer Mannheim) and 500 µl 10 mg/ml Lysozyme (Sigma), and incubated on ice for 20 minutes. 600 µl 10% Triton-X100 was added, and the mixture was incubated on ice for at least 10 minutes. The cell suspension was then sonicated for two 20 second bursts on ice and inclusion bodies were recovered by centrifugation in eppendorf tubes. Inclusion bodies were stored at -20°C until use. Bacteria containing the plasmids prLH100 and prLH101 produced the proteins

LH100p and LH101p, respectively.

Nickel affinity purification of fusion proteins for polyclonal antisera production

Because both fusion proteins were expressed in the insoluble fraction, inclusion bodies were solubilized using 8M urea-50 mM glycine pH9. Proteins in the solubilized inclusion bodies were bound to a column containing nickel bound to agarose (Qiagen). The column was first washed with 8M urea-0.1 M NaH_2PO_4-10 mM Tris (UPT) pH 8, then with UPT pH 6. Proteins were eluted with UPT pH 5.9, then UPT pH 4.7. LH101p eluted at pH 4.7. LH100p eluted at both pH 5.9 and pH 4.7, with more protein eluting at pH 4.7. Polyclonal antisera against LH100p and LH101p were raised in mice as previously described (Ou, et al., 1993).

Affinity purification of *lin-15* specific antisera

Between 2 to 4 milligrams of fusion protein was expressed and purified on a nickel affinity column as described above. Fusion proteins were then further purified, essentially as described (Deshaies and Schekman, 1990). Proteins eluted from the nickel column were electrophoresed on SDS-polyacrylamide gels. After electrophoresis, gels were stained with 4M NaOAc. Upon visualization, proteins were excised and electroeluted at room temperature in an Elutrap (Scheicher and Schuell) according to manufacturer's directions. Eluted proteins were dialyzed at room temperature against 3 changes of 300 mM NaCl, 20 mM HEPES [pH 7.7], 0.25 mM EDTA, 0.05% SDS, 2 mM DTT. Fusion proteins were then coupled to 1 ml activated affigel-15 (BioRad) by incubating at room temperature for

50 minutes, followed by addition of 100 µl of 1.0 M ethanolamine and incubation overnight at room temperature to quench coupling. Beads were washed successively with 7M urea, 1 M NaCl, 0.5 M NaCl, 0.1 M glycine [pH 2.5], 50 mM Tris-HCl [pH 7.4], and 150 mM NaCl. The anti-LIN-15B serum was first heated to 56°C to inactivate complement and passed over the LIN-15B fusion protein matrix. The matrix was washed successively with 10 mM Tris [pH 7.4], 500 mM NaCl-10 mM Tris [pH 7.4], and 10 mM Tris [pH7.4]. Antibodies were eluted using 10 ml 100 mM glycine pH 2.5 and collected in a tube containing 1 ml 1 M Tris [pH 8.5], essentially as described (Harlow and Lane, 1988). The anti-LIN-15B antibodies were dialyzed against PBS and then against TBS (100 mM Tris, 0.9% NaCl) at pH 8.2. Antibody concentrations were determined by measuring A_{280} using a spectrophotometer.

Purification of *C. elegans* protein extracts

A 5 cm plate's worth of mixed stage hermaphrodite *C. elegans* was washed off with 2 ml M9 buffer. Animals were pelleted and washed 2 times with M9. After the final wash, animals were resuspended in 200 to 500 µl of SDS-PAGE sample buffer (2% SDS, 10% glycerol, 100 mM DTT, 60 mM Tris [pH 6.8], and 0.001% bromophenol blue) and boiled for 10 minutes. Lysate was spun for 15 minutes to remove insoluble material and stored at -20°C until use.

Immunoblots

C. elegans protein extract was electrophoresed onto SDS-polyacrylamide gels and transferred onto nitrocellulose membrane according

to standard procedures (Harlow and Lane, 1988). Membranes were blocked using 3% 1x crystallized/lyophilized BSA (Sigma), followed by TTBS (100 mM Tris [pH 7.4], 0.9% NaCl, 0.1 % Tween 20). Membranes were then incubated with anti-LH101p antisera at 1 µg/ml, followed by a biotinylated goat anti-mouse IgG secondary antibody (Vector Labs) at 0.5 µg/ml. Afterwards, the Vectastain Elite (Vector Labs) avidin/biotin complex was added. Protein was visualized using diaminobenzidine ([DAB]; Sigma) and 0.04% NiCl. The anti-LIN-15B antisera and secondary antibodies were preabsorbed on nitrocellulose membranes containing protein extracts prepared from *lin-15(e1763)* animals; these membranes were blocked as described above before addition of antisera.

Immunohistochemistry

C. elegans animals were fixed and stained as described (Finney and Ruvkun, 1990) with slight modifications. 10-15 L4 larvae were placed on NGM agar in a 5 cm plate with seeded with the *E. coli* OP50 strain, as previously described (Brenner, 1974, Sulston and Hodgkin, 1988). N2 L4 larvae were allowed to mature and propagate at 20°C for 3 days; other strains were grown until all stages of development were well represented on each plate. At that time, 2 to 3 plates of mixed staged animals were collected using 2 ml M9 buffer and placed in a siliconized 1.7 ml microfuge tube. Animals were spun down and placed on ice; this results in 30 to 100 µl of animals per tube. Ice cold fixative (1% paraformaldehyde, 80mM KCl, 20 mM NaCl, 10 mM EGTA, 5 mM spermidine-HCl, 14 mM Na PIPES [pH 7.4], 25% methanol) was added to the animals, which were frozen on dry ice and stored at -70°C until use.

Tubes were thawed and fixed overnight at 4°C. All volumes used are 1 ml unless otherwise noted; all incubations and washes were performed at room temperature unless otherwise noted. Animals were rinsed twice in ice cold Tris-Triton buffer (100 mM Tris [pH 7.4], 1% Triton X-100), 1 mM EDTA) and incubated for 2 hours at 37°C in 2% β-mercaptoethanol in Tris-Triton buffer. Animals were washed twice in borate buffer (25 mM $NaBO_3$ [pH 9.2]), incubated for 15 minutes at in 30 mM DTT in borate buffer, washed twice in borate buffer, and oxidized for 15 minutes in 1% H_2O_2 in borate buffer. Animals were then washed once with borate buffer, once with TBST (100 mM Tris [pH 7.4], 0.9% NaCl, 0.5% Tween 20), and blocked in 1% 1x crystallized/lyophilized BSA (Sigma) in TBST with 4 drops of Avidin from the Avidin/Biotin Blocking Kit (Vector Labs) for at least 30 minutes. Animals were then washed twice with TBST and blocked again in 1% 1x crystallized/lyophilized BSA in TBST with 4 drops of Biotin from the Avidin/Biotin Blocking Kit (Vector Labs). Animals were then washed once with TBST and incubated in preadsorbed (see below) primary antisera diluted in TBST to a volume of 200µl overnight. anti-LH100p antibodies were used at 50 µg/ml; anti-LH101p antibodies was used at 20 µg/ml.

Animals were washed 3 times with TBST and incubated with a 1:50 dilution of preadsorbed biotinylated secondary antibody (see below) in TBST to a volume of 200 µl for 1 hour. Animals were then washed 3 times and the Vectastain Elite avidin/biotin complex (Vector Labs) which had been prepared according to the manufacturer's instructions, was added for 30 minutes. Afterwards, animals were washed 3 times with TBST. The staining was visualized by incubating animals with Vector VIP Peroxidase Substrate prepared according to manufacturer's instructions (Vector Labs) for 6-10

minutes, followed by two washes with ddH_2O. Animals were mounted onto 5% Noble Agar pads for examination. Photomicrographs were taken on Kodak Royal Gold film, ASA 25 on a Zeiss Axioplan.

Animals were prepared for preadsorption as desribed above, up to the second blocking step and the TBST wash following it. Then, either purified primary antibody or a 150 μg/ml solution of biotinylated secondary antibody diluted in TBST was added and incubated for at least 12 hours at room temperature. Primary antibodies were preadsorbed to *lin-15(e1763)* animals; secondary antibodies were preadsorbed to N2 animals. After preadsorption, animals were centrifuged for 30 minutes at 4°C and antibodies were removed and stored at 4°C until use.

RESULTS

*lin-15*B encodes a protein of 170 kD

From the nucleotide sequence of *lin-15*, we predicted that *lin-15* encodes a protein of 163 kD (Figure 1). We raised antisera against two distinct regions of the LIN-15B predicted protein by producing bacterially expressed LIN-15B proteins that were tagged with six histidine (6-His) residues (see Materials and Methods). The fusion protein LH100p is a 6-His fusion protein containing amino acids 534 to 922 from the middle of LIN-15B (see double underlined portion of Figure 1). The fusion protein LH101p is a 6-His fusion protein containing amino acids 6 to 224 from the N-terminus of LIN-15B (see single underlined portion of Figure 1).

Using the anti-LH101p antisera on immunoblots of wild-type mixed

stage *C. elegans* protein extract, we detected a band of approximately 170 kD (Figure 2A). This band was not detected in the absence of primary antisera (Figure 2B). This band was also not detected on immunoblots containing protein extract from *lin-15(e1763)* animals; *lin-15(e1763)* animals contain a deletion of the *lin-15* locus and can not produce any LIN-15 protein (Huang, et al., 1994, Clark, et al., 1994). We were able to compete away the binding of the anti-LH101p antisera to this 170 kD band by first incubating the anti-LH101p antisera with 1 μg/ml of the bacterially expressed LH101p fusion protein before applying the antisera to the immunoblot (Figure 2C). Thus, we conclude that the anti-LH101p antisera recognizes a LIN-15B protein.

LIN-15B is a nuclear protein

Antisera against both LH100p and LH101p were used on fixed whole *C. elegans* animals to determine LIN-15B expression. Both antisera showed LIN-15B to be a nuclear protein when applied to wild-type animals (Figure 3 A and B; Figure 4 A, B, and D). No nuclear staining was seen in animals of the *lin-15(e1763)* null strain (Figure 3D; Figure 4 C and E), indicating that we are detecting LIN-15B.

LIN-15B is broadly expressed

Anti-LH100p antisera used on wild-type animals showed LIN-15B to be broadly expressed (Figure 4A, B, and D). We were able to identify nuclear staining in the oocytes, maturing sperm, intestine, epidermal cells, seam cells, vulval precursor cell granddaughters, and body muscle cells. LIN-15B is likely not ubiquitously expressed, as we were able to identify cells that did not have nuclear staining when exposed to anti-LH100p antisera; these cells

were the pharyngeal cells and the developing germline. The nuclear staining seen in wild-type animals was not seen in animals of the *lin-15(e1763)* null strain (Figure 4, C and E). Staining using the anti-LH101p antisera on wild-type animals was only detectable in the oocyte nuclei (Figure 3 A, B, and C); again, this staining was not seen in *lin-15(e1763)* animals (Figure 3D). The difference in staining of the two antisera could either reflect that the anti-LH100p antisera is more sensitive, or that there is a difference in the usage of the exons containing the epitopes contained in LH100p and LH101p.

DISCUSSION

The LIN-15B protein

Through the use of antisera against portions of the LIN-15B predicted protein, we have determined that *lin-15*B encodes a nuclear protein of approximately 170 kD and that LIN-15B is broadly expressed in *C. elegans*. LIN-15B expression is detected in nuclei. However, examination of the protein sequence did not identify any motifs associated with nuclear proteins such as nuclear localization signals.

LIN-15B and vulval differentiation

The predicted protein elucidated from the sequence of the *lin-15* locus did not reveal any mechanism of LIN-15B action. Genetically, *lin-15* acts upstream of *let-23*, because animals carrying mutant alleles of both *lin-15* and *let-23* display the *let-23* phenotype; these animals are Vulvaless. *lin-15* acts in parallel to the inductive signal because *lin-15* animals without a

gonad (and thus no inductive signal) are still Multivulva, indicating that the *lin-15* phenotype is independent of the signal. Since we see LIN-15B expression in the nucleus, the negative regulation of *let-23* by *lin-15* is probably indirect, as LET-23 is likely a membrane bound protein. Thus, LIN-15B may regulate some molecule(s) that act directly on LET-23.

Candidates genes for LIN-15B regulation are the other members of the class B syn Muv (synthetic Multivulva; defined above in Introduction) pathway including *lin-9*, *lin-35*, *lin-36*, and *lin-37* (Ferguson and Horvitz, 1989). *lin-9*, *lin-35*, *lin-36* and *lin-37* act in the same pathway as LIN-15B because they are all capable of synergizing with the class A syn Muvs; animals defective in both a class B syn Muv and a class A syn Muv are Multivulva. So far, it has not been possible to order the syn Muvs in a pathway because their loss-of-function phenotypes are all identical and gain-of-function mutations have not been isolated. *lin-9* (G. Beitel and H.R. Horvitz, personal communication) and *lin-36* (J. Thomas and H.R. Horvitz, personal communication) have been cloned and encode novel proteins; their molecular identities are not informative about their order in the pathway. Now that LIN-15B antibodies exist, their use on animals mutant for other class B syn Muvs may help us order them with respect to *lin-15*B.

Genetic mosaic analysis led to the proposal that *lin-15* acts in the epidermis surrounding the vulval precursor cells (Herman and Hedgecock, 1990). This conclusion was drawn because 10 out of 33 animals that were mutant for *lin-15* in the P_1 lineage (P_1 is one of the two blastomeres in the two celled *C. elegans* embryo) were wild-type, while no animals (n=13) that were mutant for *lin-15* in the AB lineage (AB is the other blastomere at the two cell stage; VPCs originate from the AB lineage) were wild-type. Thus,

lin-15 appeared to be necessary in both AB and P_1 derivatives with a stronger requirement for *lin-15* in the AB lineage. Furthermore, some animals with *lin-15* activity in the AB lineage (and not the P_1 lineage) had mutant vulvae, indicating that wild-type *lin-15* activity in the VPCs was not always sufficient for the formation of a wild-type vulva. From these data, the authors proposed that *lin-15* was needed in *hyp7*, the syncitial epidermis surrounding the VPCs, because *hyp7* is derived from both AB and P_1 progeny and because *hyp7* is a tissue nearby the VPCs.

Now that we know LIN-15B is a nuclear protein expressed in *hyp7* (among other tissues), it is clear that the need for *lin-15* in *hyp7* can be fulfilled. Interestingly, *lin-36*, a class B syn Muv, has been shown to act cell-autonomously in the VPCs by mosaic studies (J. Thomas and H.R. Horvitz, personal communication). One interpretation of these results is that LIN-15B is involved in sending a signal that is received by the VPCs and transduced by LIN-36. However, one caveat to the *lin-15* mosaic experiment is that it was carried out before it was known that *lin-15* encodes two proteins; thus, an allele of *lin-15* that disrupts both functions was used. We do not know if both *lin-15*A and *lin-15*B act in the same tissue. The results from the mosaic experiment are consistent with one product acting in AB progeny (which include the VPCs) and the other one acting in derivatives of both AB and P_1. The wide expression of LIN-15B does not narrow the possibilities; the answer awaits mosaic studies using *lin-15*A and *lin-15*B alone.

Broad expression of LIN-15B

One reason why LIN-15B is broadly expressed could be that it

regulates receptor tyrosine kinases in addition to LET-23. The only other characterized cell fate transformation in *lin-15* hermaphrodites is the transformation of the P11 cell to the P12 cell; *let-23* is also involved in this decision because *let-23* mutant animals show the opposite transformation of the P12 cell sometimes adopting the P11 cell fate (Aroian and Sternberg, 1991, Fixsen, et al., 1985). The *egl-15* gene, which encodes an FGF receptor homolog, is the only other *C. elegans* receptor tyrosine kinase in which mutations have been identified. Its expression is currently unknown and it does not appear to interact genetically with *lin-15* (M. Stern, personal communication).

Another possible explanation for LIN-15B's broad expression is that LET-23 is also expressed broadly; in other words, the negative signal mediated by *lin-15*B is needed to prevent inappropriate basal activity of LET-23 in tissues where it is not needed. This is not unlikely, as receptors are often expressed more broadly than where they are needed. For example, although the *Drosophila* Torso tyrosine kinase is broadly expressed (Sprenger and Nüsslein-Volhard, 1992), localized ligand expression restricts its effects to the embryo's termini. The receptors for activin and FGF, two substances thought to be involved in mesoderm induction in *Xenopus*, are expressed uniformly in the embryonic stages (reviewed in Slack, 1993). The determination of LET-23 expression will help address this possibility.

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Figure 1. The LIN-15B protein. A conceptual translation of LIN-15B from Huang, et al., 1994. The underlined portion represents the part of LIN-15B used to make LH101p; the double underlined portion represents the part of LIN-15B used to make LH100p.

