Title
The role of the lin-22 gene in patterning the C. elegans lateral ectoderm

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Publication Date
1995

Peer reviewed|Thesis/dissertation

The role of the lin-22 gene in patterning the C. elegans lateral ectoderm

by

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DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Biochemistry

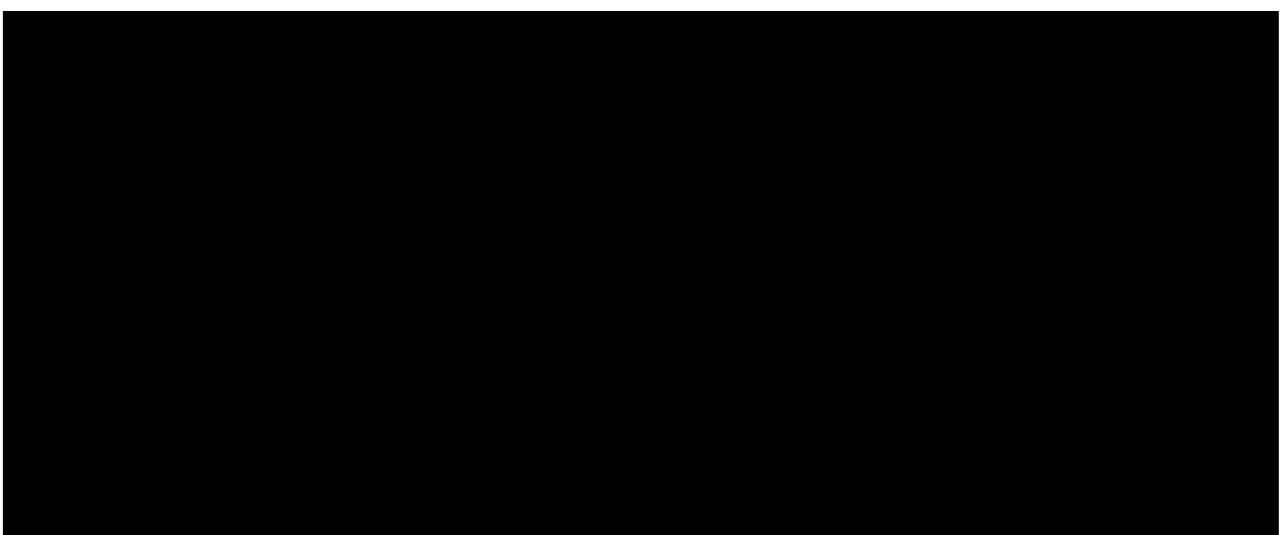
in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA

San Francisco



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I would like to dedicate this work to my grandfather,

Lucas Wrischnik

ACKNOWLEDGMENTS

After several years of hellish luck, I finally had a big breakthrough in my cloning efforts. I called my parents and regaled them with the boring details. After a while my Mom asked just one question, "Is this good?" I said that it was. The next day there was a huge bouquet of flowers waiting for me in lab. It didn't really matter what I had actually done, since they didn't really know, but my parents were just happy for me because I was happy. Throughout graduate school, and my whole life, my parents have been my biggest supporters. I will never be able to thank them enough for all they have done for me. I'm lucky to have such wonderful people as parents and friends.

Of course, even with my parents solidly behind me I've needed my local support group. I never would have made it through the first years without my classmates Josh Miller, Joe Ogas, and especially Joanna Gilbert. They helped keep me sane (or as close to that as I will ever get).

I also have to thank everyone who has ever been a member of the Kenyon Lab. If I had to be somewhere for this long, I'm sure glad the Kenyon Lab was the place, and for this I have to thank the chief herself. Cynthia seems to attract the smartest and nicest people to her lab, and this, along with her boundless enthusiasm for science, has made the Kenyon Lab a wonderful place to be. Of the people who have left, I want to thank Michael Müller for his fabulous cappuccinos, Bruce Wang for his wry humor, and especially the little Texas powerhouse, aka Nomi Robinson, for being such a good buddy. And, well , I hate to admit it, but I even miss you, Waring. I wanted to beat the hell out of you a couple of times, but you taught me so much, and you were a good friend.

I would also like to thank Deborah Cowing for her mighty organizational skills and her level head, Jennie Dorman for brightening up our little corner of the lab, and Craig Hunter and Julin Maloof for all the scientific help they've given me. Finally, I have to thank Steve Salser for putting up with me for all these years. I can't begin to describe how much help and support Steve has given me. I will miss him so much more than I could ever say in an acknowledgments page.

And where would I be without Supriya Shivakumar (the correct response being "in a lot less trouble"). Supriya, along with Joanna, have been the two best friends I've ever had. Graduate school was worth it just to have met the two of you. Let's see, I think there's someone else in the Kenyon lab that I'm forgetting, again.....Oh yes, and thank you, Gregg Jongeward, for being patient, and kind, and the bravest guy in the whole wide world.

ABSTRACT

The role of the *lin-22* gene in patterning the *C. elegans* lateral ectoderm

Lisa A. Wrischnik

This thesis describes the analysis of *lin-22*, a gene which regulates both neurogenesis and anterior-posterior patterning in *Caenorhabditis elegans*. Mutations in *lin-22* alter the fates of cells within the lateral ectoderm, a row of cells stretching from the head into the tail on both the right and left sides of the animal. In wild type males, the cells in the anterior adopt epidermal fates, while the cells in the posterior generate neuronal structures. *lin-22*(+) acts to inhibit both neurogenesis and cell proliferation in the anterior cells, and mutations in *lin-22* cause the anterior cells to behave like their posterior counterparts and make neuronal structures instead of epidermis.

In an attempt to understand the molecular function of *lin-22*, the gene was cloned. *lin-22* is a basic helix-loop-helix protein that is homologous to the *hairy* gene of *Drosophila*. *hairy* functions as both a pair-rule gene, acting early to regulate metameric patterning of the *Drosophila* embryo, and as a negative regulator of proneural genes in the adult peripheral nervous system.

lin-22 acts upstream of the *C. elegans* proneural gene *lin-32*, which turns out to be homologous to the proneural genes of the *Drosophila achaete-scute* complex. This illustrates the conservation of function between *lin-22* and *hairy*. However, we have shown that *lin-22* also regulates the Hox gene *mab-5*, a gene that, like *lin-22*, is also required for the correct anteroposterior patterning of the lateral ectoderm. In a *lin-22* mutant, *mab-5* expression expands from the posterior into the anterior. This may reflect a unique activity for a *hairy*-like gene in creating differences between anterior and posterior body regions by regulating the domain of Hox gene expression.

Table of Contents

List of Tables

List of Figures

CHAPTER 1

Introduction

INTRODUCTION

Neurogenesis in *Drosophila*

The generation of the correct numbers and types of neurons in the correct place is critical for the function and development of most organisms. The study of neurogenesis will teach us about the specification of particular cell fates in particular locations. In *Drosophila*, there is a well known cascade of gene activity that ensures that neurons are made in the correct location both embryonically and post-embryonically. The activities of the proneural genes of the *Achaete-scute* complex (AS-C) define the general regions in which neurons can be made in the embryonic CNS (the *achaete*, *scute*, and *lethal-of-scute* genes) and adult PNS (*achaete* and *scute*) (Cubas, *et al.*, 1991; Martin, *et al.*, 1991; Skeath and Carroll, 1991). All three genes are members of the basic helix-loop-helix (bHLH) class of transcriptional regulators (Villares and Cabrera, 1987). The HLH domains of these proteins are used in dimer formation, whereas the basic region is required for DNA binding (Murre, *et al.*, 1989a). Members of this large group of proteins also includes the family of myogenic determinants such as *MyoD*, *myogenin*, *myf*, members of the *Enhancer of split* complex, and the proto-oncogene *c-Myc* (Murre, *et al.*, 1989a; Murre, *et al.*, 1989b). HLH proteins appear to function primarily as heterodimers with other HLH proteins within their family (Murre, *et al.*, 1989b).

The members of the *Drosophila* AS-C are expressed in and define the cells of the proneural cluster (Cabrera, *et al.*, 1987; Cubas, *et al.*, 1991). Within this cluster, a single cell will become a neuroblast. A lateral inhibitory signal, involving the products of the *Notch* and *Delta* genes and members of the

Enhancer-of-split complex [*E(spl)*], among others, is responsible for singling out this sensory organ precursor (for review see (Ghysen, *et al.*, 1993)). Once proneural gene expression is established, cells expressing the highest levels of AS-C genes exhibit the greatest activity of the *Delta* protein, a transmembrane signaling molecule. *Delta* protein then signals neighboring cells, activating the *Notch* transmembrane receptor and leading to a decrease of AS-C expression in those cells. Negative regulation of the AS-C genes requires the function of an additional set of bHLH proteins, the members of the *E(spl)* complex. This feedback loop continues until only a single cell, the future neuroblast, expresses AS-C genes. At this point, genes such as *deadpan* and *asense* (also encoding bHLH proteins) may act in the differentiation of this neuroblast into the correct sensory structures (Bier, *et al.*, 1992; Jan and Jan, 1993).