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   1 MQTLKTARLTSNPASIPTSSSSSAISAAAIQKTLDAVNRPPAVRASGILR
  51 HRTLPAPTQETAHHLDADPKTTELMARFFISQGIPFECAHEPAFLELMKH
 101 VDPNCVIPPTNVTKKLVDKISTSSKPQVNYTKTVGPLSVTIDICGDEDEK
 151 YLAFSIHYFEDLYERKNAIYLRKLLLTELDSNSLLTNIRRSVNSYSFSNV
 201 KFTNIVCPNEEICKLVEESAVVKRYNVCFYNYVTRFVADLMEIEEFSSGL
 251 TQLRTFVRYMKQNSDMYSKFRRMQLQKNAELDIPSIDSGDWHSTAIFLTR
 301 CLVWHDTFTEFCGKLDILHYIDNETFNHLIYLQRLLQQCMKHCRELSIPN
 351 NSISQVVPAIMSIRNFIASNSMGYRFQKRIRDSFTTSFKEITSGPSQDRY
 401 DIATLLDPRFAYRDTVYTAQTWRSLEKKVIDDFVNSDLQNDKNFYQDISI
 451 LNQEQRYDIIKKEFAYYRQTSFVERPEENENSNHWWGMRQTDMEFLAVIA
 501 REYLASPAVSIDAGYYFGNGGKFQHICHTYSHQRLENCLALAGNYQTFRG
 551 KGASVDVISQSMIETLNNTASRLQKQVHLGLYAHGVDNISSDRDVQSIVG
 601 HHYPPMPTVANYDIPHVPKEEEKPPVANLQSTSSPATSSPTIIRPRAAPP
 651 PRTLAQGRPIPLNGKELKAVPIRQIPLQVRPLPPRPANVPIVPRPTVPQQ
 701 FIKAPAPKPITLQAVVCSIPEKEIKKETEDVALLEKIKDEPLDEDDFNHP
 751 STDPVPNRTTASSQGPSSYPRKIVVLASKLPTSQSSSPSTATSAQARSHV
 801 TTAQLIRCGPSEGTVPQKIHSHNFVQKFAQKQNFVHKYALNSQDHTGRLN
 851 QTVPMRAALRLPNSEQKSGAPSSINGKVQRDDFKLEPLDDFNGEPDYDNL
 901 IGAQRLMYSDNLNDASAEDAFARHRVTMEFQKRRACNRRCAVCGHLEIHE
 951 RLKNVTIENEKLLIMLGCIYRGEFTLGQAQLFMARESKTYICRLHFLETL
1001 DEIYQMLRLKSADDILICPLDLIQNALITVSALRPHIIASQLRKILHDFA
1051 ERNNHLRETPAELKKLGQQYFDYREPEPEPERNDVDEQEIIPKLFRQPRK
1101 QVLEADQHDGTVKVIEQEDFKLPTVKPSENEECDNPGVCCFCSKRGDRGG
1151 MLRVPRSEERLARWVDKLGPEFEARLHTNTENLICRSHFPDAAFSSRGRL
1201 LKGMIPDAAPEKVETTYIIQGNNFLKLKERKSGTDKNSAIDLANMLNPDG
1251 VEYTQEEEEEEEYEEMSRSPTEETSDDEPSQAAVYNNAPVIKRTYRKREL
1301 SNEDGPLNLVTPPAHTPNPRGRPRKYPKNSVTPEAEKSLTDYDYNPGTSQ
1351 RRALKKGYVQLEDGEIVGEDCEYVPEKTPSGRLIRQAVARRSFAFADEEE
1401 EEEEYEESPIVKKPKIAGRPVGRPRKDANKLPTPTPPSNE*
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Figure 2. LIN-15B is a 170 kD protein. All three panels are strips from immunoblots containing mixed stage N2 protein. A. An immunoblot probed with purified antisera against LH101p. The LIN-15B protein migrates at 170 kD. B. An immunoblot with no primary antisera added to it; no LIN-15B is detected. C. An immunoblot probed with purified antisera against LH101p that has been preadsorbed to 1μg/ml of LH101p; no LIN-15B is detected.

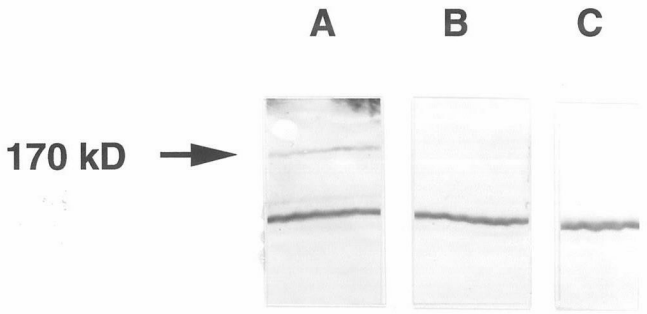
A
B
C
170 kD

Figure 3. Animals stained with purified anti-LH101p antisera. A. An N2 adult animal; arrowheads indicate oocyte staining. B. Oocytes dissected from an N2 animal. C. N2 larvae; no staining is apparent. The staining in the pharynx and the intestine are background; this staining is seen in *lin-15(e1763)* animals. D. A *lin-15(e1763)* adult animal. Arrowheads point to two representative oocytes which show no nuclear staining.

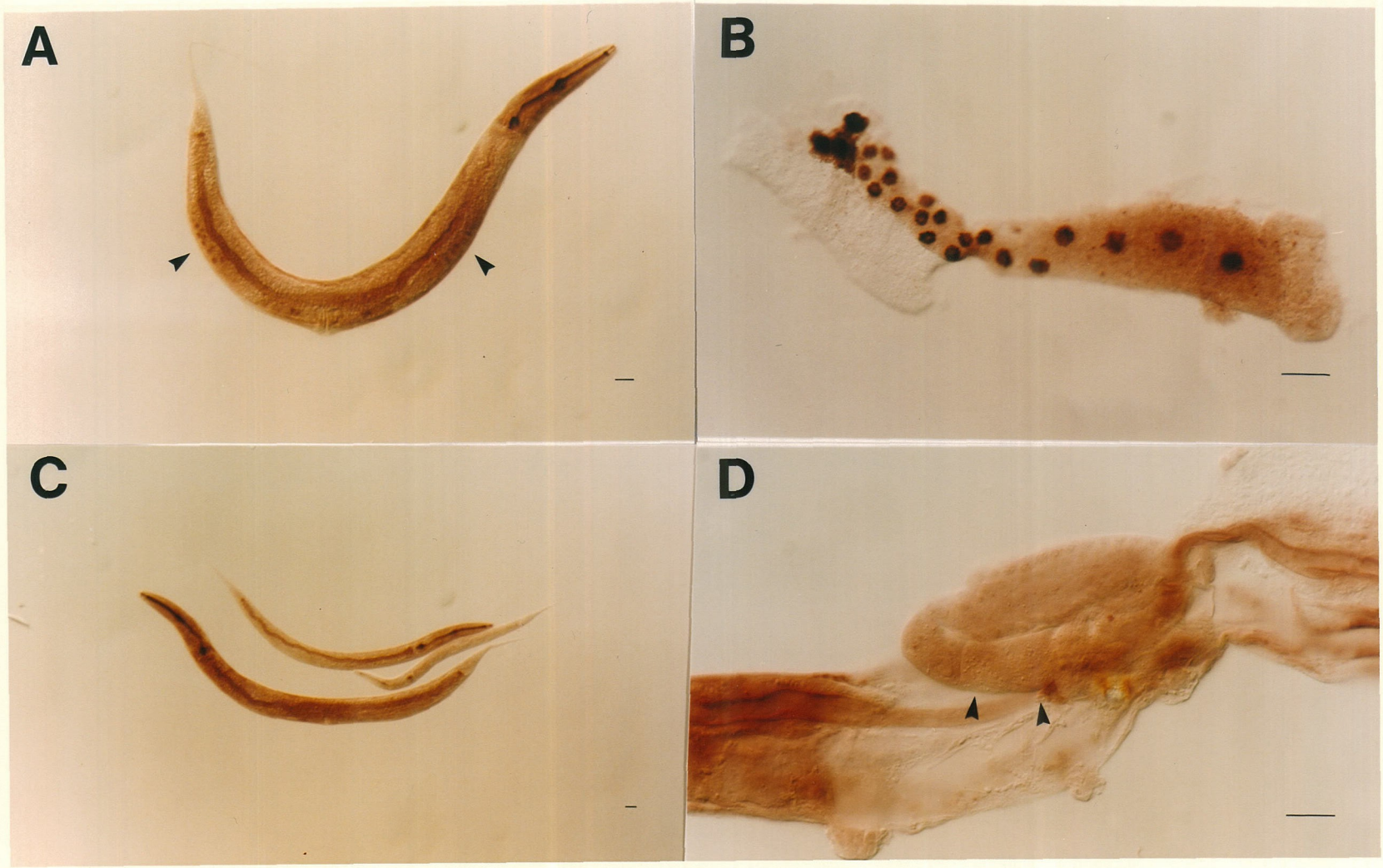
A
B
C
D

Figure 4. Animals stained with purified anti-LH100p antisera. A. An N2 L3 larval animal, dorsal side up. The nuclei along the sides are the hypodermal nuclei; arrowheads point to representatives. B. An N2 L3 larval animal. Arrowheads point to vulval precursor cell granddaughters. The white arrow point to intestinal nuclei. C. A *lin-15(e1763)* animal. Arrowheads point to the vulval precursor cell progeny; no nuclear staining is apparent. D. An N2 L4 animal, slightly ventral side up. Arrow points to the vulva. E. A *lin-15(e1763)* animal, ventral side up. Arrow points to the vulva.


A
B
C
D
E

Chapter 4

Interactions between a negative signal and an inductive signal allow for appropriate fate specification and patterning in the *Caenorhabditis elegans* vulva

Linda S. Huang and Paul W. Sternberg

ABSTRACT

The *C. elegans* vulva is made from 22 cells which originate from three vulval precursor cells. Six multipotent cells in the ventral epidermis are able to adopt vulva fates; a signal originating from the anchor cell in the gonad induces only three cells to adopt vulval fates in normal development. The three induced vulval precursor cells adopt two different fates, with the cell closest to the anchor cell adopting a 1° fate and the other two flanking cells, a 2° fate. The signal from the gonad is the LIN-3 growth factor; the vulval precursor cells receive the LIN-3 signal through a receptor-tyrosine kinase encoded by *let-23*. *lin-15* is a negative regulator of vulval development. *lin-15* acts non-autonomously, in parallel to the *lin-3* signal, acting genetically at the step of *let-23*. Here, we demonstrate that we can alter the number of vulval precursor cells adopting vulval fates by varying the amount of signalling by the receptor through varying either the *lin-3* inductive signal or the *lin-15* negative signal. We also demonstrate that varying the amount of signal transduced by the receptor can affect the fate of the vulval cells. In addition, these studies suggest that during normal vulval development, a limited amount of signal is presented to the vulval precursor cells, which have an excess of receptor.

INTRODUCTION

Appropriate pattern formation in development often requires the integration of multiple signals. For example, dorsal pattern formation in the *Drosophila* embryo requires *decapentaplegic* (*dpp*) (Irish and Gelbart, 1987), a TGF-β family member (Padgett, et al., 1987). Acting antagonistically to *dpp*

in patterning the dorsal region is *short gastrulation* (*sog*) (Ferguson and Anderson, 1992, Wharton, et al., 1993), an integral membrane protein that is a potential precursor protein for a novel class of cysteine-type secreted factors (François, et al., 1994). Similarly, the development of mammalian somites has been proposed to use three signals, a long range ventralizing signal from notochord and the floor plate as well as a dorsalizing short-range signal from the surface ectoderm and a long-range dorsalizing signal from the dorsal neural tube. These act together to pattern the somites into dermomyotome and sclerotome (Fan and Tessier-Lavigne, 1994).

These and other systems provide a way to study the integration of multiple signals in pattern formation. However, because these systems examine the effect of multiple signals over a field of cells, they do not necessarily allow the study of how multiple signals are processed within a single cell. The simple and invariant development of *C. elegans* has provided for the study of inductive signalling between an identified signalling cell (or cells) and a few potential receiving cells; extensions on these studies allow for the study of how multiple signals are processed in an identified cell. Studies of the cells of the *C. elegans* B lineage in the male tail has allowed both the identification of multiple signals involved in patterning these cells (Chamberlin and Sternberg, 1993) as well as identification of genes involved in the signalling processes (Chamberlin and Sternberg, 1994). The *C. elegans* vulva provides another system for studying how multiple signals affect cell fate determination.

During *C. elegans* vulva development, the anchor cell in the gonad sends a signal to vulval precursor cells (VPCs) in the underlying ventral epidermis (Sulston and White, 1980, Kimble, 1981, Thomas, et al., 1990). The six VPCs

(which are cells P3.p, P4.p, P5.p, P6.p, P7.p, and P8.p) are competent to respond to the anchor cell signal (Sulston and White, 1980, Sternberg and Horvitz, 1986). During normal development, the three cells closest to the anchor cell are induced to form vulval tissue; the other three competent VPCs (P3.p, P4.p, and P8.p) are not induced and form non-specialized ventral epidermis (Sulston and Horvitz, 1977). Of these three cells, the one closest to the anchor cell (P6.p) adopts a 1° fate and the other two (P5.p and P7.p) adopt 2° fates (Sulston and Horvitz, 1977); 1° fates and 2° fates can be distinguished by their morphology and their characteristic pattern of cell divisions (Sternberg and Horvitz, 1986, Katz, et al., 1995). The three cells being induced and the pattern of fates they adopt are the same in every wild-type *C. elegans* animal. This invariant development is due to at least three signals working together to specify this pattern: 1) the LIN-3 inductive signal from the anchor cell (Ferguson and Horvitz, 1985, Thomas, et al., 1990, Hill and Sternberg, 1992) 2) a lateral signal from the induced VPCs to its neighbors that inhibits adjacent cells from adopting the same vulval fates, mediated by *lin-12* (Greenwald, et al., 1983, Sternberg, 1988) and 3) a negative signal from a cell other than the VPC that acts in parallel to the anchor cell signal, mediated by *lin-15* (Ferguson and Horvitz, 1985, Ferguson, et al., 1987, Herman and Hedgecock, 1990, Huang, et al., 1994, Clark, et al., 1994).

The inductive signal and the lateral signal work together to specify the invariant 1° and 2° fates seen in wild-type animals (Sternberg and Horvitz, 1989). The inductive signal likely acts in a graded fashion to predispose the VPCs to adopt 1° and 2° fates, such that P6.p receives the most signal and becomes 1° while P5.p and P7.p receive some signal such that they are

induced and adopt a 2° fate (Sternberg and Horvitz, 1986, Katz, et al., 1995). The lateral signal acts to enforce these fates; the inductive signal likely causes P6.p to send more lateral signal to its neighbors, inhibiting them from adopting 1° fates by stimulating them to adopt 2° fates (Katz, et al., 1995, Simske and Kim, 1995, Koga and Oshima, submitted).

The relationship between the inductive signal and the negative signal has not been explored as extensively. In this study, we use a sensitized system to study how modulating the inductive signal and the negative signal can affect the fates of the VPCs. We can do this because the molecular machinery allowing the VPCs to respond to the inductive signal has been well characterized and a number of molecular and genetic tools are available. The LIN-3 signal is transduced in a manner similar to the mammalian EGF-signalling pathway (reviewed by Fantl, et al., 1993, Sternberg, 1993); LIN-3 is received by the VPCs through a receptor tyrosine kinase encoded by *let-23* (Aroian, et al., 1990).

In this study, we impair the VPCs' ability to transduce signal by using reduction-of-function *let-23* alleles that retain their ability to signal. By increasing the amount of inductive signal or, by removing the negative regulation in the reduction-of-function *let-23* background, we sensitize the system by reducing the VPCs responsiveness to inductive signal compared to a normal VPC. In this sensitized background, changes in *let-23* mediated signalling can be measured in the VPCs through their choice to be vulval or non-vulval as well as their choice to adopt a 1° or 2° vulval cell fate. We increase the amount of inductive signal by overexpressing *lin-3* through the integrated transgene *syIs1* (J. Liu, personal communication) and remove the negative signal genetically by using the *lin-15* null allele, *e1763* (Ferguson

and Horvitz, 1985, Huang, et al., 1994); we demonstrate that removal of the negative signal is similar to overexpressing the inductive signal. Using this system, we can study at a single cell level how a negative signal is used antagonistically to regulate development. By using *lin-15(e1763)* to vary receptor activity, we can both alter the number of VPCs adopting vulval fate and the fate of the VPCs. In addition, these studies also reveal an excess of LET-23 on the VPCs.

MATERIALS AND METHODS

Strains and culture conditions

Nematodes were cultured as previously described (Brenner, 1974, Sulston and Hodgkin, 1988). All strains were grown at 20°C unless otherwise noted. The *C. elegans* strains used in this study are listed in Table 1. PS1477 and PS1559 were constructed using standard genetic methods (Hodgkin, et al., 1988). *syIs1* is a transgene containing wild-type *lin-3* DNA in the form of pRH9 (Hill and Sternberg, 1992) and wild-type *unc-31* DNA (R. Hoskins, personal communication), integrated on the X chromosome by X-ray irradiation (J. Liu, personal communication).

Statistical analysis

In order to determine whether or not the differences in amount of vulval differentiation seen in the various strains was significant, we calculated χ^2 using the null hypothesis that any two sets of data were the same. χ^2 was determined for two classes of events: animals that had no vulval

differentiation vs. animals with vulval differentiation. χ^2 was calculated by the following formula:

Given the data in the form of:

genotype	# animals with differentiation	# animals without diff'n	totals
X	a	b	e
Z	c	d	f
totals	g	h	i

$$\chi^2 = \frac{(ad-bc)^2 i}{e f g h}$$; degree of freedom=1,

as in Fisher, (1948).

Vulval differentiation, vulval lineages, and photomicroscopy

Vulval differentiation and vulval lineages were scored by mounting living animals on 5% noble agar pads and examining them under Nomarski microscopy as previously described (Sulston and Horvitz, 1977, Sternberg and Horvitz, 1981). The number of cells undergoing vulval differentiation was tabulated by examining the number of vulval cells versus large non-vulval epidermal cells after the late L3 larval stage, the time after vulval differentiation occurs in wild-type animals. The percentage of cells undergoing vulval differentiation was determined as previously described (Aroian and Sternberg, 1991), with 100% indicating wild-type amount of three cell vulval differentiation, 0% indicating no vulval differentiation, and 200% indicating all six VPCs differentiating. Photomicrographs were taken on Kodak technical pan film on a Zeiss Axioskop under Nomarski optics with a 100x objective.

RESULTS

Extent of vulval differentiation in wild-type and single mutant animals

Figure 1 shows the extent of vulval differentiation in wild-type and single mutant animals. Wild-type animals have three VPCs that differentiate into vulval tissue (Figure 1A; Figure 3A). On the other hand, animals carrying the *lin-15* null allele *e1763* essentially always have six VPCs differentiating into vulval tissue (Figure 1B; Figure 3B). VPCs of animals with the *syIs1* integrated transgene behave similarly to *lin-15(e1763)* animals, with all VPCs adopting vulval fates (Figure 1, panel B).

The *let-23* alleles used in this study are *sy10* and *sy97* ((Aroian and Sternberg, 1991). We chose these alleles because they are severe reduction-of-function alleles and because they have been molecularly characterized and retain an intact kinase domain. The *sy97* allele harbors a point mutation in a 3' splice acceptor that would result in C-terminal truncations of the LET-23 protein without disrupting the kinase domain (Aroian, et al., 1994), allowing it to function in one tissue (Aroian and Sternberg, 1991) and retaining its ability to respond to inductive signal (Huang, et al., 1994). The *sy10* allele harbors a point mutation in a conserved cysteine of unknown function in the extracellular domain of the protein (Aroian, et al., 1994). The extent of vulval differentiation in animals carrying these alleles is shown in Figure 1C and Figure 3C for *let-23(sy97)* and Figure 1E for *let-23(sy10)*. Essentially no vulval differentiation is seen in animals mutant for either *let-23(sy97)* or *let-23(sy10)*; these results are not significantly different from the amount of vulval differentiation previously reported (Aroian and Sternberg, 1991) (0%

for *sy97*; 1.4% for *sy10*; n=20 for each strain).

let-23(sy97) and *let-23(sy10)* demonstrate more activity upon removal of *lin-15* mediated negative regulation

Figure 2 illustrates the extent of vulval differentiation in double mutant animals. When negative regulation is eliminated by removal of *lin-15*, the activities of the *let-23* reduction-of-function alleles appear to be unmasked; more vulval differentiation is seen than in animals defective for *let-23* alone (Figure 2C vs. Figure 1C, $p < 0.5\%$; Figure 2B vs. Figure 1E, $p << 0.5\%$; see Materials and Methods; also see Figure 3C and D). This differentiation is likely inductive signal dependent, as all differentiation seen is centered around the anchor cell (Table 2), which resides dorsal to P6.p in wild-type animals. For *let-23(sy97)*, the centering of differentiation around the anchor cell is consistent with the dependence of vulval differentiation in *let-23(sy97); lin-15(e1763)* animals on the inductive signal (Huang, et al., 1994). Interestingly, more differentiation is seen in the *let-23(sy10)*; *lin-15(e1763)* mutant animals than in *let-23(sy97); lin-15(e1763)* animals (Figure 2B vs Figure 2C). Whether this reflects a quantitative difference in the mutant receptors' abilities to signal or a qualitative difference in the ability of the receptors' abilities to be negatively regulated by *lin-15* cannot be distinguished from this experiment. Also, removal of *lin-15* mediated negative regulation is similar to providing more signal using *syIs1* in a *let-23(sy97)* mutant background (Figure 2A vs. Figure 2C; p between 50% and 10%). This similarity is consistent with the hypothesis that *lin-15* acts to regulate the receptor in parallel to the inductive signal.

The receptors on the surface of a VPC are likely not at maximal occupancy during normal inductive signalling

The behavior of the VPCs in the *syIs1; let-23(sy97)* double mutant animals suggests that the receptors on the VPCs are not maximally occupied during normal signalling. *let-23(sy97)* mutants receive a normal amount of the *lin-3* signal; these animals show 1% vulval differentiation (Figure 1D). We believe that animals with the *syIs1* transgene are overexpressing *lin-3* because these animals have greater than wild-type vulval differentiation (Figure 1, panel C). The comparison of vulval differentiation in *let-23(sy97)* animals (Figure 1C) with the *let-23(sy97); syIs1* double mutant animals (Figure 2A) show that the double mutant animals have significantly more animals displaying vulval differentiation ($p < 1\%$; see Materials and Methods for details). Thus, the receptors on the VPCs appear to be capable of binding to more *lin-3* signal than is provided under normal circumstances. It must be noted that because we do not know the time course of *lin-3* expression by *syIs1*, it could be that the receptors are being exposed to more *lin-3* over time. However, if this is the case, it would suggest that the receptors persist and thus were not maximally occupied during their existence.

We originally undertook this experiment to examine whether *lin-15* could negatively regulate activated receptors as well as unstimulated receptors; it is believed that *lin-15* can regulate a receptor's basal activity because the *lin-15* phenotype is inductive signal independent (Ferguson, et al., 1987, Sternberg and Horvitz, 1989). We had previously observed that in *let-23(sy97); lin-15(e1763)* animals, vulval differentiation was dependent on the signal (Huang, et al., 1994); these results are consistent with the differentiation seen in these animals being centered around the anchor cell

(Table 2). Because removal of *lin-15* in a *let-23(sy97)* background led to significantly more vulval differentiation than in a *let-23(sy97)* background (Figure 2C vs. Figure 1D; $p < 0.5\%$), *lin-15* activity was preventing this differentiation in the *let-23(sy97)* single mutant animals. Since that differentiation was signal dependent, if all the receptors were occupied by ligand, *lin-15* then must be capable of regulating stimulated receptors as well as unstimulated receptors. However, since we find that not all receptors are occupied during signalling, we cannot conclude whether or not *lin-15* can regulate ligand bound receptors.

VPC fates are dependent on the amount of receptor activation

We demonstrate above that activation of signalling-impaired receptors can occur through either removal of negative regulation or overexpression of the inductive signal. The number of vulval cells differentiating in these double mutants is substantially less than in wild-type animals. Thus, these VPCs are likely to have less *let-23* activity than in wild-type animals. We believe that the number of VPCs differentiating in a given animal of a given genotype is a reflection of the amount of receptor activation in the VPCs (also see Katz, et al., 1995). Therefore, we examined the vulval cell fates in the *let-23(sy97); lin-15(e1763)* double mutants to see if the amount of receptor activation is reflected in the cell fates adopted by the induced cells.

Cell lineage analysis in a *let-23(sy97); lin-15(e1763)* animal that had three vulval cells induced showed P6.p, which normally adopts a 1° fate in wild-type animals, adopting an intermediate fate (Table 3); an intermediate fate is a fate that has both 1° and 2° characteristics (Katz, et al., 1995). When only one vulval cell is induced in the *let-23(sy97); lin-15(e1763)*

animals, these cells adopt 2° fates (Table 3 and Figure 4). Conversely, in *lin-15* mutant animals, an isolated cell adopts a 1° fate (Sternberg, 1988). This indicates that *lin-15* does not prevent isolated cells from adopting 1° fates and is consistent with the hypothesis that *let-23(sy97); lin-15(e1763)* animals have decreased signalling levels. Thus, although these mutant receptors are capable of functioning, they are functioning at a reduced level and transducing signal weakly.

DISCUSSION

In this study, we use mutant forms of LET-23 to create a sensitized system to study the interaction between the negative signal and the inductive signal. Normally, animals carrying a mutation in *lin-15*, a mediator of the negative signal, have all six VPCs adopting vulval fates. By combining *lin-15* mutations with *let-23* alleles that severely inhibit its ability to signal, we create situations where few VPCs adopt vulval fates. The *let-23* alleles used are severe loss-of-function alleles, with 1% or less vulval differentiation. When the *sy97* or *sy10* mutant alleles are combined with a null allele of *lin-15*, we see vulval differentiation increase to 14% and 87%, respectively. The removal of negative regulation on the receptor by use of a *lin-15* null allows detection of the activity of the mutant LET-23 proteins; this activity is likely signal dependent due to the centering of differentiated cells around the signalling source. We find that removal of the *lin-15* mediated negative signal is similar to overexpression of the *lin-3* signal. We also find that wild-type levels of inductive signal likely do not occupy all receptors during inductive signalling. Furthermore, we find that when a single VPC

differentiates in a *let-23(sy97); lin-15(e1763)* mutant animal, this VPC is capable of adopting a 2° fate.

lin-15 and the negative signal

The localization of LIN-15B using antibodies (see Chapter 3) demonstrates that LIN-15B is a nuclear protein; the localization of LIN-15A is currently unknown. LIN-15B's nucelar localization indicates its effect on LET-23 is likely indirect. However, these studies show that the information encoded by *lin-15* does antagonize the inductive signal and is apparently processed at the receptor. Thus, even if there is no direct interaction of the negative signal with LET-23, the information sent by the *lin-15* mediated negative signal and the information encoded by LET-23 must be maintained in a proportional fashion until the negative signalling and the inductive signalling pathways converge. Previous work using *lin-15* (which is on the X chromosome) for dosage compensation studies demonstrate that the levels of *lin-15* activity are important. Increasing the dose of hypomorphic alleles of *lin-15* by varying the chromosome composition or by introducing dosage compensation mutations that elevate X chromosome expression leads to suppression of vulval defects (Meneely and Wood, 1987). Thus, changes in levels of *lin-15* activity, and not merely its presence or absence, can have measurable effects.

Levels of receptor activation and mechanisms of inductive signalling

Because we can observe a single VPC adopting a 2° fate in *let-23(sy97); lin-15(e1763)* animals in the absence of neighbors that adopt 1° fates, we know that LET-23 can transduce graded information. This ability is

consistent with the VPCs' ability to respond to different levels of LIN-3 signal by assuming different fates (Katz, et al., 1995); in order for this to occur, LET-23 must be able to transduce this information in a graded fashion. This also demonstrates that the branch point between adopting a 2° fate or adopting a 1° fate must be somewhere at or downstream of LET-23; it will be informative to examine the fates of cells using downstream components (i.e., *sem-5, let-60, lin-45*) in a similar manner to determine the branch point.

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Table 1. *C. elegans* strains used in this study

Strain	Genotype	Reference or source
N2	wild-type	Brenner, 1974
CB3278	*lin-15(e1763)*	Ferguson and Horvitz, 1985
	let-23(sy97)	Aroian and Sternberg, 1991
PS1123	*syIs1*	Jing Liu (unpublished)
PS302	*let-23(sy10) unc-4(e120)/mnC1 (dpy-10 unc-52)*	Aroian and Sternberg, 1991
PS295	*let-23(sy97) unc-4(e120)/mnC1 (dpy-10 unc-52)*	Aroian and Sternberg, 1991
PS294	*let-23(sy97)/mnC1 (dpy-10 unc-52)*	Aroian and Sternberg, 1991
PS1033	*let-23(sy97); lin-15(e1763)*	Huang, et al., 1994
PS1559	*let-23(sy10) unc-4(e120)/mnC1 (dpy-10 unc-52); lin-15(e1763)*	this study
PS1477	*let-23(sy97) unc-4(e120)/mnC1 (dpy-10 unc-52); lin-15(e1763)*	this study
PS1303	*let-23(sy97); syIs1*	Jing Liu (unpublished)

Table 2. Profile of induced cells.

genotype	number of animals	number of animals with induced cell									
		P3.p	P4.p		P5.p		P6.p		P7.p		P8.p
			a	p	a	p	a	p	a	p	
N2 (wild-type)	20	-	-	-	20	20	20	20	20	20	-
let-23(sy97); lin-15(e1763)	143	-	-	-	8	11	38	42	12	7	-
let-23(sy10); lin-15(e1763)	18	-	4	6	13	16	18	18	14	14	-
let-23(sy97); syIs1	50	-	-	-	1	2	10	9	3	1	-

Table 3. Lineage analysis of *let-23(sy97)*; *lin-15(e1763)*

P3.p	P4.p	P5.p	P6.p	P7.p	P8.p
S S	S S	LLTN	TT?D	NTLL	S S
S S	S S	S S	LL?N	S S	S S
S	S S	S S	TNOL	S S	S S
S S	S S	S S	LONT	S S	S S

Nomenclature as in (Sternberg and Horvitz, 1986) as modified by (Katz, et al., 1995). A wild type animal would have P3.p=S or S S, P4.p=S S, P5.p =LLTN, P6.p=TTTT, P7.p=NTLL, P8.p=S S. S refers to a non-specialized epidermal cell fate. L, T, and O refer to the axis of cell division. An N cell is a specialized cell that doesn't divide, a "?" indicates that the cell division was not observed, and a D indicates that the cell divided but the axis of division could not be determined. An underline indicates a cell that adheres to the ventral cuticle.

Figure 1. Vulval differentiation in single mutant animals. Each graph shows the distribution of animals of a particular genotype that have a certain number of VPCs that adopt vulval fates instead of non-specialized epidermal fates. Although there are six VPCs, sometimes only one daughter of a VPC will adopt vulval fates while the other daughter adopts a non-specialized epidermal fate. When only one daughter differentiates, the VPC is counted as 1/2 a VPC, resulting in the stippled bars which lie between the whole numbered cells. §Because *sy10* animals are sterile (Aroian and Sternberg, 1991), *unc-4(e120) let-23(sy10)* animals were picked segregating from an *unc-4(e120) let-23(sy10) / mnC1 [dpy-10 unc-52]* parent.

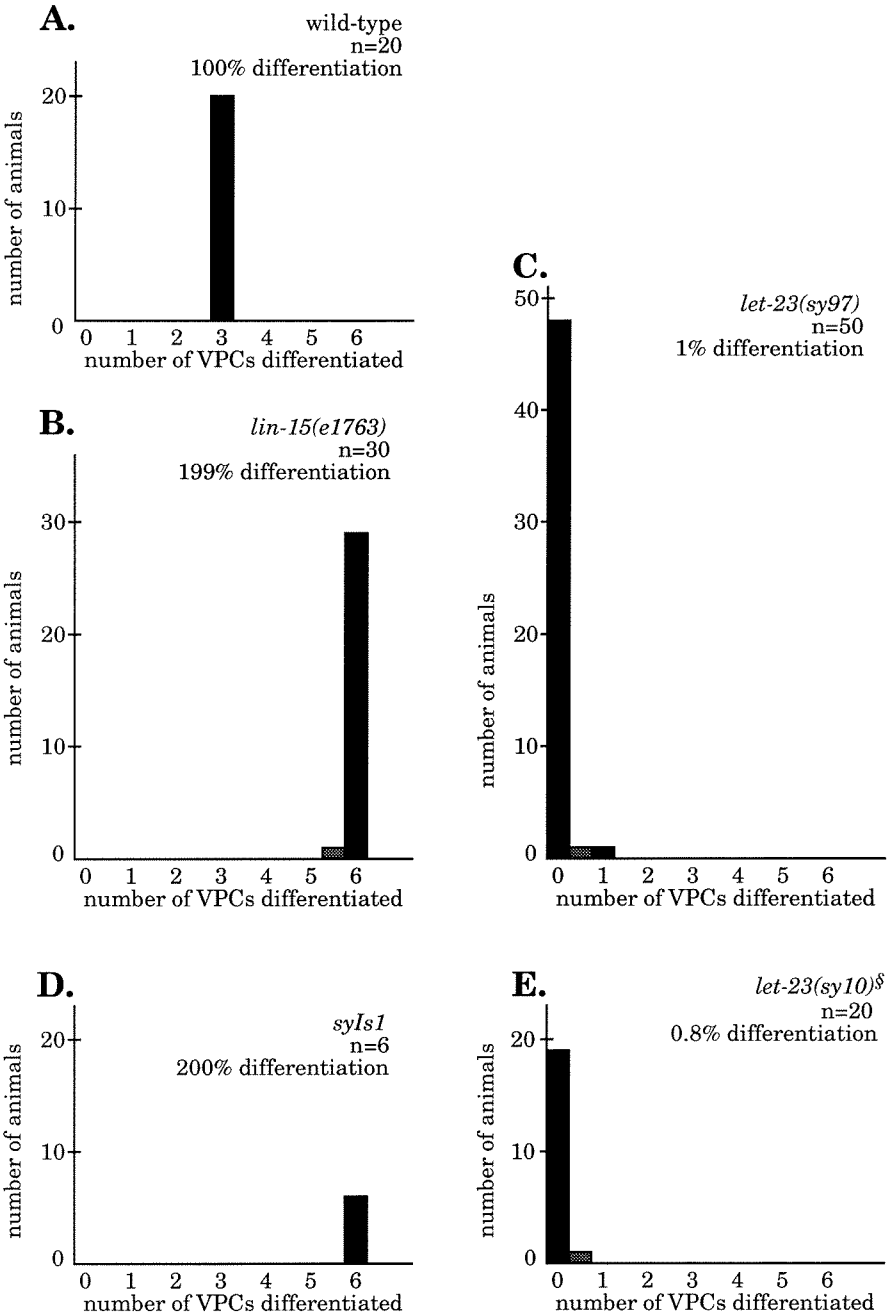
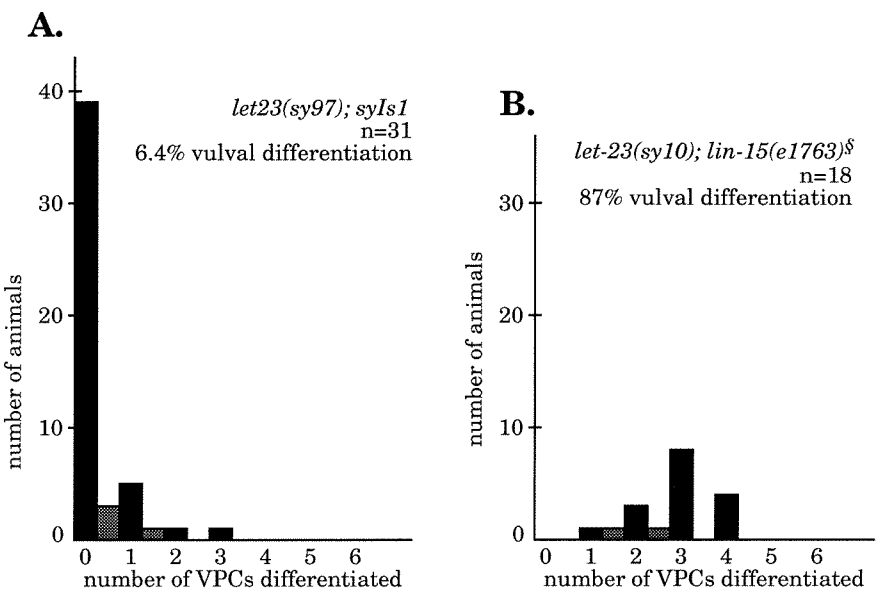

A.
wild-type
n=20
100% differentiation
number of animals
20
10
0
0 1 2 3 4 5 6
number of VPCs differentiated
B.
lin-15(e1763)
n=30
199% differentiation
number of animals
30
20
10
0
0 1 2 3 4 5 6
number of VPCs differentiated
C.
let-23(sy97)
n=50
1% differentiation
number of animals
50
40
30
20
10
0
0 1 2 3 4 5 6
number of VPCs differentiated
D.
syIs1
n=6
200% differentiation
number of animals
20
10
0
0 1 2 3 4 5 6
number of VPCs differentiated
E.
let-23(sy10)§
n=20
0.8% differentiation
number of animals
20
10
0
0 1 2 3 4 5 6
number of VPCs differentiated

Figure 2. Vulval differentiation in double mutant animals. Each graph shows the distribution of animals of a particular double mutant genotype that have a certain number of VPCs adopting vulval fates. See Figure 1 legend for details regarding 1/2 cell differentiation. §Because *let-23(sy10)* animals are sterile, *let-23(sy10); lin-15(e1763)* animals were picked as Unc animals segregating from an *unc-4(e120) let-23(sy10) / mnC1 [dpy-10 unc-52]; lin-15(e1763)* parent. The difference seen between *let-23(sy10); lin-15(e1763)* and *let-23(sy97); lin-15(e1763)* is not due to a maternal contribution of *let-23* because no maternal contribution of *let-23* has been observed (Aroian and Sternberg, 1991) and 2 of 10 *unc-4(e120) let-23(sy97); lin-15(e1763)* animals segregating from an *unc-4(e120) let-23(sy97) / mnC1 [dpy-10 unc-52]; lin-15(e1763)* parent displayed vulval differentiation, similar to the amount of differentiation seen in the *let-23(sy97); lin-15(e1763)* strain.