bHLH proteins are also important in the negative regulation of neurogenesis

One of the most important aspects of positioning neuroblasts is the proper placement of proneural gene expression. Positional cues gleaned from the embryonic patterning elements of the anterior-posterior and dorsal-ventral axes are thought to play a role in regulating the expression of the proteins of the AS-C (Martin, *et al.*, 1991; Doe, 1992; Skeath, *et al.*, 1992). Negative regulation is also important postembryonically in defining the domains of activity of these proteins. Two such negative regulators have been studied; these are the products of the *extramachrochaete* (*emc*) and *hairy* genes. *emc* is a helix-loop-helix protein that lacks the basic domain needed by these proteins for DNA binding (Ellis, *et al.*, 1990). *emc* inhibits *scute*

function by forming inactive heterodimers with the *scute* protein (Van, *et al.*, 1991), and its expression is thought to be complementary to the *achaete-scute* pattern (Cubas and Modolell, 1992).

hairy acts as a negative regulator of the proneural gene *achaete*

A second negative regulator of proneural genes is the *hairy* gene, also a member of the bHLH family of proteins (Ish, *et al.*, 1985; Rushlow, *et al.*, 1989). It is this gene and its homologs that are most closely related to the *lin-22* gene, the focus of this thesis. *hairy* plays an important role in postembryonic neuronal patterning by binding directly to the upstream region of *achaete* and inhibiting its expression (Ohsako, *et al.*, 1994; Van, *et al.*, 1994). In the leg imaginal disc loss of *hairy* function allows the domain of expression of *achaete* to expand into the region formerly expressing *hairy*. This results in the generation of ectopic neuroblasts (extra bristles) on the leg (Orenic, *et al.*, 1993). *hairy* also plays an important role in patterning neurogenesis in the eye imaginal disc, where it acts in concert with *emc* to negatively regulate progression of the morphogenetic furrow (Brown, *et al.*, 1995). Here, *hairy* may act to block photoreceptor differentiation by preventing progression of the cell cycle, thus inhibiting a cell proliferation event required for later differentiation.

hairy functions early in the metameric patterning of the _Drosophila_ embryo

In addition to its role in repressing postembryonic neurogenesis, *hairy* functions in the *Drosophila* embryo as a primary pair-rule gene (Howard and Ingham, 1986; Hooper, *et al.*, 1989), transmitting upstream positional information (i.e.. from the gap genes) to other pair-rule genes and ultimately to targets further downstream, such as the segment polarity and HOX genes. The anterior-posterior (A-P) axis of the *Drosophila* embryo is initially specified through the actions of the *bicoid* and *nanos* gene products (Struhl, *et al.*, 1989; Lehmann and Nusslein, 1991; Curtis, *et al.*, 1995). These proteins act in morphogenetic gradients to define the anterior and posterior poles, respectively, of the embryo. These protein gradients establish the expression patterns of the various gap genes, so called because removal of their function leads to large gaps in the A-P segmentation pattern (Eldon and Pirrotta, 1991; Kraut and Levine, 1991; Pankratz, *et al.*, 1992; Struhl, *et al.*, 1992). Examples of gap genes include *giant*, *knirps*, *hunchback*, and *Kruppel*. Differences in the concentrations of these gap gene products leads to the activation of the pair-rule genes, including *hairy*, in seven stripes along the A-P axis, corresponding to seven alternating two-segment units (Pankratz, *et al.*, 1990). For example, the presence of *Kruppel* activity is thought to be necessary for the activation of particular *hairy* stripes. The pair-rule genes, in turn, help to pattern the segment polarity genes, which are expressed in and establish the correct A-P polarity of each segment of the embryo (Warrior and Levine, 1990; Benedyk, *et al.*, 1994; Mullen and DiNardo, 1995).

As a primary pair-rule gene, *hairy* functions to regulate the activities of other pair-rule genes. For example, *hairy* is known to regulate such pair-rule

genes as *even-skipped*, *runt*, and *fushi tarazu* (Howard and Ingham, 1986; Carroll, *et al.*, 1988; Kellerman, *et al.*, 1990). Ultimately, the combined activities of the gap, pair-rule, and segment polarity genes regulate the numerous homeotic genes (the Hox genes) responsible for patterning specific body regions along the A-P axis (Jack and McGinnis, 1990). Hox genes are a class of homeobox-containing transcriptional regulators that pattern the A-P axis in virtually all animals examined (for review, see (McGinnis and Krumlauf, 1992)). These genes are found in clusters, and are organized in the genome in the same order in which their domains of expression are found along the A-P axis. Although *hairy* is an important regulator of metameric patterning, *hairy* is only indirectly responsible for the initial specification of proneural gene expression through its actions on other pair-rule, segment polarity, and Hox genes (Macias, *et al.*, 1994).

Several homologs of *hairy* have been identified in other organisms

Homologs of *hairy* have now been identified in a number of organisms, including *Drosophila* itself. The closest *hairy* homolog in *Drosophila* is the *deadpan* (*dpn*) gene. *dpn* is a negative regulator of AS-C genes, and is expressed in the embryo in a pair-rule pattern as well (although its role in embryonic patterning is unknown (Bier, *et al.*, 1992). Another *hairy* homolog has been found in the short germ-band insect *Tribolium*, where it is expressed in an embryonic pattern suggestive of an important role in segmental A-P patterning (Sommer and Tautz, 1993). The role of *hairy* in regulating neurogenesis in *Tribolium* is not yet known.

Several genes homologous to *hairy* and the *E(spl)* genes have also been discovered in rats and mice. These vertebrate homologs are not expressed in

patterns that suggest conclusive roles for them in axial patterning (Ishibashi, *et al.*, 1993; Feder, *et al.*, 1994; Takebayashi, *et al.*, 1994). However, these vertebrate homologs do appear to play a role in regulating neurogenesis. The rat homolog *HES-5* is specifically expressed in the nervous system (Akazawa, *et al.*, 1992) while rat and mouse *HES-3* are found only in the cerebellar Purkinje cell layer (Sakagami, *et al.*, 1994). Even though it is expressed in a multitude of tissue types, in vitro experiments with rat *HES-1* suggest that it can repress the NGF-induced neuronal differentiation of PC12 cells in tissue culture (Feder, *et al.*, 1993). In addition, overexpression of *HES-1* in a portion of the mouse brain results in the formation of fewer neurons and glial cells (Ishibashi, *et al.*, 1994). These data support the important role of *hairy*-like genes in regulating vertebrate neurogenesis. This is not surprising, given that numerous vertebrate homologs of the AS-C genes, targets of *hairy* regulation, have been identified and implicated in vertebrate neurogenesis as well (Franco, *et al.*, 1993; Guillemot and Joyner, 1993; Guillemot, *et al.*, 1993).

Mutations in the *C. elegans* gene *lin-22* result in the formation of ectopic neuroblasts

A single mutation in the *C. elegans lin-22* gene was first identified and characterized by W. Fixsen (Horvitz, *et al.*, 1983; Fixsen, *et al.*, 1985; Fixsen, 1985). The primary effect of this mutation is seen in the V cells, a row of 6 hypodermal blast cells that extend along both sides of the worm (see Fig 1-1). These cells generate some of the most obvious anterior-posterior patterning elements found in *C. elegans* ((Sulston and Horvitz, 1977; Sulston, 1983). The anterior cells adopt epidermal fates and generate lateral alae, four parallel

ridges in the adult worm cuticle that extend from the head to the tail. The posterior V cells of the male produce rays, sensory structures that are used in male mating, and a single sensory structure called a postdeirid. In the hermaphrodite, the postdeirid is the only posterior sensory structure generated by the V cells. In a *lin-22* mutant, the anterior V cells that would normally make epidermal structures adopt the neuronal fates of the posterior V cells (Fig. 1-2). This leads to the formation of ectopic postdeirids in the anterior of the hermaphrodites and ectopic postdeirids and sensory rays in the male.

Thus, *lin-22* mutant animals display a phenotype remarkably similar to that of negative regulators of proneural genes: the formation of ectopic neuroblasts. The only other evidence that *lin-22* was involved in neurogenesis was that *lin-22* was shown to act genetically upstream of the gene *lin-32* (Kenyon and Hedgecock, pers. comm.; Zhao and Emmons, pers. comm.), which is required for the formation of the V cell-derived neuroblasts (Zhao and Emmons, 1995). Formally, the *lin-22* phenotype can be classified as an anterior-to-posterior homeotic transformation, since anterior cells are adopting posterior cell fates. It was this aspect of the phenotype that led to the proposal that *lin-22* was a new homeotic gene (Horvitz, *et al.*, 1983).

This interpretation of the *lin-22* phenotype was supported by the work of Kenyon (1986) and Costa et al. (1988) on the *C. elegans* gene *mab-5*. Mutations in *mab-5* cause numerous cell types of the posterior body region to adopt the fates of their anterior counterparts. In particular, the *mab-5* mutation results in the posterior cells V5 and V6 adopting the anterior V cell fates and generating epidermal cells instead of sensory rays in the male (Kenyon, 1986). Therefore, *mab-5* and *lin-22* promote opposite cell fates among the V cells. Kenyon (1986) also saw that increasing levels of *mab-5*

activity in a *lin-22* mutant background let to an increase in sensory ray production, but that this effect was not seen in *lin-22*(+) animals. This further suggested that *lin-22*(+) activity was antagonistic to *mab-5* function. Subsequently, *mab-5* was found to be homologous to the *Antennapedia* gene of *Drosophila*, a member of the Hox family of A-P patterning genes (Costa, *et al.*, 1988). Because of its phenotype and its relationship to *mab-5*, it was thought that *lin-22* could be another Hox patterning gene.

Cell signaling is required to specify the correct pattern of V cell fates

A series of experiments involving the laser ablation of cells in the *C. elegans* lateral ectoderm suggested that both the formation of the V5 postdeirid and the generation of alae by the V cells depend on signals sent from neighboring cells (Sulston and White, 1980; Waring and Kenyon, 1990). Thus, cell signals play an important role in influencing the neuronal vs. epidermal fate decisions of the V cells. However, the relationship between these signals and *lin-22* activity, which regulates the same cell fate decisions, was unclear, and it was proposed that perhaps signals could regulate the activity of genes such as *lin-22* and *mab-5*, which play a role in patterning the V cells (Waring and Kenyon, 1990). Chapter 2 is a further analysis of the role of cell signals in promoting postdeirid formation, and we see that postdeirid-inducing signals are present among the anterior V cells and not just found locally next to the V5 cell. These signals are unaffected by a *lin-22* mutation, and are actually required for the generation of ectopic postdeirids by the anterior V cells. These observations support the idea that *lin-22* does not affect the signaling mechanism itself, but that *lin-22*(+) normally functions in

the cells anterior to V5 to prevent them, directly or indirectly, from responding to postdeirid-inducing signals.

lin-22, a bHLH protein with homology to the _Drosophila_ gene _hairy_, regulates both neurogenesis and A-P patterning

Because of the interesting phenotype of the *lin-22* mutation and the fascinating relationship between *lin-22* and *mab-5*, I decided to pursue a more extensive analysis of this gene. Chapters 2 and 4 contain further genetic analysis of the original and two additional *lin-22* alleles. Chapter 3 addresses the key question of *lin-22* function: what protein product does the *lin-22* gene encode? The role of *lin-22* as a regulator of worm proneural genes is supported by the discovery that *lin-22* encodes a bHLH protein that is most similar to the *hairy* gene of *Drosophila* and its homologs in other organisms. Further evidence for this role for *lin-22* comes from the recent cloning of the *lin-32* gene, which was found to be a *C. elegans* AS-C homolog (Zhao and Emmons, 1995).

Although *lin-22* is not a Hox gene, Chapter 3 shows that *lin-22* nonetheless is an important regulator of Hox gene function. An analysis of the Mab-5 protein expression pattern in a *lin-22* mutant shows that loss of *lin-22* function allows Mab-5 expression to expand into the V cells of the anterior. Certain pair-rule genes are thought to be direct regulators of HOX gene function, but this has not been seen for the *hairy* gene of *Drosophila* (Macias, *et al.*, 1994). Hox gene regulation has not even been suggested for the members of the *E(spl)* complex. The potential regulation of both a proneural

gene and a Hox gene by *lin-22* in the *C. elegans* lateral epidermis may reflect a novel activity for a *hairy*-like bHLH protein if *lin-22* directly regulates *mab-5*.

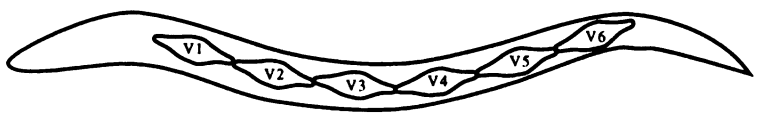
Data in Chapters 3 and 4 also address such issues as the role of *lin-22* in regulating cell proliferation and the effects of *lin-22* activity on the remaining *C. elegans* Hox genes. Thus, the continuing study of *lin-22* has lead to new insights about the role of bHLH proteins in neuronal and axial patterning.

Figure 1-1. The lineages and adult structures generated by the *C. elegans* lateral ectoderm.

a. View of the six V cells on the left side of the lateral ectoderm of *C. elegans* at hatching. There are six V cells along the right side as well.
b. V cell lineages in a wild type male (Sulston and Horvitz, 1977). The vertical axis represents time and the horizontal axis represents the direction of cell division, with anterior to the left. Circles indicate cells that will fuse with hyp7, a large epidermal syncytium. The stages at which the various divisions occur are indicated. The anterior cells V1-V4 generate cuticular ridges called alae. The most posterior cell, V6, makes five sensory rays. The V5 cell is unique in generating a second neuronal structure called a postdeirid, as well as alae from an anterior lineage and a single ray from a posterior lineage. The actual ray precursor lineage is replaced by an arrow.
c. Drawing of an adult male *C. elegans*. The alae extend from the head to the fan of the tail, while the six V rays are located in the fan itself. The postdeirid is located in the posterior of the animal.

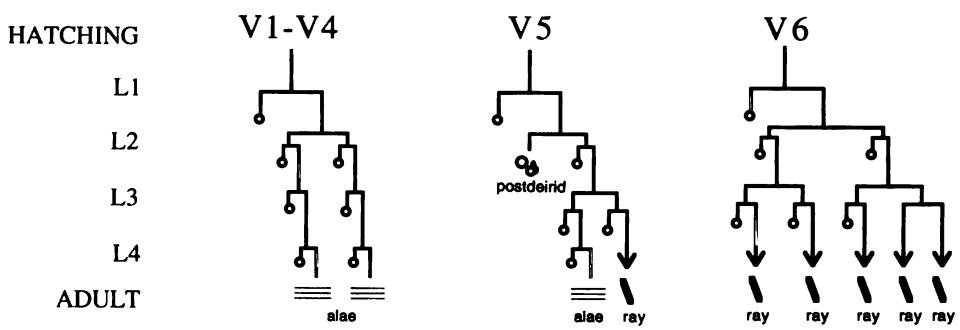
Figure 1-1

a.



L1 HATCHLING

b.



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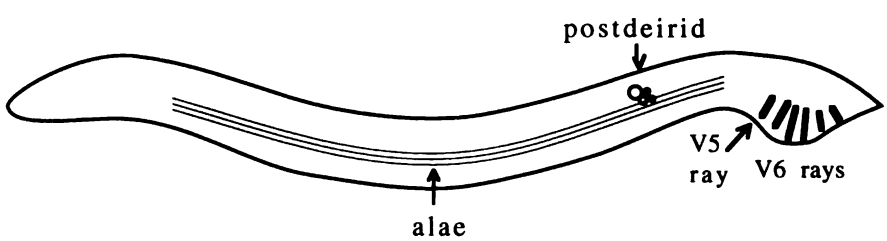


ADULT MALE

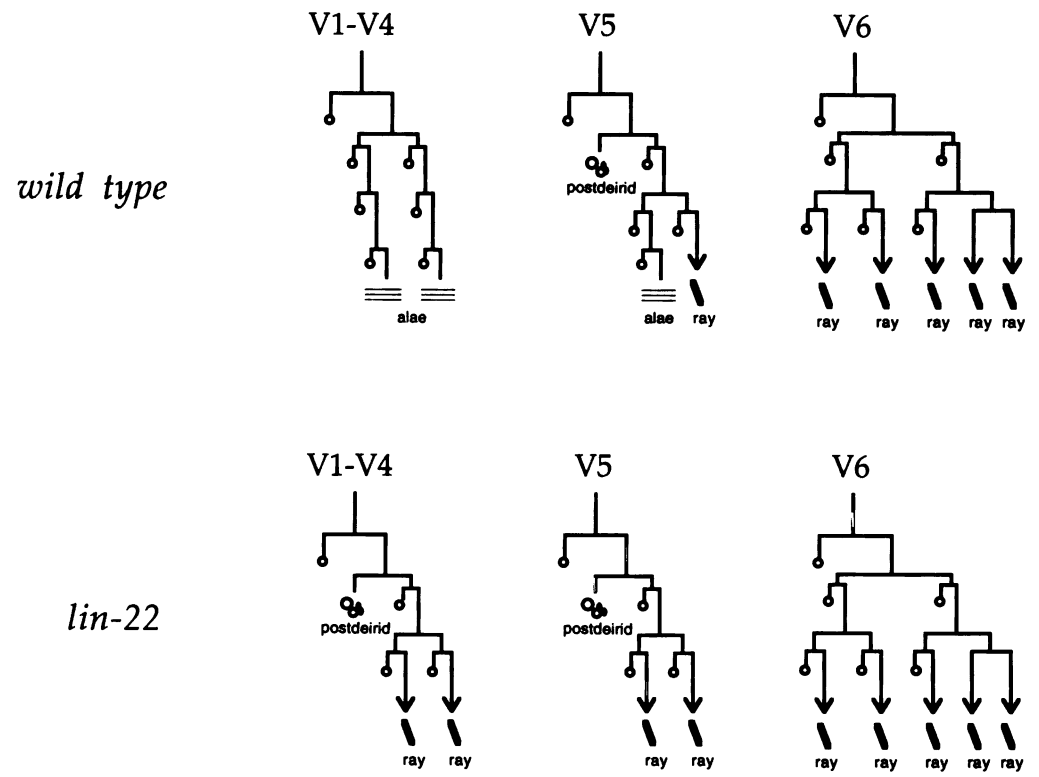
Figure 1-2. The V cell lineages and the adult phenotype of the wild type male compared to those of a *lin*-22 mutant male.

a. The V cell lineages of a wild type male are shown above the canonical V cell lineages of a *lin*-22 male (Fixsen, 1985; for actual *lin*-22 lineages see Figure 3-1). In the V5 lineage of a *lin*-22 male, the anterior branch that would normally generate alae now makes a ray instead. In addition, the anterior V cells V1-V4 adopt this altered V5 cell fate and make ectopic postdeirids and rays in the anterior.