A.
let23(sy97); syIs1
n=31
6.4% vulval differentiation
number of animals
0
10
20
30
40
0 1 2 3 4 5 6
number of VPCs differentiated
B.
let-23(sy10); lin-15(e1763)§
n=18
87% vulval differentiation
number of animals
0
10
20
30
0 1 2 3 4 5 6
number of VPCs differentiated

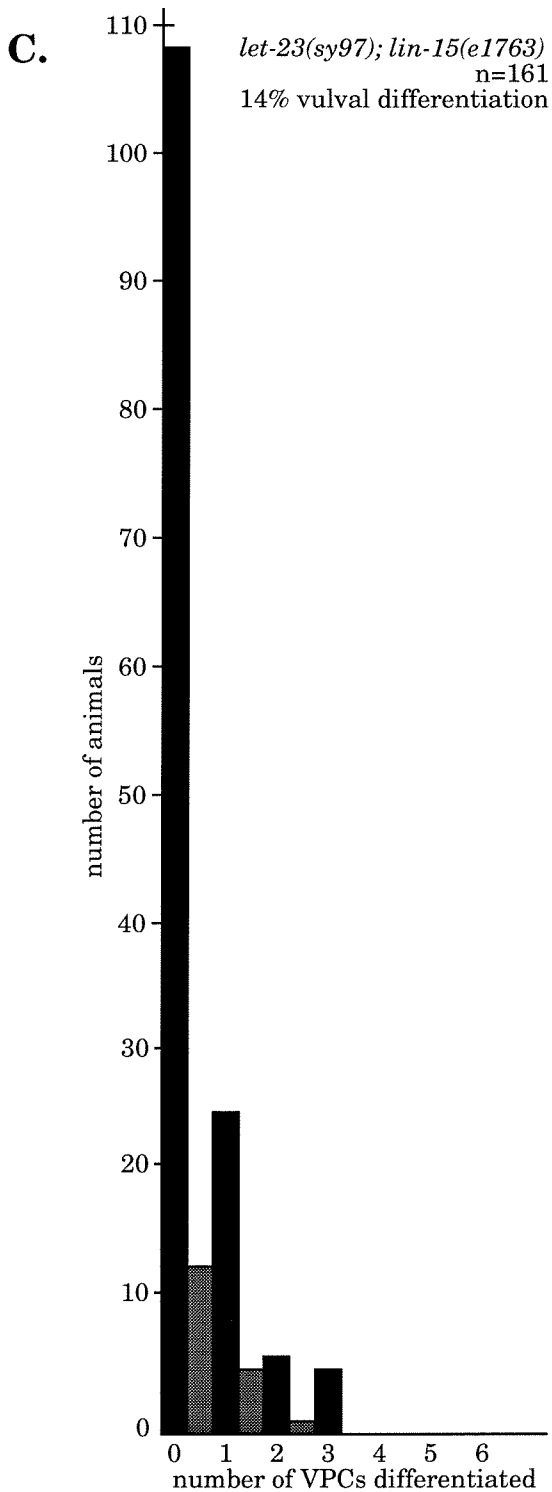

C.
let-23(sy97); lin-15(e1763)
n=161
14% vulval differentiation
number of animals
110
100
90
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6
number of VPCs differentiated

Figure 3. Vulval differentiation after two rounds of division of a VPC. White arrowheads point at cells which have adopted a non-specialized epidermal fate, black arrowheads point at cells which have adopted vulval fates, and white arrow point at the anchor cell. Genotypes: A. N2, B. *lin-15(e1763)*, C. *let-23(sy97)*, and D. *let-23(sy97); lin-15(e1763)*.

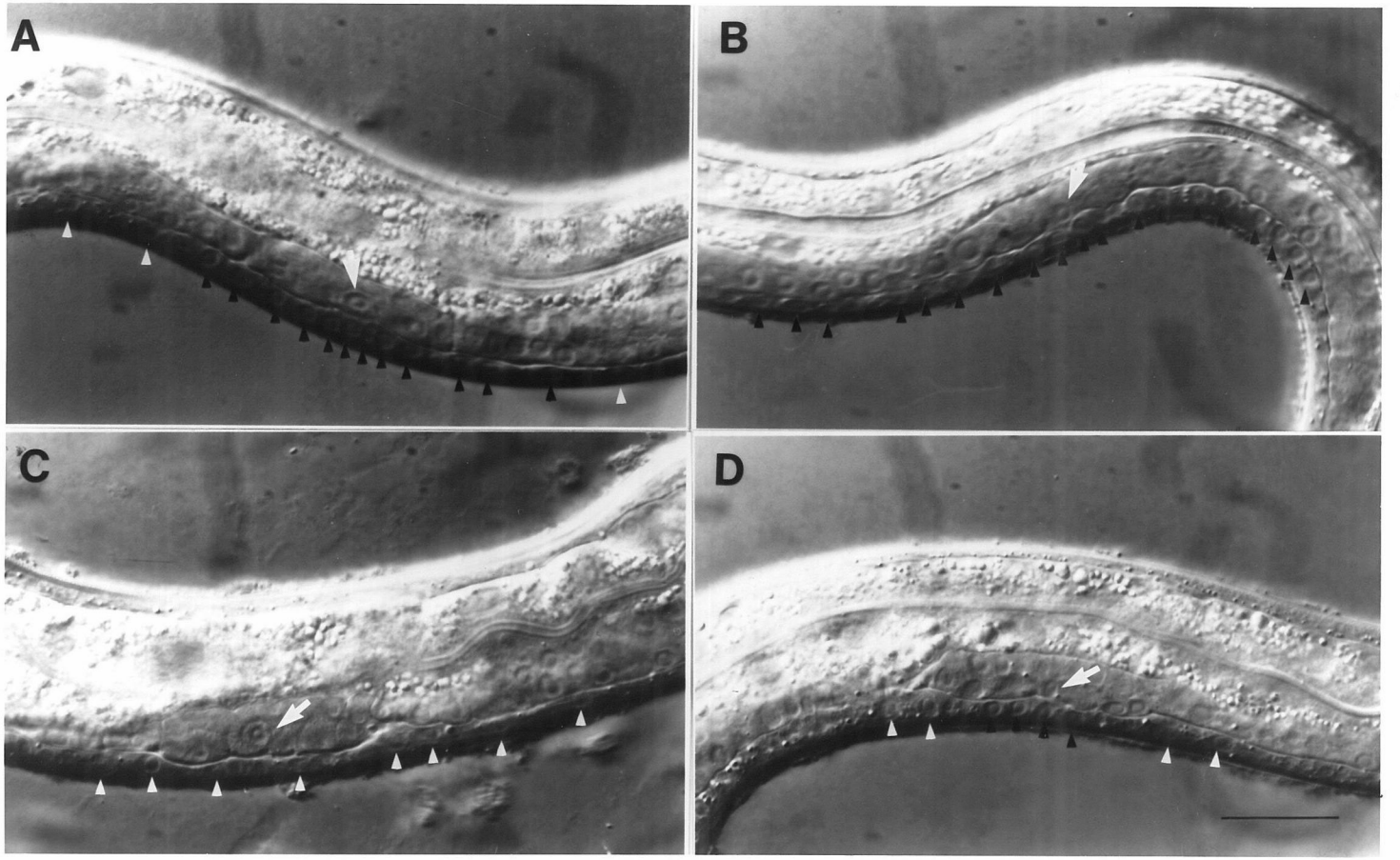
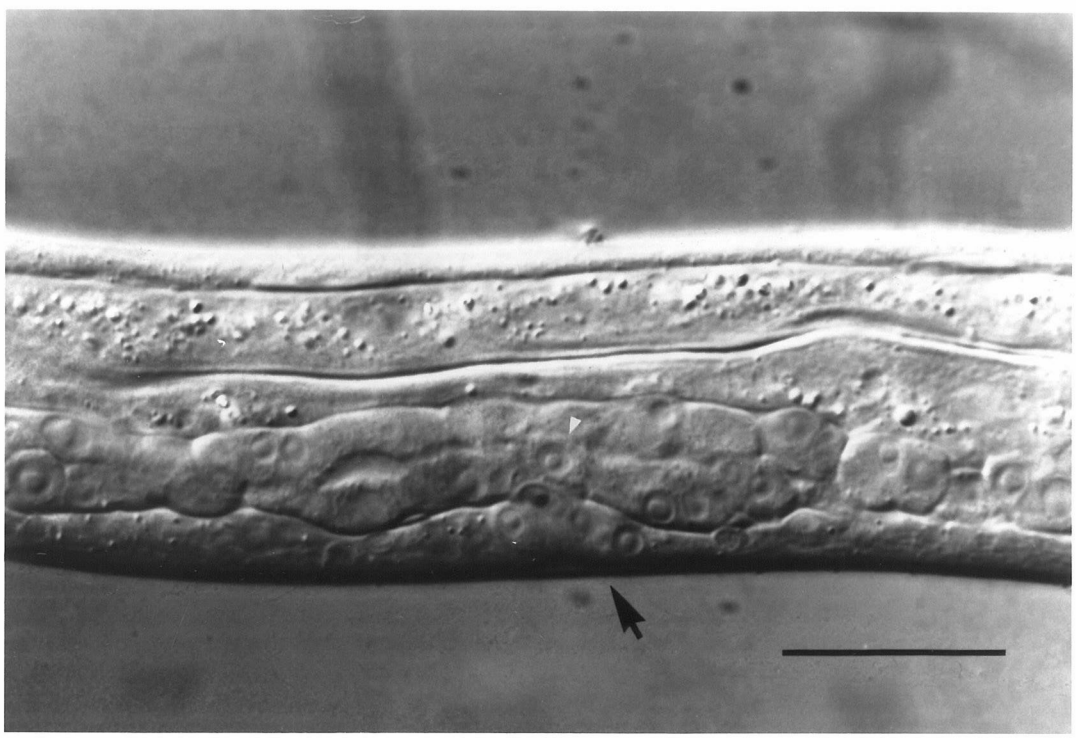

A
B
C
D

Figure 4. A single VPC adopting a 2° fate. This *let-23(sy97); lin-15(e1763)* animal's VPC cell lineage was followed; the final result is on the bottom line of Table 3. In this picture, the VPC has finished its cell divisions and is undergoing morphogenesis. The black arrow points to the progeny of the induced VPC; an asymmetric morphology can be observed. The white arrow points to the anchor cell. When this animal reached adulthood, a small bump was seen at the vulva characteristic of a 2° fate. Furthermore, this animal was unable to lay eggs and resulted in a bag of worms when its eggs hatched internally.



Chapter 5

Summary

In my graduate work, I have investigated the mechanisms used in *C. elegans* vulval differentiation through a molecular and genetic analysis of the *lin-15* locus. I chose to focus on the *lin-15* locus for two reasons. First, it was a negative regulator of the inductive signalling process that occurs during *C. elegans* vulval development (Ferguson and Horvitz, 1985, Sternberg and Horvitz, 1986, Ferguson, et al., 1987). Thus, the study of *lin-15* would elucidate its role in signal regulation.

Second, the *lin-15* locus was a complex locus, genetically defined to have two separate activities, A and B (Ferguson and Horvitz, 1985, Ferguson and Horvitz, 1989). The A and B activities of *lin-15* indicated that it acted in the negative regulation of the inductive event in concert with two sets of genes, the class A and class B genes (which have A and B activity, respectively), known as the synthetic Multivulva genes. These loci are synthetic because animals carrying mutations in either a class A gene or a class B gene have no phenotype. However, a double mutant animal defective in both A function and B function display the Multivulva phenotype characteristic of excessive vulval differentiation. Of all the known class A and class B genes, *lin-15* was the only gene known to have both A and B activity; all other genes had either A or B activity. One possibility for why *lin-15* had both activities was that it encoded a bifunctional protein that had an A and a B domain that could be independently mutated. If this were true, *lin-15* would be a good candidate for a regulator of the A and B pathways; it could either be an upstream gene that regulated the A and B activities, or, a downstream target of the A and B pathways. Therefore, the study of *lin-15* would lead to an understanding of how the A and B negative regulatory pathways function.

The work in this thesis demonstrates that *lin-15* has both A and B activity because it encodes two different gene products that correspond to the A and B functions (Chapter 2). Thus, instead of encoding a bifunctional protein, the *lin-15* locus can be thought of as two very closely linked A and B synthetic Multivulva loci. These gene products are hydrophilic, novel and contain no known motifs. The inductive process regulated by *lin-15* involves a growth factor mediated receptor tyrosine kinase signalling pathway (reviewed in Chapter 1). Therefore, we have identified a novel regulatory mechanism of this pathway.

Epistasis studies (Ferguson, et al., 1987, Chapter 2) demonstrate *lin-15* works upstream of the receptor tyrosine kinase encoded by *let-23*. Ablation studies that remove the signalling source (Sternberg, 1988, Chapter 2) demonstrate the *lin-15* phenotype to be signal independent. These results are consistent with genetic studies demonstrating *lin-15* modulation of *let-23* signalling (Chapter 4). Mosaic studies (Herman and Hedgecock, 1990) suggest that *lin-15* acts in the epidermis surrounding the receiving cells. Thus, the negative regulation mediated by *lin-15* influences the receiving cells from a different tissue, acting early in the signalling pathway at the receptor, on the basal activity of the receptor, and in parallel to the inductive signal.

This regulation is mediated by *lin-15*, but likely not due to a direct interaction of LIN-15 with LET-23 because the LIN-15B protein is a nuclear protein that is broadly expressed (Chapter 3). Why LIN-15B is broadly expressed is currently unknown; one possibility is that it reflects broad expression of LET-23, as there is much precedent for receptors being expressed more broadly than where they actually function (for example, see

Banerjee, et al., 1987, Tomlinson and Ready, 1987, Sprenger and Nüsslein-Volhard, 1992, Slack, 1993). The ability to asses LIN-15B expression should allow the ordering of LIN-15B in relation to the other class B synthetic Multivulva genes; this could not be performed using standard genetic methods because the phenotype of a class B mutant alone is wild-type. Thus, the work in Chapter 3 lays the foundation for analyzing the other genes in this negative regulatory pathway, with the goal of understanding how this negative pathway functions and the identification of the component(s) of this pathway that directly interacts with LET-23.

This method of negative regulation of a receptor tyrosine kinase is likely not limited to *C. elegans*. Whether or not direct homologs of *lin-15* exist in other organisms, the activity represented by *lin-15* probably does. I believe this for several reasons. *let-23* is homologous to other receptor tyrosine kinases, showing 44%, 40.6%, 31.6%, 32%, 28.6%, and 32% identity at the amino acid level to the human EGF receptor, the *Drosophila torso* tyrosine kinase, the human cellular *src* product, the human cellular *abl* produce, the human insulin receptor, and the mouse PDGF receptor, respectively (Aroian, et al., 1990). Analysis of *let-23* mutant alleles demonstrate that some alleles have mutations in residues characteristic of tyrosine kinase receptors, likely reflecting a functional conservation of receptor activities (Aroian, et al., 1994). Furthermore, many components that interact with LET-23 are conserved (see Chapter 1 for details). The similarities of LET-23 to other receptor tyrosine kinases suggest that any activity seen in LET-23 is generalizable to other receptor tyrosine kinases. Thus, a basal level of activity of receptor tyrosine kinases is likely to exist in other systems. If this activity exists, it is most likely regulated; this

regulation would be the *lin-15*-like activity.

The study of *lin-15* will lead insights into this novel mechanism of receptor regulation, potentially providing information regarding the questions raised in Chapter 1 regarding the use of multiple signals to specify cell fate and the different methods for regulating signalling, as well as questions not yet formulated. The next century of inductive studies have only just begun.

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Appendix 1

Genetic dissection of developmental pathways

Linda S. Huang and Paul W. Sternberg

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A major tool of developmental geneticists is the ordering of genes in functional pathways. In this chapter, we explain the logic behind constructing pathways, starting from the knowledge of the relevant phenotypes associated with the genes of interest, assuming that careful analysis of the phenotype has been carried out. We discuss the construction and interpretation of phenotypes of double mutants, screening for and analysis of extragenic suppressors, as well as issues regarding complex pathways and genetic redundancy. Avery and Wasserman (1992) have provided a brief theoretical discussion of epistasis analysis; here we explain the more practical aspects of how models of developmental pathways are built in *C. elegans*.

EPISTASIS ANALYSIS

Epistasis is the masking of the phenotype of one mutant by the phenotype of a mutant in another locus. Hence, epistasis analysis can be used to determine a functional order of action of two genes, regardless of the directness of the interaction. However, epistasis analysis is most informative when the genes analyzed control a common process. Thus, it is important to determine the relationships between the mutations of interest before embarking on the construction of a formal genetic pathway.

Two different kinds of pathways exist (Figure 1). Historically, these have been distinguished as regulatory pathways and assembly or metabolic pathways. As both types of these pathways require "regulation," for the purpose of this chapter we will refer to the classical regulatory pathway as a

switch regulation pathway and the classical assembly pathway as a substrate dependent pathway. The switch regulation pathway involves genes or gene products that can be turned "on" or "off"; these different states of the genes (or their products) involved determine the outcome of the pathway. Mutations in genes involved in this type of pathway will have two distinct and opposite phenotypes, and will also have the ability to bypass the requirement for upstream genes. The second type of pathway involves a substrate, where an obligate series of sequential steps are required to generate the final outcome; classical examples of this second type of pathway include metabolic pathways and bacteriophage morphogenesis. Mutations in genes involved in the substrate dependent pathway will have phenotypes that suggest a progression of events. Sometimes determining which of the two types of pathways the genes of interest are involved in requires extensive phenotypic analysis of the mutations involved.