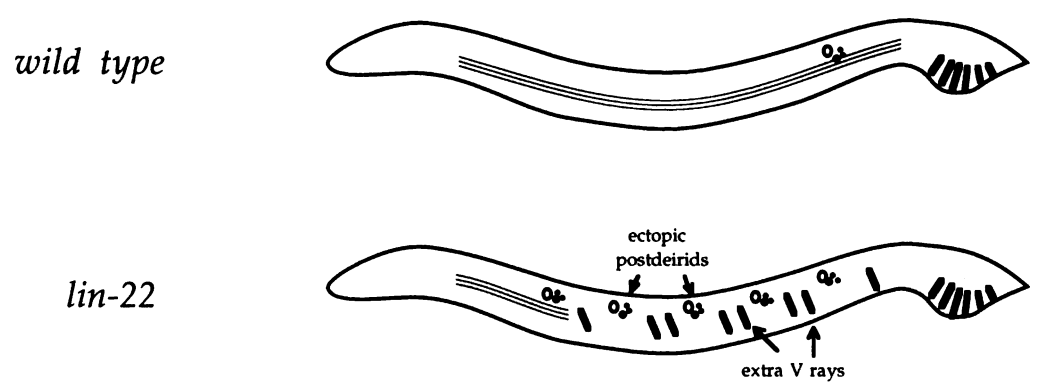
b. Drawings of the adult phenotype of *lin*-22 compared to wild type. The generation of ectopic postdeirids and rays in the anterior of a *lin*-22 male results in the concomitant loss of epidermal alae.

Figure 1-2

a.


wild type
V1-V4
V5
V6
postdeirid
alae
ray
lin-22
V1-V4
V5
V6
postdeirid
postdeirid
ray

b.


wild type
lin-22
ectopic
postdeirids
extra V rays

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CHAPTER 2

The role of *lin*-22 in signaling among the cells of the *C. elegans* lateral ectoderm

CHAPTER 2

is derived from the (1992) *Development* paper entitled

Cell Signals Allow the Expression of a Pre-existent Neural Pattern in *C. elegans*

by

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1. Analysis of the*pal-1* and *pal-1; lin-22* phenotypes, and all ablation experiments, were carried out by D. Waring
2. Isolation of *lin-22(mu2)* and *lin-22(mu5)* by C. Kenyon
3. Genetic analysis of the three *lin-22* alleles carried out by L. Wrischnik

Abstract

In *C. elegans*, a simple pattern develops within a row of epidermal precursor cells, V1-V6. One cell, V5, gives rise to a neuroblast called the postdeirid neuroblast, while the other V cells produce epidermal cells instead. Here we show that in order for V5 to produce the postdeirid it must be in close or direct contact with neighboring V cells. Signaling between V cells induces the formation of the neuroblast. However, which of the V cells makes postdeirids is not determined by the signals but rather by the action of the homeotic *lin-22* and *pal-1* genes. These genes prevent V cells in specific body regions from responding to intercellular signals and producing postdeirids. This is a clear example of cell signals playing a permissive rather than an instructive role in neuroblast induction.

Introduction

A major question in the fields of cell signaling and development is what roles intercellular signals play in pattern formation. In some instances, the position of a signal determines where a structure is formed. For example, in *C. elegans* the position of a signaling cell in the gonad determines which epidermal cells initiate vulval development (Kimble, 1981). In *Xenopus*, the position of the organizer, which itself is induced by signals from nearby cells, determines the future positions of structures along the dorso-ventral and antero-posterior axes (reviewed in Gerhart et al, 1989). Previously we described a case in which cell signals play a different role in pattern formation. In the development of a pattern of sensory rays and epidermal structures (called alae) in *C. elegans*, cell-cell signals induce epidermal structures. However, the signals themselves are not targeted to specific precursor cells. Instead, only certain precursor cells have the potential to respond to the signals (Waring and Kenyon, 1990; Waring and Kenyon, 1991).

The same precursor cells that ultimately generate the ray/alae pattern, the V cells, give rise to another neural pattern earlier in development. This is a simple pattern consisting of a single neuroblast, the postdeirid, within a row of epidermal cells (see Figure 1A) (Sulston and Horvitz, 1977). Laser ablation experiments have shown that some type of cell-extrinsic signal is required for V5 to make a postdeirid neuroblast instead of behaving like the other V cells and producing only epidermal cells (Sulston and White, 1980). However, the signaling events required for the postdeirid and the ray/alae patterns are distinguishable, since ablation of precursor cells at certain stages affects one pattern but not the other. Two genes involved in ray pattern formation, the homeobox-containing gene *pal-1* (Waring and Kenyon, 1990;

Waring and Kenyon, 1991) and the gene *lin-22* (Horvitz et al., 1983) also affect postdeirid formation.

Here we use genetics and laser microsurgery to investigate the roles of cell-extrinsic signals and the genes *lin-22* and *pal-1* in postdeirid pattern formation. The results indicate that in order to make a postdeirid, a V cell must be in close or direct contact with each of its V cell neighbors, which raises the possibility that it must lie within an intact epithelium. We find that the genes *lin-22* and *pal-1* prevent cells other than V5 from being signaled by their neighbors to produce postdeirids. In these mutants, additional V cells produce postdeirids in a signal-dependent fashion. Mosaic analysis and laser microsurgery indicate that *pal-1* and probably also *lin-22* function within individual V cells to prevent them from responding to postdeirid-inducing signals. Thus, postdeirid pattern formation requires both cell signals to induce the neuroblast and also pattern formation genes to create a prepattern of cells that are competent to respond to the signals.

The postdeirid and ray/alae pattern formation systems differ from one another in many ways. For example, the gene *lin-22* has opposite effects on cell signals in the two cases. Nevertheless, there is a fundamental similarity between them: in both, patterns arise through a mechanism that creates intrinsic differences in the ability of cells to respond to intercellular signals. This basic strategy can thus be used to produce very different patterns of neurons even within the same tissue.

Materials and Methods

General Procedures and Strains

Methods for routine culturing and genetic analysis were described in Brenner (1974). All experiments were performed at 20°C. The complete genotype of the *pal-1; lin-22* mutant strain was *unc-79(e1068) pal-1(e2091); lin-22(mu2); him-5(e1490)*

Observation of Postdeirids

Late L2 or early L3 animals were examined for the presence of postdeirids (wild-type and ectopic). Postdeirids were recognized by their cell morphology and position relative to other cells. By examining animals at this stage, it is possible to determine which cells were derived from each V cell and the type of lineage that had taken place (postdeirid, seam cell, or hybrid lineage). In the case of hybrid lineages, it was sometimes not possible to be sure of the origin of all cells, but it was always clear that some type of hybrid lineage had occurred (see Figure 2-1G). Sodium azide (2mM) was used in many cases to anesthetize the animals while they were being examined.

Isolation of *lin-22* mutations

The *lin-22(mu2)* and *lin-22(mu5)* alleles were isolated by screening for males that had undergone the alae-to-ray transformation characteristic of *n372* animals (Kenyon, 1986). F2 male progeny from EMS mutagenized *him-5(e1490)* animals were examined under a dissecting microscope using oblique illumination for the absence of adult alae, and then examined with Nomarski optics for the presence of ectopic ray papillae and ray cell groups. (The *him-5* mutation increases the frequency of male self-progeny). From 5402 F2 male progeny of EMS-mutagenized animals, two independently isolated males were found that displayed an alae-to-ray transformation. One of these males produced progeny when crossed to *unc-17(e245)* hermaphrodites. Complementation tests indicated that this animal contained a new *lin-22* mutation, *mu2*. The second new *lin-22* allele, *mu5*, was isolated in a complementation screen by mating mutagenized *him-5(e1490)* males with *lin-22(n372) unc-17(e245)* hermaphrodites and examining 4267 F1 non-Unc cross progeny for the alae-to-ray transformation. In this screen, a single animal with this phenotype was identified. The new mutation was recovered by outcrossing and shown to be a *lin-22* allele by complementation tests with *n372*. In C. elegans, the frequency with which null (or severe reduction-of-function) alleles arise in most genes following standard EMS mutagenesis protocols is thought to be approximately 1/2000 haploid genomes. Because of the small sample size, the *lin-22* mutation frequency cannot be determined accurately from these data; however, the results indicate that these alleles do not correspond to rare, highly specific alterations in protein sequence.

Genetic Analysis of *lin-22*

lin-22; him-5(e1490) animals were examined using Nomarski optics for the presence of a postdeirid among the descendents of each V cell. A dominance test was carried out in which heterozygous *lin-22/+* animals were generated for each allele by crossing *lin-22; him-5(e1490)* males to *unc-17(e245) dpy-13(e184)* and *dpy-17(e164); him-5(e1490)* hermaphrodites. Cross progeny were then scored for postdeirid formation. In these crosses, the wild-type copy of *lin-22* was always derived from the mother. To test whether there is a maternal effect of *lin-22*, we generated *n372/+* animals in which the mutant *lin-22* allele came from the mother. These *n372/+* heterozygotes were produced by crossing *him-5(e1490)* males to *lin-22(n372) unc-17(e245) osm-3(p802); e1490* hermaphrodites (the *osm-3* mutation affects dye uptake in head and tail neurons), *lin-22(n372) unc-17(e245) osm-3(p802) dpy-13(e184); e1490* hermaphrodites, and *lin-22(n372) unc-33(e204), e1490* hermaphrodites. Cross progeny were then examined for postdeirids. When the mutant *n372* allele was provided by the mother, ectopic postdeirid lineages were generated in 4/122 animals (3%) as compared to the 11/250 (4%) animals that made extra postdeirids when the *n372* allele was provided by the father, indicating that there is no maternal effect on postdeirid production.

lin-22 Gene Dosage Analysis: Animals of the genotype *n372/n372/+* were generated by crossing single males of the genotype *mDp1(lin-22+); n372 e245 p802; e1490* to *n372 e245 p802 e184; e1490* hermaphrodites. non-Unc F1 cross progeny, which should carry the duplication *mDp1*, were scored for postdeirids. The *mDp1; n372 e245 p802; e1490* strain is itself *n372/n372/+*; however, we did not want to examine this strain directly because non-Unc *n372/+* recombinants are easily generated and could bias the results of a

dosage analysis. To insure that the original male actually carried the free duplication and not a recombinant chromosome, all hermaphrodite F1 cross progeny examined for postdeirids were subsequently allowed to self-fertilize. The F2 progeny of these hermaphrodites were scored for the presence of wild type, Unc and Unc Dpy phenotypes. This confirmed the presence of *mDp1* and both of the *n372* mutant chromosomes.