Consider for example, vulval differentiation in *C. elegans*. In wild-type animals, three of six equipotential vulval precursor cells differentiate to form the vulva. These six precursor cells are a subset of the 12 "Pn.p" cells (the posterior daughters of the twelve ectoblasts, P1 through P12). Although loss-of-function mutants in *lin-26* (*lin*=lineage abnormal), *lin-39*, and *let-23* (*let*=lethal) are all vulvaless and have the same phenotype when examined under a dissecting microscope, these genes are involved in very different aspects of vulval differentiation. *lin-26* is involved in the formation of the Pn.p cells, *lin-39* is involved in determining which of the Pn.p cells become potential vulval precursor cells, and *let-23* is involved in the fate decision regarding which Pn.p cells will actually differentiate into vulval tissue. Phenotypic analysis under higher magnification, Nomarski optics

demonstrates that the vulvaless phenotype of animals mutant in *lin-26* is due to the absence of Pn.p cells; in *lin-39* mutants, the vulval precursor cells are not generated; and in *let-23* mutants, the vulval precursor cells are not differentiating. In this example, the Pn.p cells are the substrate; *lin-26*, *lin-39*, and *let-23* act to change the state of this cell. A mutation in each of these genes represents a step in the pathway that provides the appropriate context or substrate for the next step. Here, arrows are used to show a progression of events from one step to the next (Figure 1A). Epistasis analysis of substrate dependent pathways will confirm the order of events seen by phenotypic analysis.

On the other hand, mutations involved in a switch regulation pathway should all have the same phenotype or the direct opposite phenotype (Figure 1B); in effect, the mutations will represent two states of an event, either ON or OFF. An example of this type of pathway in *C. elegans* involves the *tra-1* (*tra*=tra nsforming) and *her-1* (*her*=her maphroditization) genes, which are needed for proper sex determination (reviewed by Villeneuve and Meyer, 1990); these genes are part of a negative regulatory pathway and thus bars are used instead of arrowheads to symbolize the relationship between them. In *C. elegans,* the sex of the animals is determined by the number of X chromosomes in an animal; an XX animal is hermaphroditic while an XO animal is male. The *her-1* loss-of-function mutation causes feminization of XO animals without affecting XX animals. The *tra-1* loss-of-function mutation causes the opposite phenotype, masculinization of XX animals without affecting XO animals. These genes are involved in a switch regulation pathway because XO animals can be either one of two states, male or hermaphrodite; the same is true for XX animals.

The definition of a switch regulation pathway versus a substrate dependent pathway depends on the phenotype studied or the event assayed. In the study of the pathway containing the receptor tyrosine kinase encoded by *let-23*, its effects on the decision of the vulval precursor cells to take on vulval versus non-vulval fates constitutes a switch regulatory pathway. However, synthesis of the LET-23 receptor would constitute a substrate dependent pathway; one event would lead to another and the presence of LET-23 is required. Biochemical experiments do not necessarily define a substrate dependent pathway. Studies on how ligand activation of LET-23 leads to a phosphorylation cascade of downstream targets would be a switch regulation pathway; two distinct states are being analyzed, for example, phosphorylated and non-phosphorylated. Further, an obligate substrate is not needed in this pathway, as activation of a downstream target can bypass the need for LET-23 (e.g., a gain-of-function mutation in the *let-60 ras* gene will activate the pathway even in the absence of LET-23). It is imperative to distinguish whether the mutations of interest affect a switch regulation versus a substrate dependent pathway, as the logic used to order the genes involved is different and thus the resultant pathway constructed can be very different. The ordering of genes in a substrate dependent pathway can be determined through an examination of the state of the substrate (i.e., the state of the Pn.p cell given above); epistasis analysis serves to confirm what is inferred from the phenotypic analysis or to imply the sequence of events. In the next section of this chapter, we will consider the logic behind constructing a switch regulation pathway, where pure phenotypic analysis will not allow gene ordering.

EPISTASIS ANALYSIS OF SWITCH REGULATION PATHWAYS

Double mutant construction

Once it is determined that the mutations of interest might constitute a switch regulation pathway, double mutants can be constructed to determine the epistatic relationships between the two mutations. The *epistatic* mutation is the one whose phenotype is displayed in the doubly mutant animal; the mutation whose phenotype is not displayed is *hypostatic* to the other. Thus, an epistasis test can only be performed on two mutations in different loci and with opposite phenotypes; epistatic relationships cannot be determined using mutations with the same phenotypes. However, genes with mutations of the same phenotype can be ordered by epistasis analysis using a mutation in a gene that lies between the two and that causes the opposite phenotype.

We outline three basic strategies for constructing double mutants in Figure 2. The first method is the simplest (Figure 2A), as it involves taking animals carrying the single mutations and using them directly to make the double mutants. Epistasis is determined by the self-progeny of the F2 animals. If animals of phenotype A produce progeny of phenotype A and B while animals of phenotype B only produce progeny of phenotype B, gene B is epistatic to gene A. Gene A would be epistatic to gene B if the opposite were true. Furthermore, the animals of phenotype B from parents of phenotype A will be the desired double mutant; these animals can be cultured to maintain the doubly mutant strain. Although this method is the simplest, it cannot be used if gene A and gene B are tightly linked on the same chromosome.

The second method used for construction involves using a closely

linked marker in *cis* to (on the same chromosome as) each gene of interest (Figure 2B). The construction then involves making the strain carrying both marked chromosomes. The advantage of this method is that the strain can be constructed by simply following the mutations used as markers without regard to the phenotypes in interest. Once the doubly marked animal is obtained, the phenotype of interest can be determined. If the doubly marked animal has phenotype A, gene A is epistatic to gene B and *vice versa*. However, the control experiment must be done to construct double mutants with just the two markers, to make sure there is no effect on the phenotype of interest. Sometimes this method cannot be used because the markers will occlude scoring the phenotypes of interest.

The best method for determining epistatic relationships involves using linked markers in *trans* to (on the homologous chromosome as) the mutations of interest (Figure 2C). This will eliminate non-specific marker effects, while ensuring that the double mutation of the two genes of interest is actually being constructed. The markers should be close to the gene of interest to reduce the possibility of recombination. These markers can either be mutations in an unrelated gene or, better yet, balancer chromosomes (see chapter X by Edgley, et al., for an explanation of how to use balancers). This construction is similar to the first construction described, except that doubly mutant animals can be ascertained by the methods described above and also by their failure to segregate the *trans* markers.

A combination of methods can also be used. No matter which method is used to construct the double mutant, it is important to demonstrate that the presumptive doubly mutant animal actually carries both mutations of interest. One way that this can be done is by allowing males to mate with the

doubly mutant hermaphrodites and picking F1 heterozygous animals. Animals of both phenotype A and phenotype B should be produced by the heterozygous animals, demonstrating that both mutations were present in the putative doubly mutant animal. Also, F1 heterozygous males can be picked and outcrossed to test whether each carries the A and B mutation.

Interpretation of epistasis

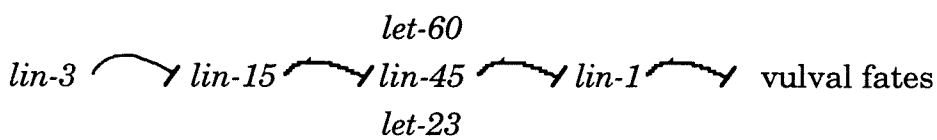
In a switch regulation pathway, the epistatic gene is the downstream gene because these genes are negatively regulating each other. This rule can be derived from analysis of the single and double mutant phenotypes, as illustrated in the epistasis of two genes involved in sex determination, *tra-1* and *her-1*. As mentioned above, the *tra-1* mutation transforms XX animals into males while the *her-1* mutation transforms XO animals into hermaphrodites. The *tra-1; her-1* double mutant has the *tra-1* phenotype; XX animals are males. *tra-1* is epistatic to *her-1* and thus downstream of *her-1*. *tra-1* cannot be upstream of *her-1* because the wild-type activity of *tra-1* is to activate the hermaphrodite program of development while the wild-type activity of *her-1* is to activate the male program of development. If *tra-1* were upstream of *her-1*, inactivation of *tra-1* would eliminate the repression of *her-1*, activating the male program of development. Inactivation of *her-1* would lead to the inability to activate the male program of development, resulting in the hermaphrodite program of development. Removal of both *tra-1* and *her-1* would make *tra-1* unable to repress *her-1* (which does not matter because *her-1* is inactivated by mutation) and *her-1* unable to activate the male program of development. This would result in the double mutant exhibiting the *her-1* phenotype. However, the experimental evidence says

this is not true, so *tra-1* is downstream of *her-1*; that is, inactivation of *tra-1* obviates the need for *her-1.*

Comprehensive double mutant analysis with genes involved in a common process allows the construction of a pathway (see Figure 3). For example, during vulval induction, three of six vulval precursor cells (VPCs) normally adopt vulval fates while the other three adopt non-vulval hypodermal fates. By removing the inductive signal through ablation of the signalling cell, the three VPCs that normally become vulva now adopt non-vulval epidermal fates. Mutations that mimic the signalling-cell ablation phenotype (all six VPCs adopt non-vulval hypodermal fates, a.k.a. vulvaless (Vul) mutations) include *let-23, lin-3, let-60,* and *lin-45.* Mutations also exist that have the opposite phenotype (all six VPCs adopt vulval fates, a.k.a. multivulva (Muv) mutations); these include *lin-15* and *lin-1*. Double mutation construction using Muv and Vul mutations yield the following results:

lin-1 is epistatic to *let-23*	*let-23* is epistatic to *lin-15*
lin-1 is epistatic to *lin-3*	*lin-15* is epistatic to *lin-3*
lin-1 is epistatic to *let-60*	*let-60* is epistatic to *lin-15*
lin-1 is epistatic to *lin-45*	*lin-45* is epistatic to *lin-15*

Since two states are being assayed, the ability of the VPCs to adopt vulval fates versus their ability to adopt non-vulval fates, the following pathway can be constructed, using the rule of the epistatic gene being the downstream gene:



The importance of using null alleles

The examples of epistasis given above result from double mutant analyses using severe loss-of-function recessive alleles. Epistasis analysis is based upon the assumption that the mutations in the genes involved remove the function of that gene in the cell, tissue, or process being analyzed. Thus, it is important to determine by dosage analysis whether the alleles involved are hypermorphs (increased-function), hypomorphs (reduction-of-function), amorphs ("null;" complete loss-of-function) or neomorphs (novel function) [see Muller (1932) for details; see Sternberg (1990) and Greenwald & Horvitz (1980) for a discussion regarding the determination of a gene's null phenotype].

Null mutations are important for epistasis because the logic behind epistasis analysis is only valid if the two mutations used can be presumed to have no assayable activity. For example, consider two genes, A and B, in a regulatory pathway where there exists a null mutation in gene A but there exists no null mutations in gene B, only a hypomorphic mutation. If A is epistatic to B, this would not be as critical, since the normal function of B is to negatively regulate A. In this case, A is downstream of B. So, even if B has residual activity, the double mutant animals do not have functional A activity so the residual B activity cannot regulate A activity.

On the other hand, if B is downstream of A, the double mutant animal may display a misleading phenotype because the B mutation used contains residual activity and the doubly mutant animal has no A activity to regulate this residual activity. Since B and A have opposite phenotypes, having some wild-type B activity will lead to some expression of the A mutant phenotype, depending on how much activity the hypomorphic B mutation retains.

Therefore, the outcome of an epistasis experiment where one or both of the mutations used are not null alleles could be co-expression, where both A and B phenotypes are expressed, or, a misleading double mutant phenotype, where the animal displays the A phenotype.

A practical example of this phenomenon can be found in Ferguson et al. (1987). Epistasis analysis using the *lin-15* allele, *n309*, and the *let-23* allele, *n1045*, resulted in the *let-23(n1045); lin-15(n309)* doubly mutant animals expressing either the Muv or the Vul phenotype. It is now known that *let-23(n1045)* makes some wild-type product (Aroian et al., 1994) and that *lin-15(n309)* is not a complete deletion of the locus (Huang et al., 1994; Clark et al., 1994). It is not possible to use a complete null allele of *let-23* to analyze its phenotype in late larval development (without mosaic analysis) because, as the name implies, a *let-23* null is lethal and would not survive to make the vulva. However, the *sy97* allele of *let-23* is a severe loss of function allele with respect to the vulva, such that vulval differentiation is essentially not seen (see Aroian and Sternberg (1991) for an example of tissue-specific activities of a genetic locus). Molecular analysis also identified a *lin-15* allele, *e1763*, whose lesion removes more coding sequence than *n309*. When epistasis analysis is performed using *let-23(sy97)* and *lin-15(e1763)*, all the doubly mutant animals display a Vul phenotype (Huang et al., 1994). Of course, the *let-23(sy97)* allele did not exist in 1987 and the molecular nature of the *lin-15* alleles was not known, so the epistasis was performed using the best of what existed and was known at the time.

Use of dominant mutations

As mentioned above, epistasis analysis cannot be performed using

mutations which display the same phenotype. This can lead to subsets of the pathway where the order of some genes is not known. Sometimes these genes can be ordered through the use of gain-of-function alleles, which will display the opposite phenotype of the loss-of-function mutations. In these cases, it is important to know that the gain-of-function mutation in the gene of interest increases the normal activity of the gene and does not confer a novel activity.

In the example of the pathway regulating vulval differentiation given above, the genes *let-60, let-23,* and *lin-45* cannot be separated by epistasis using hypomorphic alleles, as all display the Vul phenotype. However, there exists a gain-of-function allele of *let-60*; animals carrying this allele display the Muv phenotype. Double mutant construction using the *let-60* gain-of-function allele and the *lin-45* and *let-23* hypomorphic alleles gives the following results:

genotype	phenotype
lin-45; let-60(gf)	Vul
let-23; let-60(gf)	Muv

Therefore, the *let-60* gain-of-function allele can be used to split the order of action of *let-23* and *lin-45*. These results imply the order:

let-23 ⟶ *let-60* ⟶ *lin-45*

Arrows are used because the wild-type function of the genes is to positively regulate each other. The gain-of-function phenotype of *let-60* results in the opposite phenotype of hypomorphic *let-60* alleles and allowed the ordering of these genes using the logic used for epistasis for switch regulation pathways.

Complex pathways

Epistasis analysis does not always result in simple interpretations of

gene order. Sometimes this is because the mutations used are not null alleles; the consequences of this are discussed above. Sometimes this is because the mutations used are not part of a switch regulation pathway but instead, part of a complex pathway that combines both switch regulation processes and substrate dependent processes. In fact, complex pathways are more often the rule than the exception. Development proceeds through a series of steps; often, a set of decisions about a fate leads to an execution of a particular fate, which may then lead to decisions about another fate. Development can be viewed as a set of substrate dependent pathways leading to switch regulation pathways which lead to more substrate dependent pathways, and so on.

Because the logic of epistasis differs for switch regulation and substrate dependent pathways, it is important to determine if the mutations involved in a complex pathway are all involved in the same step of the pathway being studied. This is not always obvious and may require careful phenotypic analysis. For example, animals carrying the mutation *n300* are Vul when examined under a dissecting microscope. Because animals carrying the *lin-15* mutation are Muv, superficially, Muv and Vul are opposite phenotypes, the epistasis analysis of these two genes seems simple. The doubly mutant animal of genotype *n300; lin-15* is Vul (Ferguson, et al., 1987); the simple interpretation is that *n300* is downstream of *lin-15*. However, this is not the case. Careful examination of the *n300* phenotype reveals its involvement in the generation of the VPCs; *n300* animals are Vul because the VPCs are not there and thus cannot make a vulva. The correct interpretation is *n300* is upstream of *lin-15*, operating in the pathway required to generate the VPCs and not in the regulation of VPC fate, as

lin-15 does.

The process of programmed cell death is an excellent example of a complex pathway (reviewed in Ellis, et al., 1991a, see Figure 4). In *C. elegans*, 131 of the 1030 cells generated during development of the adult hermaphrodite undergo programmed cell death. Programmed cell death is a complex process, first involving the specification of whether or not a cell will die, followed by the process of killing the cell, and finishing with the engulfment and degradation of cell corpses. Mutations have been found in genes affecting each of these processes. For example, the genes *ced-3* (*ced*=cell death abnormal), *ced-4*, and *ced-9* are involved in the killing process (Ellis, et al., 1991b; Hengartner and Horvitz, 1992). Genes involved in the engulfment of cell corpses include *ced-1*, *ced-2*, *ced-5*, *ced-6*, *ced-7*, *ced-8*, and *ced-10*; mutations in these genes causes cell corpses to persist. The process of cell death thus consists of:

specification of death → killing of cells → engulfment of cell corpses

Epistasis can only be interpreted using the logic discussed for switch regulation pathways for genes involved in a common process; *ced-3* and *ced-1* are involved in different processes and thus phenotypic analysis will elucidate their order relative to each other.

ced-3, *ced-4*, and *ced-9* are involved in regulating the killing of cells. In animals without *ced-3* or *ced-4* function, cells survive that normally die by programmed cell death. Animals without *ced-9* function die. When *ced-9* mutant animals also lack either *ced-3* or *ced-4*, they live, suggesting that functional *ced-3* or *ced-4* is necessary for the *ced-9* gene product to function. This interpretation is consistent with the gain-of-function phenotype of *ced-9*, which results in the survival of cells that normally die by programmed cell

death (this is the same phenotype as loss-of-function of *ced-3* and *ced-4*). These results imply the gene order of:

ced-9 ⊣ *ced-3, ced-4*

When double mutants are made using mutations in *ced-3* and *ced-1*, a gene involved in the engulfment of cell corpses, the doubly mutant animal displays the *ced-3* phenotype. However, *ced-1* and *ced-3* are part of the same substrate dependent pathway but not part of the same switch regulation pathway. For one, *ced-3* and *ced-1* do not have the opposite phenotype from one another. Phenotypic analysis would suggest that the cell must first be specified to die by *ced-3* before its corpse can be engulfed through the activity of *ced-1*. Also, the engulfment pathway that *ced-1* regulates is not necessary for causing cell death like *ced-3*, because mutations that prevent engulfment do not prevent most cell deaths (Hedgecock, et al, 1983, Ellis, et al, 1991a). The action of *ced-3* leads to the action of *ced-1*. Thus, the pathway should be drawn as:

specification of death → *ced-9* ⊣ *ced-3, ced-4* → *ced-1*

These examples regarding *n300* and *lin-15* in the vulval differentiation pathway and the genes in the programmed cell death pathway illustrate the importance of detailed phenotypic characterization of the singly mutant animals in preventing misinterpretation of double mutant phenotypes. Once it is determined that two genes are in a switch regulation pathway, the rule that the epistatic gene resides downstream of the hypostatic gene can be simply applied. In contrast, if two genes are involved in a substrate dependent pathway, double mutant analysis again confirms the order of events that have been visualized, although in this case, the epistatic gene is the upstream gene. Whether or not two genes are involved in a switch

regulation process or a substrate dependent process can only be determined by careful analysis of the phenotypes of the mutations involved.