RESULTS

Background

The V cells are a particular type of stem cell called seam cells that divide during each of the four postembryonic larval stages, L1-L4. Each division generates one seam cell and a second cell that becomes either another seam cell, a neuroblast, or a cell that fuses with a large epidermal syncytium that covers much of the animal. The cell V5.pa (the anterior daughter of the posterior daughter of V5), which is generated during L2, becomes the postdeirid neuroblast. This cell goes on to generate the postdeirid sensillum [consisting of a neuron (PDE) and two support cells] and an additional sensory neuron (PVD) (Sulston and Horvitz, 1977; Way and Chalfie, 1988) For the purposes of this paper we will refer to the group of four cells as a postdeirid. The lineages generated by V cells in wild-type hermaphrodites are shown in Figure 1A.

The *lin-22*$^{+}$ gene prevents V1-V4 cells from producing postdeirids

In the wild type, only one V cell, V5, produces a postdeirid. The *lin-22(n372)* mutation, identified by Fixsen and Horvitz (Horvitz et al., 1983; Fixsen, 1985), causes V cells anterior to V5 to generate postdeirids (Figure 2-1B). Therefore *lin-22* is a candidate for a gene that regulates postdeirid pattern formation. The *n372* allele is semidominant; *n372/+* animals produce ectopic postdeirid lineages at a frequency of about 4%. Either a gain-of-function mutation in *lin-22* or a haplo-insufficient, loss-of-function mutation could produce a semidominant phenotype. In order to infer the wild-type function of this gene, we have therefore isolated additional *lin-22* mutations and asked whether these are gain- or loss-of function mutations by altering gene dosage.

We have isolated two additional *lin-22* alleles, *mu2* and *mu5* (see Methods). Each of these mutations causes V2-V4, and to a lesser extent V1, to generate a postdeirid. (The postdeirid is always produced by a Vn.pa cell). V6 has never been observed to produce a postdeirid. The frequency with which each V cell generates a postdeirid in homozygous *lin-22* mutants and in heterozygous *lin-22/+* animals is shown in Table 2-1. Unlike *n372*, both *mu2* and *mu5* are fully recessive.

Are these *lin-22* mutations loss-of-function or gain-of-function mutations? To learn whether the *n372* semidominant phenotype is the result of a loss- or gain-of-function mutation we have altered gene dosage using the duplication *mDp1*, which carries a copy of the wild-type *lin-22* gene (see Methods). As shown in Table 2-1, adding a wild-type *lin-22* gene copy to *n372/n372* homozygotes reduces the severity of the mutant phenotype seen in *n372/n372* homozygotes. This means that these *lin-22* mutations do not

elevate wild-type gene activity. In that case, adding a wild-type *lin-22* copy should have increased, not decreased, the frequency of ectopic postdeirids. The data are most simply explained by postulating that the *n372* allele reduces or eliminates *lin-22* gene function. *mu2* and *mu5* are fully recessive, so it is likely that these alleles also result in decreased *lin-22* activity. Thus these data argue strongly that the wild-type role of *lin-22* is to prevent V1, V2, V3, and V4 from making postdeirids.

Synopsis of Key Results:

The remainder of the experiments in the paper were carried out by David A. Waring and summarized as follows.

The *pal-1* protein functions cell-autonomously to prevent V6 from making a postdeirid

So far no single mutation has been identified that is sufficient to cause V6 to generate a postdeirid. However, in double mutants that carry both a *lin-22* mutation as well as a reduction-of-function mutation in the gene *pal-1*, V6 generates a postdeirid (Waring and Kenyon, 1990). Thus both *pal-1* and *lin-22* function to prevent V6 from making a postdeirid.

pal-1 genetic mosaics were generated in the presence of the *lin-22* mutation and *pal-1* was shown to act cell autonomously in the V6 lineage to prevent V6 from making a postdeirid.

The ectopic postdeirids that form in *lin-22* and *pal-1; lin-22* mutants require cell-extrinsic signals

Cell-extrinsic signals are known to be required in order for V5 to produce a postdeirid. When V5's posterior neighbor V6 is ablated, V5 fails to form a postdeirid. In addition, when V5's anterior neighbors are ablated, V5 fails to form a postdeirid (Sulston and White, 1980). To determine whether ectopic postdeirids in V1-V4 and V6 require cell-extrinsic signals, postdeirid

formation in *lin-22* and in *pal-1; lin-22* mutants was examined following ablation of specific V cells. It was found that the signals were still present and necessary to generate ectopic postdeirids in both *pal-1* and *lin-22* mutants. This means that the wild type function of both *lin-22* and *pal-1* is somehow to block signal-dependent postdeirid formation in cells other than V5.

Synopsis of Discussion

What role do neighboring cells play in postdeirid formation?

The experiments described here indicate that postdeirid production requires some type of signal from neighboring V cells. In experiments in which all but three V cells are killed, the central cell produces a postdeirid. Thus the normal neighbors of a V cell are sufficient to induce postdeirid formation.

The role played by the presence of neighboring V cells is not at all clear. The data suggest that in order to form a postdeirid, a V cell must be in close or direct contact with V cells on both sides of itself. Why are neighbors on both sides required? It could be that the the level of an intercellular signal produced by only one neighbor is insufficient to induce postdeirid formation, and therefore that close neighbors on each side are required. Alternatively, it is possible that a V cell receives different types of signals from its anterior and posterior neighbors.

It is also possible that disruption of the epithilium *per se* somehow changes the development of these cells. For example a V cell may simply have to be part of an intact epithelium in order to produce a postdeirid neuroblast. The *Drosophila Notch* protein, which affects the determination of many cell types, also affects cell-cell adhesion (Fehon *et al.* , 1990).

One feature of this signaling system that sets it apart from certain others is that a V cell sends postdeirid-inducing signals to its neighbor whether or not it produces a postdeirid neuroblast itself. This differs from the phenomenon of lateral inhibition, in which a cell entering a specific pathway of differentiation signals its neighbor to adopt a fate different from its own. In

cases of lateral inhibition, a cell's ability to signal its neighbor is correlated with its own developmental fate.

pal-1 and *lin-22* prevent cells other than V5 from producing postdeirids in response to intercellular signals

The gene dosage analysis of *lin-22* mutations described here, together with previous analysis of *pal-1* (Waring and Kenyon, 1990) indicates that these *lin-22* and *pal-1* mutations decrease the level of gene product, and therefore that in the wild type, both genes prevent V cells other than V5 from producing postdeirids. These two genes function in distinct spatial domains. *lin-22* functions in V1-V4 and, at least in a *pal-1* mutant, in V6, whereas *pal-1* functions only in V6.

It is clear that both *pal-1* and *lin-22* can function to inhibit postdeirid formation. How do they accomplish this? One possible model, that mutations in these genes allow signal-independent postdeirid formation, has been ruled out since signaling still takes place in both mutants. *pal-1* is a homeodomain protein, which could affect cell fate by acting on the signaling pathway directly. For example, it could repress a gene required for signal transduction, or compete with a signal-dependent transcriptional regulator for binding to a target gene. Alternatively, it could bypass the signaling pathway altogether and directly initiate an alternative cell fate.

The results of this study indicate that together *lin-22* and *pal-1* create a postdeirid prepattern within the lateral epidermis (Figure 2-2). The V cells and their daughters appear morphologically similar to one another. However, because of *lin-22* and *pal-1*, they have different developmental

potentials: only V5 is competent to be induced by extracellular signals to produce a postdeirid. As long as neighboring V cells are present, V5 will express its potential and produce a postdeirid neuroblast. It is not known how *lin-22* and *pal-1* activities are targeted to their respective domains of function, and how V5 is able to escape their effects. Whatever mechanism localizes *lin-22* and *pal-1* ultimately determines the spatial properties of the postdeirid neuroblast pattern.

Similar but distinct regulatory strategies are used in postdeirid and ray pattern formation

The postdeirid pattern is generated during the L2 stage. During the L3 stage in males, certain descendants of the V cells generate another pattern of neurons and epidermal structures called the ray/alae pattern. Rays, which are mating sensilla, are made in posterior body regions by V5 and V6. Alae, which are epidermal structures, are made in anterior body regions by anterior V cells. Intercellular signaling between V cells (or their descendants) is also required for ray pattern formation. Intercellular signals cause certain V cells to produce alae lineages instead of ray lineages. Previously we showed that the gene *pal-1* allows V6 to produce rays by preventing V6 from responding to alae-inducing signals from its neighbor T (Waring and Kenyon, 1991).

Because both the postdeirid and the ray/alae patterning systems involve communication between V cells, the following question arises: does a single signal-transduction event initiate one subprogram that generates both the postdeirid and ray patterns? This seems unlikely since the ablation of V cells themselves affects both patterns, while ablation of the daughters of

the V cells does not affect postdeirid patterning but does affect the ray decision (Fixsen et al., 1985). Thus, although the same type of signaling mechanisms may well operate in both patterning systems, the developmental decisions required for postdeirid and ray patterning are made independently of one another.

There are several differences between the postdeirid and ray pattern formation systems. One striking difference is that the regulatory relationship between *lin-22* and cell signals is opposite in the two cases. In postdeirid patterning, *lin-22,* like *pal-1,* prevents V cells from responding to signals from their neighbors. However, in ray/alae pattern formation, *lin-22* activity instead promotes the effects of intercellular signals by initiating the signal-dependent pathway, alae lineages. This suggests that cell signals function independently of genes such as *lin-22,* which appear to create intrinsic differences between the V cells.

In spite of the differences between postdeirid and ray pattern formation, the experiments presented here indicate that there is a fundamental similarity in the role that cell signals play in the two processes. In both, intercellular signals are required to specify one pattern element; however, the signals themselves do not provide positional information. Instead a spatial pattern forms because cells differ intrinsically in their ability to respond to intercellular signals. This finding is significant because it indicates that this general patterning strategy--creating a specific arrangement of precursor cells which differ in their ability to respond to intercellular signals-can generate very different types of patterns within a single tissue.

Recent studies on the role of growth factors in *Xenopus* pattern formation indicate that inductive signals may play a permissive rather than instructive role in axial patterning in vertebrates. Dorsal and ventral regions

of the ectoderm respond differently to the factor activin in culture, indicating that intercellular signals probably play a permissive role in allowing the expression of a predetermined pattern in this organism as well (Sokol and Melton, 1991). It may be that the strategy of using intercellular signals to trigger expression of a prepattern is actually widespread in development.

Figure 2-1. The lineages of V1-V6 in wild type, *lin*-22, and *pal-1; lin*-22 mutants.

A-C. The lineages of V1-V6 in wild-type and mutant hermaphrodites. In the standard *C. elegans* lineage convention anterior daughters of a cell division are drawn to the left and posterior daughters to the right. Symbols: "O"= cell that joins the hypodermal syncytium, ⊙ = Adult seam cell, "X"= cell death. Archetypal lineages of *lin-22* (Horvitz *et al.*, 1983) and *pal-1; lin-22* animals are shown, along with the wild type lineage for reference (Sulston and Horvitz, 1977). As shown in Table 2-1 and discussed in the text, individual V(1-4).pa cells in any given animal may generate a postdeirid, a hybrid lineage, or a seam cell.

D. Nomarski photomicrograph of the lateral epidermis of a *lin-22*$^{+}$*; him-5(e1490)* late L2 animal. The focal plane is sub-epidermal, so that the postdeirid cells are in focus.

E. Photomicrograph of a *lin-22(mu2); him-5(e1490)* animal.

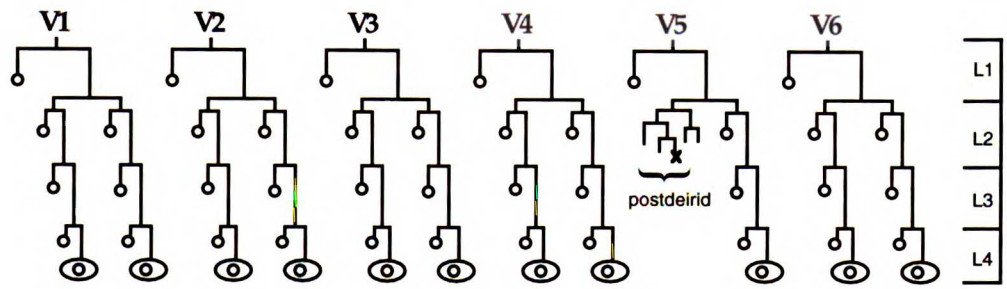
F. The wild-type L2 lineage of V5.p, showing the production of the postdeirid. The fates of the postdeirid cells are shown. PDE and PVD are neurons, PDEso and PDEsh are the postdeirid support cells (socket and sheath).

G. A typical "hybrid" Vn.pa lineage. This lineage is from a V4 cell in a *pal-1(e2091); lin-22(mu2); him-5(e1490)* animal. In this example (which is by far the most common) the V4.pa cell generated a postdeirid (as judged by lineage and cell morphology) and a seam cell which was smaller than the V.pp-derived seam cell. The cells of the postdeirid in the hybrid lineages exhibited the typical compact nuclei seen in wild type postdeirid; however, without EM studies we can not be certain of the fates of the individual cells. Other hybrid lineages have been observed that yield, for example, two neuron-like cells

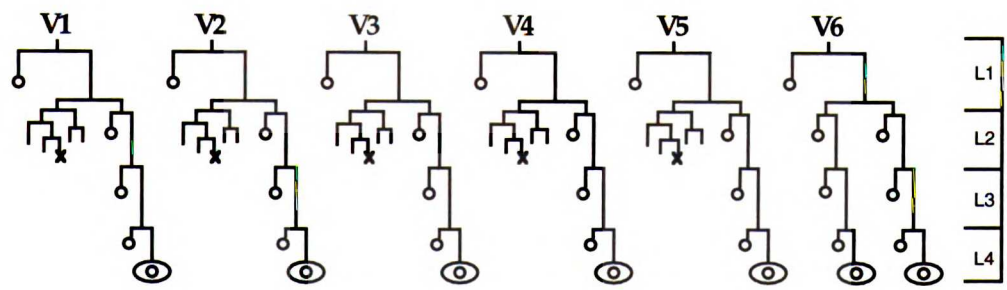
and an extra seam cell or four cells that are neither seam cells or neuron-like cells, but appear to be epidermal in character.

Figure 2-1

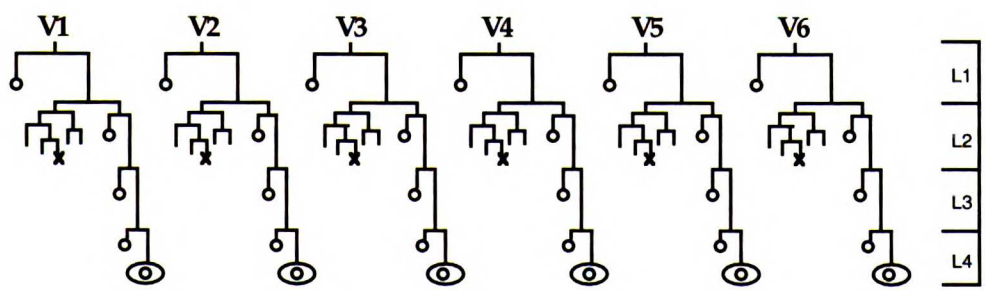
A. wild type



B. *lin-22*



C. *pal-1; lin-22*



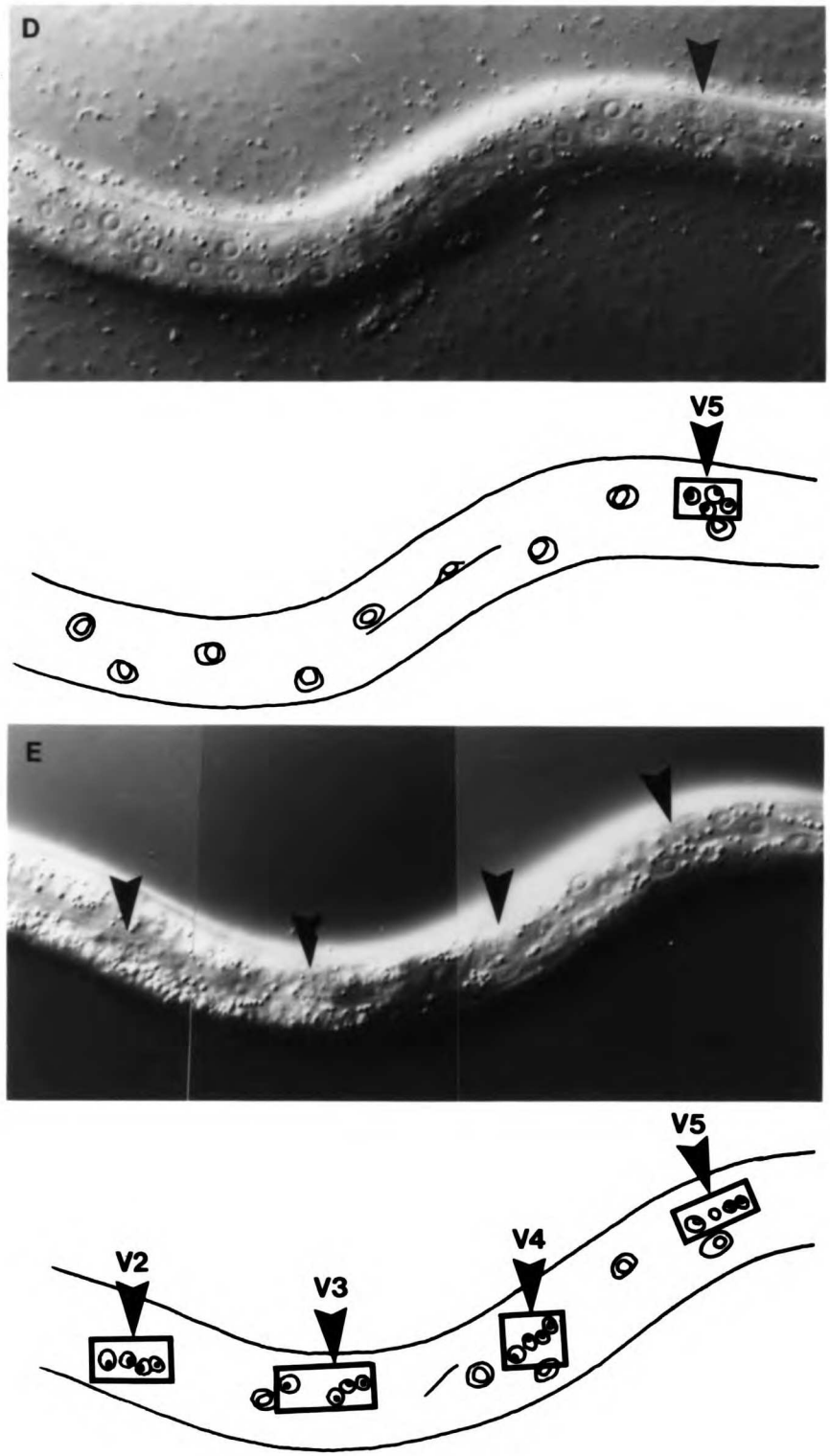
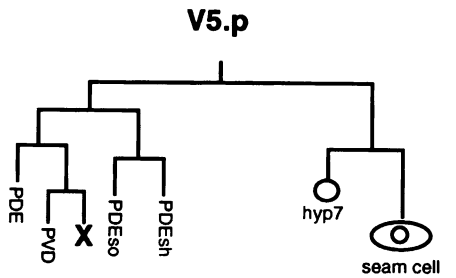