Genetic redundancy

Another factor that can complicate the interpretation of epistatic relationships is genetic redundancy. Discussions on the theoretical implications of genetic redundancy can be found in Thomas (1993) and Tautz (1992); these issues are beyond the scope of this chapter. In practice, genetic redundancy can be discovered in three ways, by backcrossing newly isolated mutants of interest, by undertaking a screen for redundant genes, or by constructing double mutants with two different genes (see Figure 5).

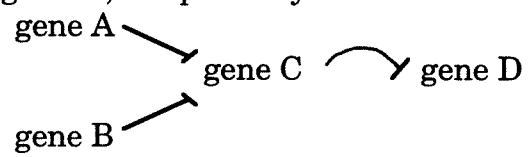
Discovering genetic redundancy by backcross involves examination of the frequency of segregation of the mutation involved from a heterozygous animal (Figure 5A). This method is most often used during the analysis of a new mutation following mutagenesis (for example, see Ferguson and Horvitz, 1989). A recessive phenotype caused by a single gene trait will usually segregate from a heterozygote animal at a frequency of 1/4. However, if two genes are required to cause the phenotype, animals expressing this phenotype will segregate from a heterozygous parent at a frequency of 1/16, assuming they are unlinked from each other. Once it is determined that two genes cause the phenotype of interest, the phenotype of each mutation alone should be determined. If these two mutations are genetically redundant, they will only cause the phenotype of interest in combination with each other and should display either a reduced phenotype alone or possibly even no phenotype.

Another method for discovering genetic redundancy involves

constructing double mutant animals using existing mutations (Figure 5B). This is usually done when mutations are recovered that display an incomplete phenotype or that display no phenotype with respect to the phenotype of interest (i.e., a silent mutation). This method was extremely informative in the discovery of the two sets of genes involved in the engulfment process during programmed cell death (Ellis, et al., 1991a) and the redundancy seen in the dauer formation pathway (Thomas, et al., 1993).

A third method for discovering genetic redundancy involves screening for new mutations that synergize with the original mutation to cause the phenotype of interest (Figure 5C, also see Ferguson and Horvitz, 1989). This is an especially useful technique if it is already known that redundancy exists in the system, through molecular information or by the discovery of genetic redundancy through other methods, as described above.

The formal interpretation of genetic redundancy is that the redundant genes are involved in parallel pathways, regulating the process of interest. For two redundant genes, A and B, that negatively regulate gene C that negatively regulates gene D, the pathway would look like:



In this case, the animal carrying mutations in both gene A and gene B would display the opposite phenotype as animals mutant in gene C and would display the same phenotype as animals mutant in gene D. Thus, when epistasis analysis is done with genetically redundant genes, both redundant genes should be eliminated because only then is the phenotype of interest seen. In this case, since gene A only partially negatively regulates gene C, even doing epistasis with a null allele of gene A will not completely eliminate

the function of that particular step; both gene A and gene B function need to be completely eliminated to create the phenotype that represents a lack of function at that step. Thus, the triply mutant animal lacking gene A, gene B, and gene C must be constructed for epistasis analysis.

Limits of epistasis

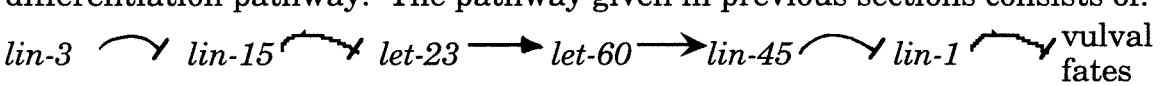
The ordering of genes in pathways using genetic epistasis is a powerful tool; however, it merely provides a working model for more phenotypic studies or molecular analysis and should not be taken as the "answer." One limitation of epistasis is that it assumes a linearity of events. This is not always the case; sometimes pathways are branched or contain multiple inputs. Also, some pathways use tissue specific regulators that are not necessarily used in other developmental processes that use a subset of these same genes. Ultimately, these issues can be resolved by careful phenotypic analysis of the genes involved in the pathway, although where to look may not be initially obvious.

One example of a branched pathway in *C. elegans* is the sex determination pathway (see Figure 6). A high X chromosome to autosome ratio (X:A) negatively regulates the *fem-1, 2,* and *3* genes, which negatively regulate the *tra-1* gene. *tra-1* functions to activate the developmental program for the female soma while repressing the developmental program for the male soma. However, the role of the *fem-1, 2,* and *3* gene products is not just to regulate *tra-1*; these genes are also responsible for promoting male germline development and negatively regulating female germline development. In the case of a high X:A ratio, inactive *fem-1, 2,* and *3* would lead to female germline development. By recognizing the branch point at

fem-1, 2, and *3* and realizing that germline development is regulated differently than somatic development, the intersex phenotype of animals carrying the *tra-1* mutation can be interpreted. XX *tra-1* mutant animals have a male soma because they have no functional *tra-1* to repress male somatic development; these XX animals also have a female germline because *fem-1, 2,* and *3* are repressed by the high X:A ratio.

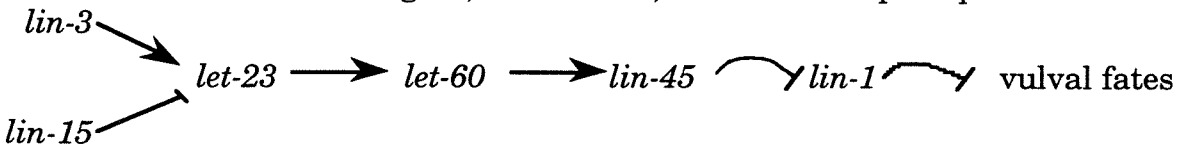
This example also illustrates the existence of tissue specific regulators of a particular pathway. Although *tra-1* is downstream of *fem-1* in somatic development, it is not so in germline development. Thus, the order of gene action should be determined by epistasis analysis with the genes involved for the particular developmental process of interest, looking at their action in the tissue of interest. Another example of this is the use of the heterochronic genes *lin-4*, *lin-14*, *lin-28*, and *lin-29* (Ambros and Moss, 1994). Animals carrying loss-of-function mutations in *lin-4* and *lin-14* have widespread defects in temporal control. By examining their effects in the temporal control of the hypodermal seam cells, a regulatory pathway was constructed with the more specifically used heterochronic genes, *lin-28* and *lin-29*. A complete description of the construction of this pathway is found in Ambros, 1989; this paper also contains an excellent explanation of how the logic of epistasis analysis was used to construct the pathway.

Another variation on branched pathways are pathways with more than one input (Figure 7). An example of this is the *C. elegans* vulval differentiation pathway. The pathway given in previous sections consists of:



However, the molecular identification of the *lin-3* gene product as a growth factor/ligand-like molecule and the *let-23* gene product as a receptor tyrosine

kinase suggests that *lin-3* encodes the ligand for *let-23* (reviewed by Sternberg, 1993). Furthermore, previous phenotypic characterization of *lin-15* demonstrated that although *lin-15* mutant animals exhibit the mutant Muv phenotype even in the absence of the inductive tissue, their VPCs are still capable of responding to the signal. Thus *lin-15* does not belong directly downstream of the *lin-3* signal, but instead, as a second input upon *let-23*:



The examples of the vulval differentiation pathway and the sex-determination pathway given above underscore the importance of careful phenotypic analysis in the construction of developmental regulatory pathways. This is not to say that epistasis analysis should not be performed until an exhaustive understanding of the mutant phenotype exists; however, pathways constructed through epistasis analysis should be considered models which can change over time, given new information from further phenotypic or molecular analysis.

Extension to other perturbations

The logic presented in this chapter can be extended to other non-genetic interactions, such as cell-cell interactions that can be dissected using cell ablations. Rather than remove the function of a gene by mutation, a cell can be removed by laser ablation (see chapter by Avery & Bargmann) or by a pharmacological agent (see chapter by Johnson & Rand). One example of using cell ablations instead of mutation for epistasis is the analysis of cell signalling during male spicule development (Chamberlin and Sternberg, 1993). Bγ and Bδ are two different cell fates adopted by certain progeny of the

B cell, a male specific blast cell involved in spicule development. Ablation of the nearby Y.p cell (the posterior daughter of the male specific blast cell, Y) leads to a defect in the Bδ lineage; ablation of the **pa** cells (the two cells, B.alpa and B.arpa, great-great granddaughters of the blast cell, B; sisters of Bγ and Bδ) leads to a defect in Bγ. As Bγ and Bδ are alternative cell fates, the ablation of the Y.p cell can be considered to cause the opposite phenotypes from that of the ablation of the **pa** cells. The double ablation of both Y.p and the **pa** cells has a defect similar to ablation of the Y.p cell alone. Using the logic of epistasis, the role of the **pa** cells might be to negatively regulate the effect of Y.p.

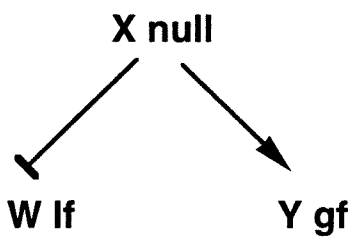
EXTRAGENIC SUPPRESSORS

To further analyze a pathway, it is often desirable to identify additional genes. One method for doing this involves starting with a wild-type animal and screening for additional mutations with the phenotypes of interest. Another method involves isolating extragenic suppressor mutations of a previously identified mutant gene in the pathway (see chapter by P. Anderson for information regarding the different mutagens available). Suppression screens can be better than direct screens because the presence of the starting mutation might suppress the lethality of the suppressor mutation, and thus allow the mutation to be recovered. Screens for mutations that enhance a partial mutant phenotype can also identify new genes.

Screens for extragenic suppressors

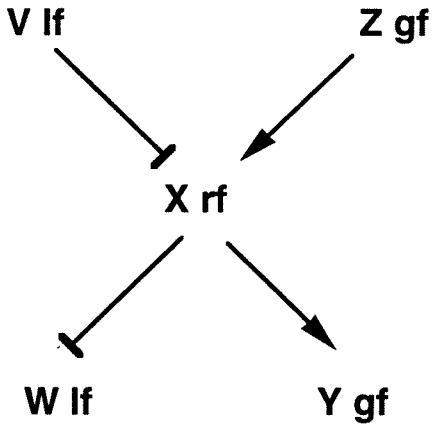
Screens for extragenic suppressors seem deceptively simple; a mutation in the pathway is chosen and a screen is performed to look for new mutations which no longer express the mutant phenotype, either in the F1 generation (for dominant suppressors) or in the F2 generation (for recessive suppressors) (see Figure 8). Although the screening process is straightforward, the key to the success of an extragenic suppressor screen involves choosing the most appropriate starting mutation for the purpose of the screen, as the choice of a starting strain affects the suppressor mutations isolated.

One consideration when choosing a starting mutation is to decide what type of mutation to suppress: a null mutation, a loss-of-function mutation, or a gain-of-function mutation. Suppression of a null mutation (X) will allow recovery of new mutations that allow bypass of the gene of interest. These will either be gain-of-function mutations of downstream genes in the same substrate dependent pathway (Y) or loss-of-function mutations of downstream negative regulators (W). However, suppression of a null mutation will not allow recovery of mutations in genes that directly interact with the starting gene's product.

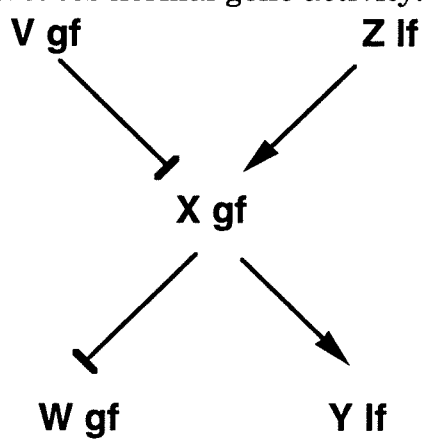


On the other hand, suppression of a reduction-of-function mutation (X) will allow recovery of mutations in genes that interact with the gene of interest. A mutation in an interacting gene will be recovered if it removes the negative regulation of an upstream interacting gene (V). Mutations that

allow bypass of the gene of interest will also be recovered (W and Y), as with using the null. Upstream genes that, when mutated, increase the activity of the loss-of-function mutation used will also be recovered (Z).



Suppression of a gain-of-function mutation can allow the subsequent recovery of the opposite spectra of alleles as suppression of a loss-of-function mutation. For example, loss-of-function mutations of downstream genes will be recovered (Y) as well as gain-of-function mutations of downstream negativly regulated genes (W). These screens can be very powerful (for example, see Wu and Han (1994) and Lackner et al. (1994) regarding suppression of the gain-of-function *let-60 ras* mutations; Riddle (1977) for suppression of dauer defective mutants; and Hodgkin (1986) for suppression of sex determination mutations); however, it is important to know that the starting mutation (X) increases normal gene activity.



Besides choosing a starting mutation for a suppression screen based on the nature of the mutation, the more information known about a particular mutation, the better the choice that can be made. For example, screens using an amber suppressible allele will not only yield suppressors of interest, but can also yield mutations in amber suppressor tRNAs. If the suppressors of interest occur at a very low frequency, the other more non-specific mutations may overwhelm the recovery of the desired mutations. Similarly, screens using alleles which are splice site mutations may yield mutations in splicing components as well as the suppressors of interest. Of course, it is not always possible to begin with knowledge of the exact defect of the mutation of interest; nonetheless one can test by construction whether a starting mutation is suppressed by known informational suppressors. e.g., *sup-7* and *smg-1* (see below for further details).

Analysis and interpretation of suppressors

Analysis of suppressor mutations involves separating the suppressors of interest from intragenic revertants and informational suppressors, testing dominance, checking for phenotypes in an otherwise wild-type background, mapping the suppressor locus, and determining the spectrum of suppression. When a suppressor mutation is isolated, its only known phenotype is that it suppresses the original mutation. Thus, no assumptions can be made about the phenotype of the suppressor on its own; it could have an unexpected phenotype, be lethal, or be silent. Because of this, strains for the analysis of suppressor mutations must carry the starting mutation to allow scoring of the suppressor phenotype. In essence, the strain with the starting mutation is the background strain for further analysis. Besides this, analysis of

suppressor mutations proceeds as for any other mutation. For example, to establish linkage with a recessive suppressor mutation, *mut/mut; sup/sup* animals can be crossed to *mut; marker* strains to construct a *mut/mut; marker/+; sup/+* heterozygote, and linkage of *sup* and *marker* observed among the progeny (*mut*=starting mutation, *sup*=suppressor mutation, *marker*=any unrelated marker) (Figure 9A). Dominance can be tested by crossing *mut; sup* males to *mut; marker* hermaphrodites and examining the phenotype of the non-marker cross-progeny (Figure 9B). Similarly, complementation of recessive suppressor mutations can be tested by crossing *sup-1; mut* males to *sup-2; mut; marker* hermaphrodites.

Intragenic revertants will be linked; extragenic linked suppressors can be separated by recombination. For analysis of intragenic revertants see Greenwald and Horvitz (1980). Such revertants can be informative with respect to the structure, function or expression of a particular gene, but are not helpful in defining a pathway.

It is important to determine the spectrum of suppression of a suppressor mutation: Does it suppress other alleles of the starting gene? If not, it might suggest informational suppression (see below) or a protein-protein interaction, depending on the nature of the alleles. Does it suppress hypomorphic or null alleles? If it only suppresses hypomorphic alleles, the suppressor might act upstream in a pathway or act in branch of the pathway. Does it suppress other phenotypes of the starting mutation? This would tell you if it acts in all pathways in which your starting mutation functions. Does it suppress mutations in other genes in the pathway, and if so, does it only suppress mutations that lie upstream? Not only can these types of data reveal whether a suppressor is informational, but they also indicate the

specificity of the suppressor gene defined. Whether specific or generally acting suppressors are of greater interest is a matter of personal taste.

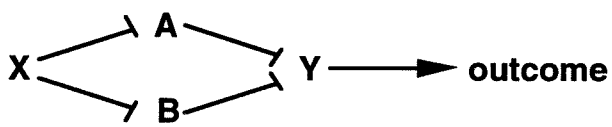
One class of extragenic suppressors are informational suppressors (reviewed by Hodgkin et al., 1987). Formally, informational suppressors are allele-specific, gene non-specific, and thus act on particular types of mutations, rather than particular types of gene products. The classic informational suppressor is an amber suppressor, e.g., *sup-7* in *C. elegans*. A new class of informational suppressors, the *smg* genes, were found by reversion of three independent phenotypes; the *smg* genes affect mRNA stability (Pulak and Anderson, 1993). The informational nature of their suppression was discovered by the realization that three different genetic screens for unrelated pathways identified the same set of suppressor mutations (Hodgkin et al., 1989).

Silent suppressors

Once the map position of a suppressor mutation is determined, markers can be used in *trans* to separate the suppressor from the original mutation so that the phenotype of the suppressor alone can be determined. The simplest suppressor mutations to interpret are those that confer a phenotype opposite to the starting mutation, with suppression by virtue of epistasis [e.g., suppression of *lin-15* multivulva by *let-60* vulvaless (Han, et al., 1990); suppression of *tra-3* masculinization by *fem-1*, *fem-2* or *fem-3* feminization (Hodgkin, 1986); suppression of dauer constitutive by dauer defective (Riddle, et al., 1981)]. However, many suppressor mutations have an unrelated phenotype or no phenotype other than suppression of the starting mutation; these are silent suppressors as they have no effect with

respect to the phenotype of interest. There are various types of experiments one can carry out but no generalizable formula is available to determine what will be the most informative types of silent suppressor mutations. Note that silent suppressors can identify genes that would otherwise not have been identified.