D
V5
E
V5
V4
V3
V2

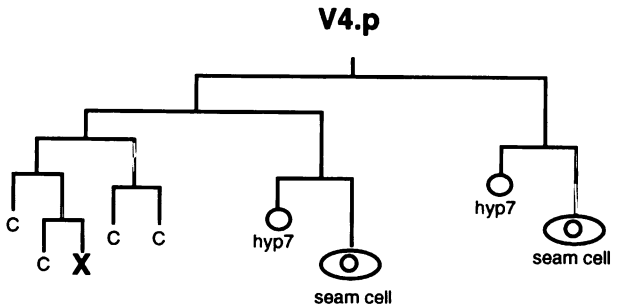
Figure 2-1

F.



wild type postdeirid lineage

G.



"hybrid" Vn.pa lineage

Figure 2-2. Genetic model for establishment of the postdeirid pattern.

In the model, *lin-22* and *pal-1* activity have the effect of preventing intercellular signals between V cells from inducing postdeirids. The products of these genes could block postdeirid induction by acting directly on components of the signal transduction pathway, or they could bypass the effects of signal transduction and act directly on downstream genes. T generates a lineage distinct from any of the V cell lineages, which does not include a postdeirid.

Figure 2-2

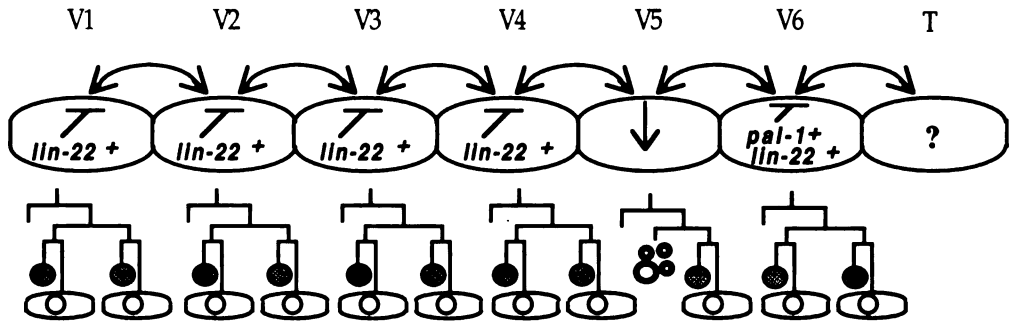


Table 2-1. Postdeirid production in *lin*-22 mutants.

The first number in each column represents the percentage of V cells that produced either a hybrid or complete postdeirid. In *lin-22* animals, Vn.pa cells sometimes generate "hybrid" lineages that display elements of both the wild-type V5.pa and wild-type (V1-V4).pa lineages (Fixsen, 1985). The percentage of V cells that produced hybrid lineages is given in parentheses. n: number of animals examined. The frequency of postdeirids in *n372/372/+* animals is much lower than in *n372/n372* animals, indicating the *n372* mutation does not elevate gene activity. However, the postdeirid frequency in *n372/n372/+* animals is slightly higher than in *n372/+* animals. This could result from some property of the duplication (for example, duplication loss, reduced levels of *lin-22*$^{+}$ gene expression from the duplication, or dosage effects of genes other than *lin-22*), or it could reflect a slight dominant-negative effect of the *n372* mutation. ("*" indicates data generated by D. Waring).

Table 2-1

	Percent Postdeirid Production in *lin-22* Mutants						
	V1	V2	V3	V4	V5	V6	n
+	**0**	**0**	**0**	**0**	**100**	**0**	105
mu5	**21** (5)	**75** (11)	**84** (25)	**90** (15)	**100**	**0**	110
mu2	**39** (5)	**94** (6)	**91** (17)	**95** (19)	**100**	**0**	100
n372	**40** (7)	**88** (10)	**90** (17)	**95** (15)	**100**	**0**	102
mu5/+	**0**	**0**	**0**	**0**	**100**	**0**	168
mu2/+	**0**	**0**	**0**	**0**	**100**	**0**	209
n372/+	**0**	**0.5** (0.3)	**1** (0.3)	**2** (0.5)	**99.7**	**0**	377
n372/n372/+	**0**	**4** (0.9)	**3** (0.9)	**12** (9)	**98**	**0**	114
mu2/mu2/+	**0**	**0.9** (0.9)	**0**	**0**	**99.1**	**0**	106
+/+/+	**0**	**0**	**0**	**0**	**100**	**0**	97
pal-1; mu2 *	**28** (3)	**56** (7)	**72** (18)	**75** (19)	**100**	**70** (15)	102

Acknowledgements

We thank members of the Kenyon lab for discussions, and Kenyon lab members, C. Goutte and J. Gilbert for comments on the manuscript. Special thanks to S. Salser for help with Table 2-1. Some nematode strains were provided by the *Caenorhabditis* Genetics Center, which is funded by the NIH National Center for Research Resources. This work was supported by the NIH. D.W. was supported by the NIH and the Lucille P. Markey Foundation. L.W. was supported by an NSF Predoctoral Fellowship and by the NIH.

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CHAPTER 3

Conserved and unique activities of a *hairy* homolog generate pattern in the *C. elegans* peripheral nervous system

prepared for publication by

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Abstract

The primary pair-rule gene *hairy* plays an important role in establishing metameric pattern in a Drosophila embryo (Carroll, *et al.*, 1988; Lawrence and Johnston, 1989). *hairy* also acts postembryonically to help pattern the peripheral nervous system (PNS) by directly regulating the proneural gene *achaete* (Skeath and Carroll, 1991; Orenic, *et al.*, 1993; Ohsako, *et al.*, 1994; Van, *et al.*, 1994). Here we define the function of a *C. elegans hairy* homolog, the *lin-22* gene. Like *hairy*, *lin-22* patterns the *C. elegans* PNS by regulating the worm *achaete-scute* homolog *lin-32* (Zhao and Emmons, 1995), suggesting that this regulatory relationship has been conserved during evolution. However, unlike *hairy*, *lin-22*plays an additional role in *C. elegans* as a regulator of Hox gene expression and function in creating anterior vs. posterior differences in body pattern.

Results and Discussion

The lateral ectoderm of *C. elegans* consists of a single row of cells, V1-V6, that extends from the head to the tail of the animal. These cells generate either epidermis or neuronal sense organs in a position-specific manner along the anterior-posterior (A-P) axis. Two different sense organs are generated by cells in the posterior (Figure 3-1): the V5 cell makes a structure called a postdeirid and, in males, V5 and V6 generate sensory structures called rays, used in male mating. The formation of these sensory rays is preceded by an extra round of cell proliferation which takes place only in the posterior cells of the male and not in the hermaphrodite (Sulston and Horvitz, 1977).

In *lin-22* mutant animals, extra neuronal structures are generated in place of epidermis (Horvitz, *et al.*, 1983; Fixsen, 1985). Hermaphrodites produce ectopic postdeirids in the anterior, whereas males make both ectopic postdeirids and ectopic sensory rays (Figure 3-1). We followed the cell lineages and found that the anterior cells in males also undergo the extra round of cell division normally found only in the posterior cells. The *lin-22* phenotype therefore resembles a transformation of the anterior cells into a posterior V5-like fate. We say "V5-like" because V5 itself has a slight change of fate in a *lin-22* mutant male, and makes two sensory rays instead of generating just one ray and an epidermal cell.