Some suppressor mutations enhance other mutations that have the opposite phenotype to the starting mutation, suggesting that they indeed control activity of the pathway of interest. Some suppressor mutations have synthetic phenotypes, i.e., the double mutant animal carrying two suppressor mutations display a phenotype opposite to the starting mutation; this is due to the redundant action of the genes defined by the suppressor mutations. The synthetic phenotype can then be used in epistasis tests. If there is clear epistasis of the suppressor double mutant with mutations in the pathway, the redundant suppressors most likely act in a parallel subset of the otherwise linear pathway, with redundant genes A and B defined by suppressors of X (see Thomas, et al., 1993 for an example). This pathway would look like:



If suppressor mutations suppress hypomorphic alleles but not null alleles, then they are more likely to be a side branch of the main pathway. These mutations are in a side branch because they are unable to bypass the null allele, and thus do not define a downstream component regulated by an upstream gene (see Lee et al., 1994 for an example). Silent suppressors that do not show synthetic phenotypes cannot be placed in a pathway using epistasis, but the spectrum of suppression of mutations in the pathway of interest can suggest their role.

PROSPECTS: use of new technologies for pathway analysis

Transgenes add to the arsenal of tools for pathway analysis: they can be used for suppression and epistasis. For example high copy number of *lin-3* provided by a transgene results in a multivulva phenotype; this phenotype is hypostatic to the *let-23* vulvaless phenotype, consistent with *lin-3* acting upstream of *let-23* (Hill and Sternberg, 1992). A powerful technique in the future will be reversion of the dominant effects of transgenes. One potential problem may be a high degree of intragenic revertants, that is, deletions of the integrated transgenes. In one case, this did not happen; rather reversion of integrated *lin-3* transgene using ethylmethylsulfonate (EMS) as the mutagen yielded suppressor mutations in expected genes such as *let-23* but not deletions of the transgene (J. Liu and P. Sternberg, unpublished observations).

In *Saccharomyces cerevisiae*, high copy suppression using plasmid libraries is a powerful tool for finding interacting or related genes. In *C. elegans*, similar analysis will be more difficult, but still possible. Due to the larger genome size compared to yeast, it would require testing on the order of 500 transgenic lines, each with multiple copies of five cosmids (assuming good representation of the genome with 2500 cosmids). The ability to create mutations in genes of interest will be a powerful tool. As genetically defined genes are cloned and homologies are seen, other molecularly defined components which may lie in the pathway of interest can be cloned through reverse genetics and engineered into a genetic mutation (see chapter by Plasterk for further details) for further analysis. Finally, from a geneticist's perspective, the genome project makes it more attractive to pursue the

characterization of silent suppressor mutations because the molecular cloning will be increasingly facile.

CONCLUSION

The determination of the functional order of genes in a developmental pathway provides a working model for the design of additional experiments. New genes can be added to the pathway once they are found, either at a branchpoint or directly in the pathway. The gene order can be refined upon receipt of more information about the phenotype of the mutations involved, the isolation of new mutations that allow more epistasis analysis to be performed, as well as more information about the molecular and biochemical nature of the pathway of interest. Eventually, individual pathways will be connected to other pathways, evolving into a network of gene interactions for developmental processes.

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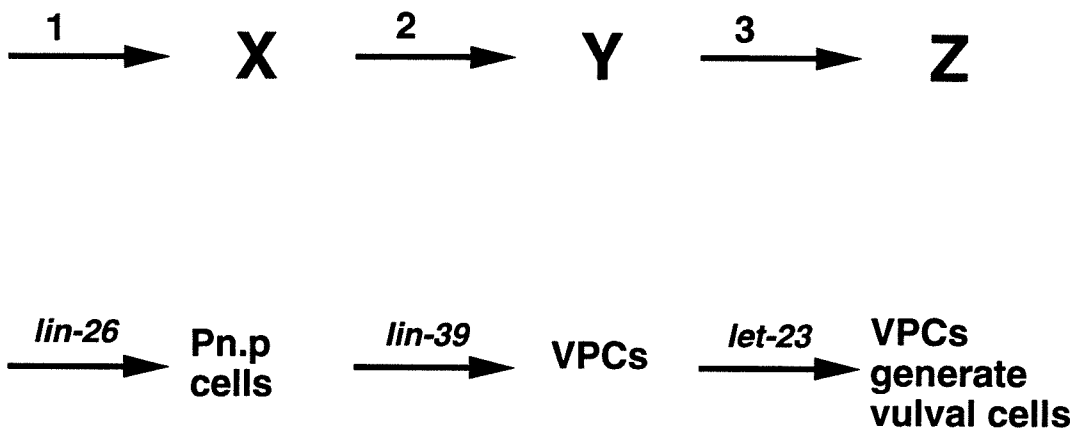
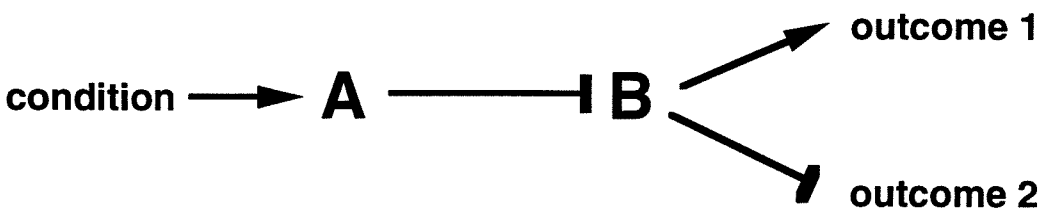
Figure 1. General types of pathways. A. Substrate Dependent pathway: vulval development. A series of genes (1, 2 and 3) are necessary to produce a series of outcomes (X, Y and Z). *lin-26* is necessary for the development of Pn.p cells. *lin-39* is necessary to select a subset of Pn.p cells to become vulval precursor cells (VPCs). *let-23* is necessary for the VPCs to generate vulval cells. **B. Switch Regulation pathway: somatic sex determination.** A formal regulatory pathway is shown with conditions regulating which of two outcomes (1 or 2) will occur. If the condition is met (e.g., presence of a signal, particular environmental conditions; chromosome constitution), outcome 1 occurs. A specific example is shown at the bottom. High ratio of X chromosomes to autosome sets results in the activation of *her-1* via a series of other genes. *her-1* activity inactivates *tra-1* via a series of other genes. *tra-1* activity results in female somatic differention; *tra-1* inactivity leads to male somatic differentiation. *lf*, loss-of-function mutation; arrows represent positive regulation; bars represent negative regulation.

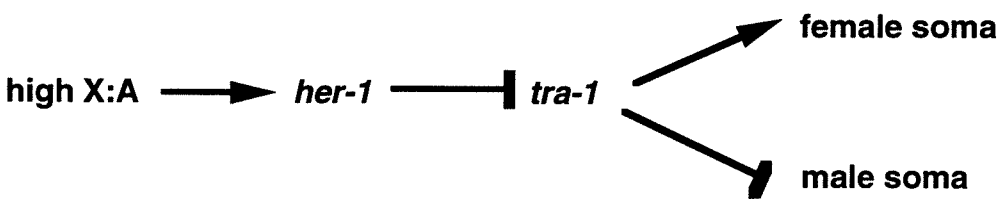
A Substrate Dependent Pathway



B Switch Regulation Pathway



Yes	ON	OFF	outcome 2
No	OFF	ON	outcome 1



her-1(lf)	female soma
tra-1(lf)	male soma

Figure 2. Construction of double mutant strains.

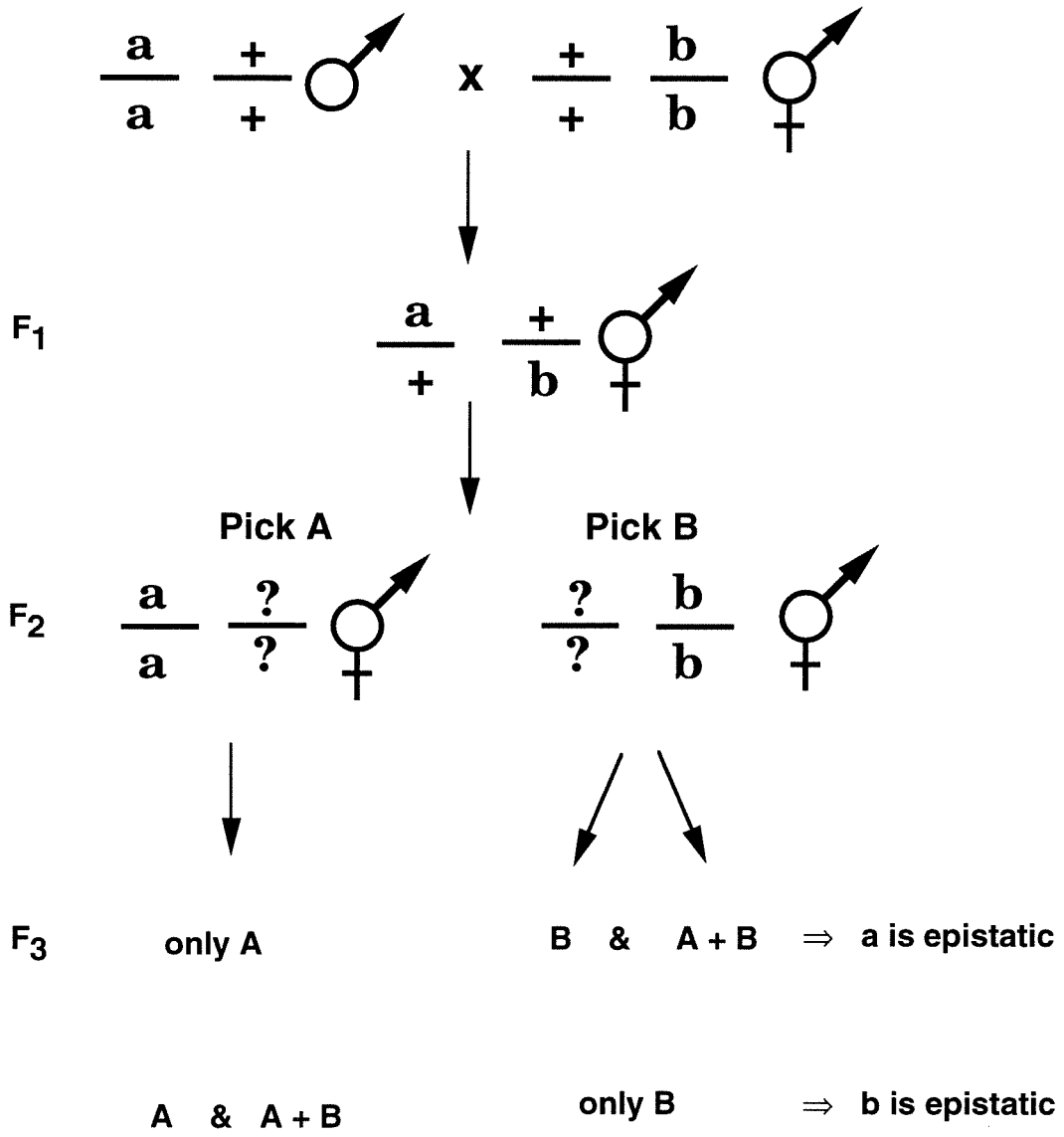
A. No markers. Animals carrying mutations in one gene of interest (gene A) with phenotype A are mated with animals carrying mutations in the other gene of interest (gene B) with phenotype B. The F1 heterozygote (gene a/+; gene b/+) will produce progeny of both phenotype A and phenotype B. Next, F2 animals of phenotype A and phenotype B are individually cultured and their F3 progeny examined to determine whether animals of phenotype A produce progeny of phenotype B or *vice versa*. By analyzing both classes, one can ensure that the desired strain will be constructed, even if one's assumption about the double mutant phenotype is incorrect. "?" indicates the allele at a given locus in a particular animal is not inferable from its phenotype or the genotype of its parents.

B. Linked markers in *cis*. The second method used for epistasis analysis involves using linked markers in *cis* to (on the same chromosome as) the genes of interest. The markers linked to the two genes should be different, closely linked to the gene of interest to avoid recombinants, not have any epistatic relationships to each other, not have any interactions with the genes of interest, and be easily scored as a double mutant. Males carrying mutation *b* with linked marker *n* are mated to hermaphrodites homozygous for mutation *a* and linked recessive marker *m*. Cross-progeny are non-M and heterozygous for *a* and b. Cross-progeny that do not produce N progeny are discarded, as they did not receive the *b n* chromosome from their father. From this heterozygote, hermaphrodites with phenotype M or N are picked, and the double mutant M N (also homozygous for *a* and *b*) is easily recognized.

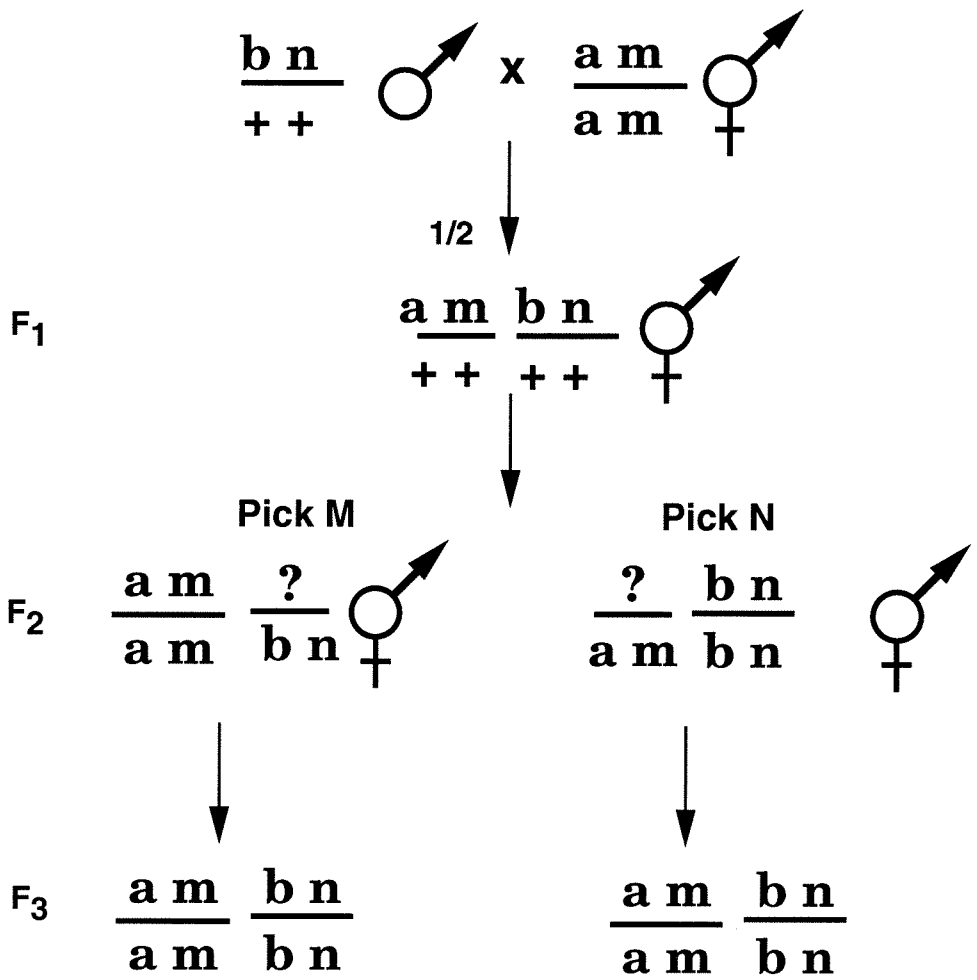
C. Linked markers in *trans*. The best method for determining

epistatic relationships involves using markers (m, n) linked in *trans* to (on the homologous chromosome as) the genes of interest. This will eliminate non-specific marker effects while ensuring that the double mutation of the two genes of interest is actually being constructed. Once again, the markers should be fairly close to the gene of interest to reduce the possibility of recombination. This construction is similar to that in part A, except that doubly mutant animals can be ascertained by the method described above and also by their failure to produce progeny carrying the markers linked in *trans*.

A

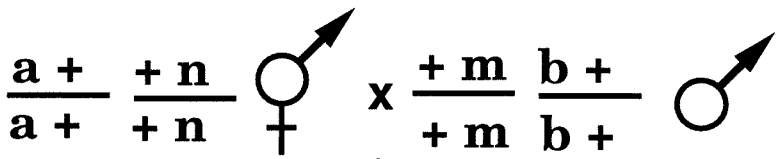

a/a + /+ x + /+ b/b
F1
a/+ +/b
Pick A
Pick B
F2
a/a ?/? ?/? b/b
F3
only A
B & A + B ⇒ a is epistatic
A & A + B
only B ⇒ b is epistatic

B



Phenotype? B ⇒ **b is epistatic**
A ⇒ **a is epistatic**

C



F_1 $\frac{a+}{+m}$ $\frac{b+}{+n}$ ♀

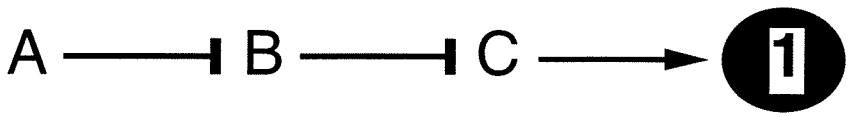
Pick non-M non-N

F_2 $\frac{a+}{+m}$ $\frac{b+}{b+}$ $\frac{a+}{a+}$ $\frac{b+}{b+}$ $\frac{a+}{+m}$ $\frac{b+}{+n}$ $\frac{a+}{a+}$ $\frac{b+}{+n}$

F_3 M | no M, no N, Phenotype? | M, N | N

B ⇒ **b is epistatic**
A ⇒ **a is epistatic**

Figure 3. Epistasis analysis. The phenotypes of a set of genotypes and the inferences are shown. The inferred pathway leading to the outcome 1 (black oval; •) is shown at the top. The default outcome is 2. Phenotypes are described in two ways in this figure: as transformations from 1 to 2 or 2 to 1, or as missing and extra structures, states or parameters. The inference from the phenotype of each genotype is shown. For example, in (ii), the absence of A leads to no 1; thus A promotes •. However, in (iii), the absence of B leads to extra 1; thus B inhibits •.



	Genotype	Phenotype: transformation	Phenotype: duplication/ deletion	Inferred Pathway
i	$\frac{+}{+}\ \frac{+}{+}\ \frac{+}{+}$	none (both 1 and 2)	none	
ii	$\frac{a}{a}\ \frac{+}{+}\ \frac{+}{+}$	1 → 2	missing 1	A → ●
iii	$\frac{+}{+}\ \frac{b}{b}\ \frac{+}{+}$	2 → 1	extra 1	B ⊣ ●
iv	$\frac{+}{+}\ \frac{+}{+}\ \frac{c}{c}$	1 → 2	missing 1	C → ●
v	$\frac{+}{+}\ \frac{b}{b}\ \frac{c}{c}$	1 → 2	missing 1	B ⊣ C → ●
vi	$\frac{a}{a}\ \frac{b}{b}\ \frac{+}{+}$	2 → 1	extra 1	A ⊣ B ⊣ ●

Figure 4. Mixed switch regulation and substrate dependent pathway: programmed cell death. An example of a mixed pathway. Some signal controls activities of either *ced-9* or *ced-3* and *ced-4*, resulting in activation of a pathway by which cells die. Inactivation of *ced-9* results in extra cells dying. Inactivation of either *ced-3* or *ced-4* results in the survival of cells that would otherwise die. Inactivation of *ced-1*, *ced-2*, etc. results in cells that die, but whose corpses are not engulfed by surrounding tissue.

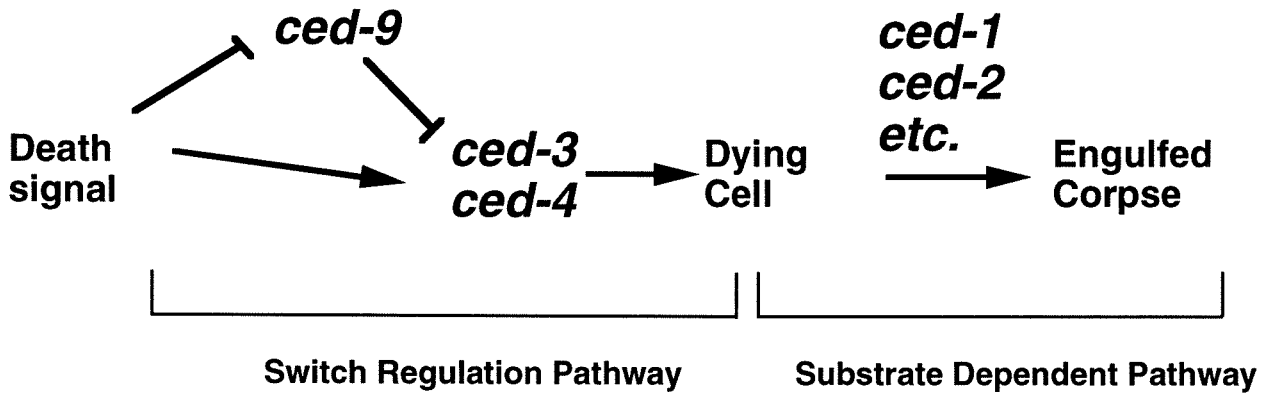
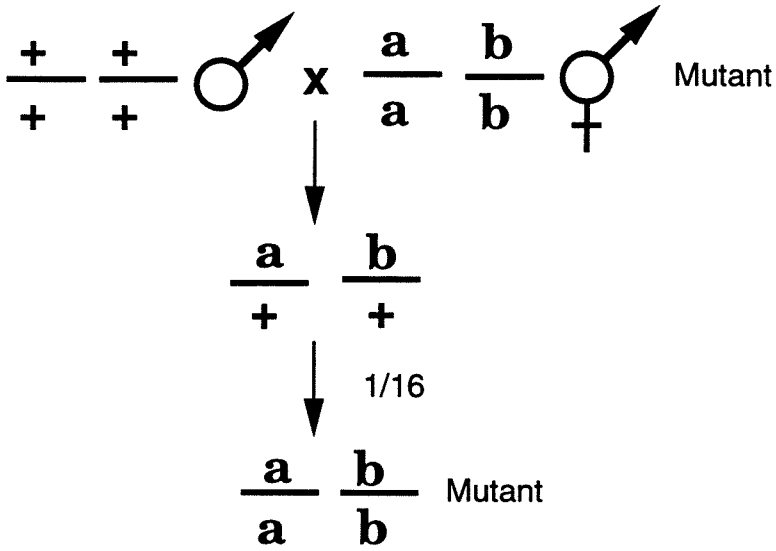
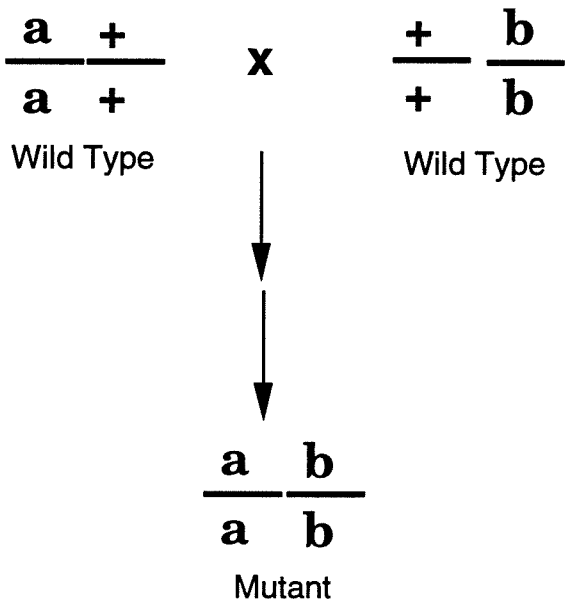

ced-9
Death signal
ced-3
ced-4
Dying Cell
ced-1
ced-2
etc.
Engulfed Corpse
Switch Regulation Pathway
Substrate Dependent Pathway

Figure 5. Synthetic phenotypes. A. Discovery by backcrossing. If two recessive mutations are responsible for the phenotype, then 1/16 progeny of the double heterozygote will display the phenotype. B. Discovery by construction. C. Discovery by mutagenesis.

A By Backcross



B By Construction



C By Mutagenesis

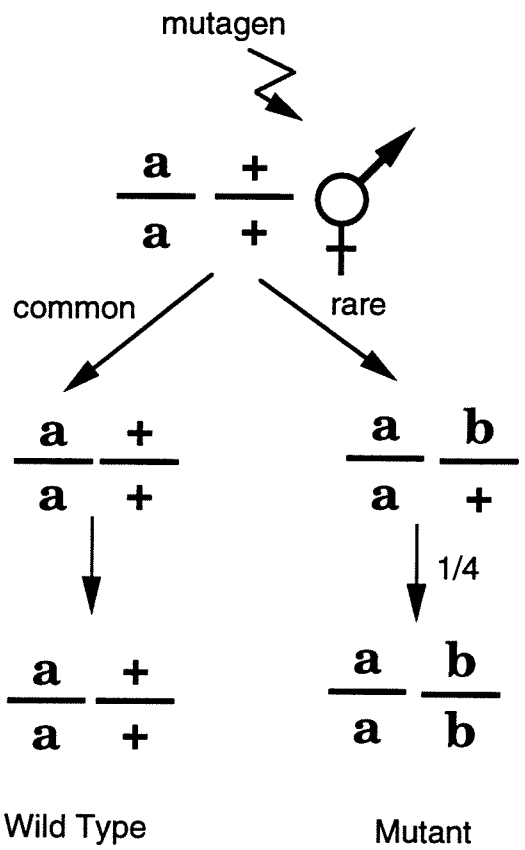


Figure 6. Branched pathway: somatic and germline sex determination. The ratio of X to autosomes determines sexual phenotype (see Villeneuve and Meyer, 1990 for review). High ratio of X chromosomes to autosomes results in repression of activity of the *fem* genes. For somatic sexual determination, the *fem* genes negatively regulate *tra-1* activity. For germline sex determination, the *fem* genes control sexual identity independently of *tra-1*.

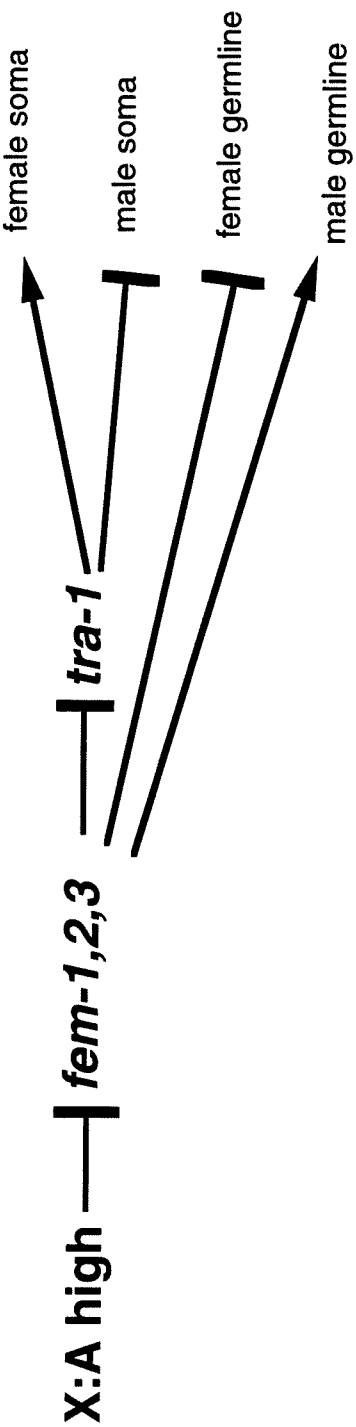
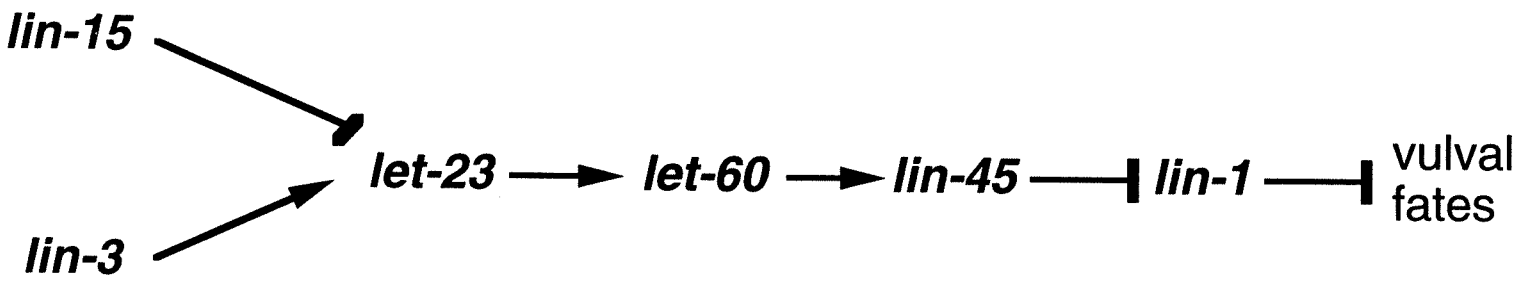

X:A high
fem-1,2,3
tra-1
female soma
male soma
female germline
male germline

Figure 7. Multiple inputs: vulval induction. A. Abbreviated pathway of vulval induction (see Sternberg, 1993 for review). B. A "truth table" schema for the combined action of *lin-15* and *lin-3* to control vulval differentiation.

A



B

lin-15	*lin-3*	vulva?
ON	ON	Yes
OFF	OFF	Yes
ON	OFF	No
OFF	ON	Yes

Figure 8. Suppressor screens. From mutant parents that have been mutagenized, the F1 progeny and F2 grandprogeny are examined for phenotypic revertants, that is animals that do not display the mutant phenotype. Each F1 examined screens two mutagenized gametes, as either the sperm or the ova could carry a mutation. If the F2 are examined, the number of mutagenized gametes is also two per F1. m, starting mutation; sup, suppressor mutation.

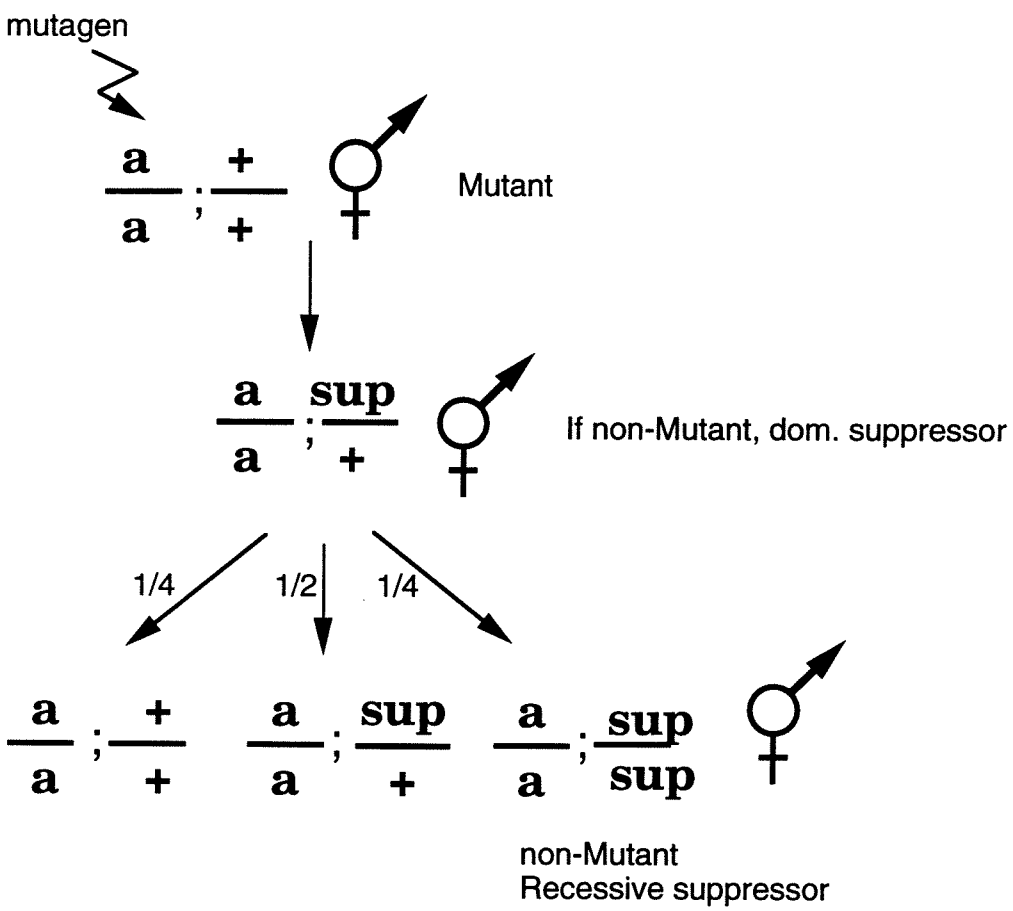
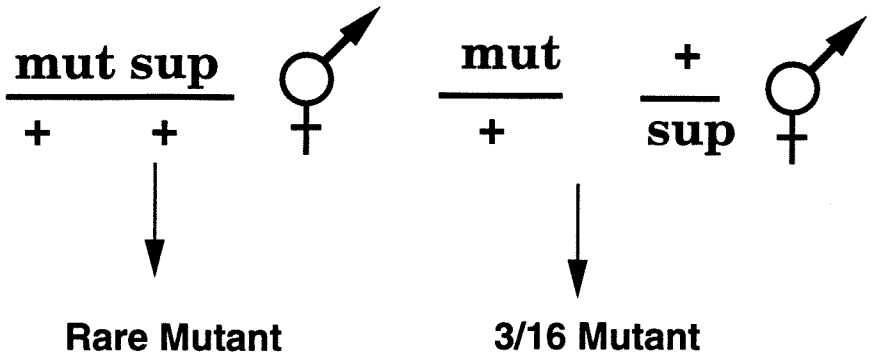
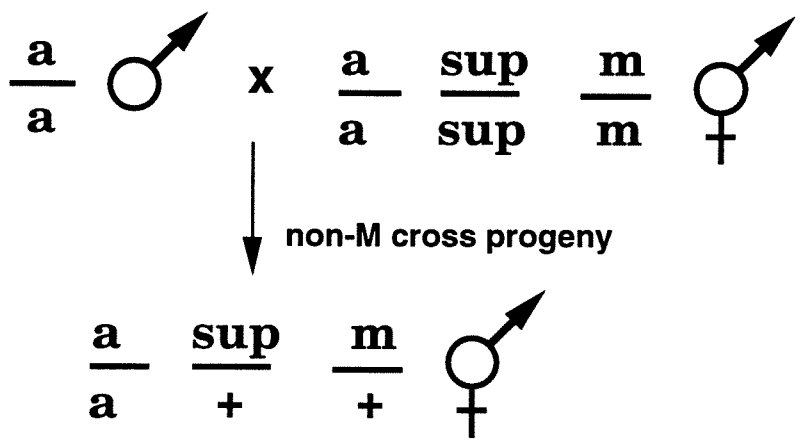

mutagen
a/a ; +/+
Mutant
a/a ; sup/+
If non-Mutant, dom. suppressor
1/4
1/2
1/4
a/a ; +/+
a/a ; sup/+
a/a ; sup/sup
non-Mutant
Recessive suppressor

Figure 9. Analysis of suppressor mutations. A. Linked versus unlinked suppressors. Assuming complete penetrance and recessivity of the *sup* mutation, only *mut* + / *mut sup* recombinants will be detected among the progeny of animals heterozygous for *mut* and *sup*. These will occur at a frequency of 2p/4 = p/2, where p is the map distance (see Sulston and Hodgkin, 1988). B. Test for dominance. Animals heterozygous for the *sup* mutation and homozygous for the starting mutation, a, are constructed to determine the phenotype.

A


mut sup
+ +
mut
+
+
sup
Rare Mutant
3/16 Mutant

B


a
a
x
a
a
sup
sup
m
m
non-M cross progeny
a
a
sup
+
m
+