We cloned the *lin-22* gene to better understand its molecular function in patterning neuroblast production in the V cells . The *lin-22* gene encodes a basic helix-loop-helix protein with striking homology to *hairy* and *hairy*-like genes from *Tribolium* and *Drosophila*, as well as *hairy* and *Enhancer of split* complex [*E(spl)*] homologs from vertebrates (Figure 3-2). The strongest *lin-22* allele (*n372*) contains a missense mutation in a glutamate residue that is

completely conserved in *hairy*, the *E(spl)* genes, and their homologs. A missense mutation of this specific glu residue is found the h^5 allele of *Drosophila* (Wainwright and Ish, 1992). We do not yet know if *lin-22* contains the universally conserved trp-arg-pro-trp tetrapeptide found at the carboxy-terminus of all *hairy*- and *E(spl)*-like proteins. This motif has been shown to be required for the direct interaction of *hairy* protein with the product of the *groucho* gene (Paroush, *et al.*, 1994).

Given that *lin-22* appears to encode a *hairy* homolog, what potential targets of *lin-22* activity can explain its role in worm development? The *C. elegans* gene *lin-32* has recently been shown to be homologous to members of the proneural AS-C genes of *Drosophila* (Zhao and Emmons, 1995) *lin-32* activity promotes the formation of neuroblasts in the posterior V cells: in *lin-32* mutants, neither the postdeirid nor the male sensory rays are made, and instead the neuroblasts that normally generate these structures adopt epidermal fates (Figure 3-3). In animals with mutations in both the *lin-22* and the *lin-32* genes, no neuronal sensory structures are made and the lateral ectoderm generates only epidermis (Figure 3-3). This indicates that the formation of the ectopic sensory structures in a *lin-22* mutant requires *lin-32* activity, and that *lin-22*, like *hairy* and the *E(spl)* genes, acts, directly or indirectly, as an upstream negative regulator of a nematode proneural gene, *lin-32*.

Can all the effects of a *lin-22* mutation be explained as a consequence of deregulated *lin-32* activity? Expression of the *lin-32* gene using a heat-shock promoter suggests that *lin-32* activity is sufficient to induce sensory structures in the lateral ectoderm of both wild type and *lin-32* mutant animals (Zhao and Emmons, 1995). Misexpression of *lin-32* in a *lin-22* mutant could therefore account for the production of the ectopic sensory structures seen in

a *lin-22* mutant. This misregulation may explain the entire extra-postdeirid phenotype of a *lin-22* hermaphrodite, but it cannot account for the observation that *lin-22; lin-32* double mutant males still undergo the ectopic round of cell proliferation caused by the loss of *lin-22* function. In addition, while the loss of *lin-32* activity causes the sensory ray neuroblasts to generate epidermal cells, *lin-32* does not affect the first step in ray development (the division of the ray precursor parent cell into what would normally be the ray neuroblast and its epidermal sister cell). In *lin-22; lin-32* double mutant males this first step in sensory ray formation still occurs in ectopic positions in the anterior of the worm (Fig. 3-3).

What remaining targets of *lin-22* repression could explain these remaining ectopic events seen in the *lin-22; lin-32* male? One good candidate is the gene *mab-5*. *mab-5* is the *C. elegans* homolog of the *Antennapedia* gene, a Hox gene involved in patterning the anterior-posterior axis in *Drosophila*. The role of *mab-5*, an Antennapedia homolog, is to specify the correct fates of cells in the posterior body region of the worm, including V5 and V6 (Kenyon, 1986; Costa, *et al.*, 1988). In particular, *mab-5* is required for both cell proliferation and sensory ray production in the posterior (Fig. 3-1c). We wondered whether deregulation of *mab-5* activity could explain the transformation of the anterior cells V1-V4 into the posterior V5-like fate seen in *lin-22* mutants, so we examined the expression pattern of the *mab-5* protein in a *lin-22* background. We found that early in the V5 lineage the *mab-5* expression pattern was the same as that found in wild type. However, in a *lin-22* mutant V5 makes two sensory rays instead of one, and we saw this reflected in extra *mab-5* staining later in the V5 lineage (Figure 3-3). As for the anterior cells V1-V4, the pattern of *mab-5* expression is now identical to that seen in the V5 cell in *lin-22* mutants.

This change in the *mab-5* expression pattern shows that *lin-22*, a *C. elegans hairy* homolog, establishes a difference between the anterior vs. posterior body region by repressing Hox gene expression outside of its normal domain of function. This activity has not been observed for *hairy* or *E(spl)* genes. *hairy* can affect Hox gene activity within specific segments, but this appears to be due to the effects of *hairy* mutations on downstream pair-rule and segment polarity genes known to be Hox regulators. For example, the hairy mutation indirectly alters the expression of the Hox gene *Abdominal-A* through its effects on *fushi tarazu* and *engrailed* (Macias, *et al.*, 1994). However, even in this example, the domain of Hox gene expression remains intact and only the levels of *Abd-A* expressed within this domain are affected.

mab-5 is required for proliferation and sensory ray precursor formation in V5 and V6, and *mab-5* alone is sufficient to generate cell proliferation and ray formation when expressed ectopically using a heat-shock promoter (S. Salser, pers. comm.). Therefore, it might be expected that the production of ectopic cell divisions and sensory rays could be dependent on *mab-5* activity as well. However, the proliferative divisions are generally unaffected by the loss of *mab-5* activity in a *lin-22* mutant (Figure 3-1c), suggesting that ectopic *mab-5* expression is not wholly responsible for the extra cell divisions seen in *lin-22*. However, *mab-5* is required for the formation of many of the ectopic sensory rays seen in *lin-22* males, since the loss of *mab-5* activity leads to the generation of far fewer rays by the lateral ectodermal cells (Figure 1c; Kenyon, 1986).

As ectopic activity of the Hox gene *mab-5* can account for most of the rays produced in a *lin-22* background, we decided to see whether other Hox gene activities might be responsible for the residual rays seen in *mab-5; lin-22* males. These genes, *lin-39* and *egl-5*, are expressed in the lateral ectoderm and

can both affect sensory ray production. Mutations in *egl-5* result in the loss of one of the V6 sensory rays and affect the identities of the remaining ones (Chisholm, 1991; Chow and Emmons, 1994). *lin-39* mutations affect only cells of the central body region, and do not normally affect the sensory rays (Clark, *et al.*, 1993). However, ectopic expression of *lin-39* can lead to the occasional formation of a sensory ray in males (Hunter and Kenyon, *Nature* in press).

We found that the residual ray production seen in a *mab-5; lin-22* male is due in part to the activities of both *lin-39* and *egl-5*. The removal of either gene activity leads to a reduction in sensory rays, but only within that gene's normal domain of function (data not shown). However, the protein expression pattern of *lin-39* appears unaffected by a mutation in *lin-22* (data not shown). This suggests that while mutations in *lin-22* may not cause an obvious change in the level or pattern of *lin-39* (and possibly *egl-5*) expression, the activity of this gene is nonetheless affected by loss of *lin-22* function. Perhaps the loss of *lin-22* affects a threshold for neuronal-versus-epidermal fate decisions, such that levels of *lin-39* and *egl-5* activity that would not normally be neural-promoting can now function to make ectopic neurons. A small number of rays are still generated randomly along the A-P axis in *lin-22* mutant animals containing all the known Hox mutations. These rays could result from the activity of the final *C. elegans* Hox gene, a *labial* homolog for which there is as yet no known mutation. Alternatively, these rays could be the products of elevated *lin-32* activity.

We have shown that a *C. elegans* gene with homology to the *hairy* gene of Drosophila regulates neuroblast formation, A-P patterning, and cell proliferation in the lateral ectoderm. Two of the potential targets of *lin-22* activity have been identified. In the neurogenesis pathway *lin-32*, an *achaete-scute* homolog, acts downstream of *lin-22* to facilitate sense organ formation.

In A-P patterning, the Hox gene *mab-5* appears to be repressed by *lin-22*. Finally, *lin-22* activity negatively regulates an additional unidentified gene function that permits or promotes cell proliferation. Loss of either *mab-5* or *lin-39* activity, or both (data not shown), does not completely remove the L3 proliferative divisions seen in *lin-22* males (although the frequency is somewhat reduced). The Hox genes may again be substituting for each other in a promiscuous manner at this step in development in a *lin-22* mutant. However, it may be that *lin-22* acts on an unidentified target gene to regulate cell proliferation in the anterior lateral ectoderm. This activity is interesting in light of the recent observation that *hairy*, acting with *extramacrochaete* (an inhibitor of the proneural gene *scute* (Van, *et al.*, 1991)), appear to act together to block cell-cycle progression and proliferation in the *Drosophila* eye imaginal disc (Brown, *et al.*, 1995). Taken together, these findings argue that the *C. elegans* PNS is patterned using a combination of conserved and, potentially, unique regulatory relationships between a *hairy* homolog and its targets.

Figure 3-1. Comparison of V cell fates in wild type, *lin-22*, *mab-5*, and *lin-32* mutant animals.

a. V cell lineages in wild type and *lin-22(n372)* hermaphrodites. Anterior is to the left. Divisions at the first (L1), second (L2), third (L3), and fourth (L4) larval stages are shown. ○ = epidermal cell fate. △ = epidermal tail seam cell fate. "X" = cell death. The formation of a sensory ray in the adult is indicated by ◥, while epidermal alae is shown as ≡. The postdeirid cells are shown bracketed. "?" = unfinished lineage. Unmarked V cell daughters fuse with hyp7, an epidermal syncytium. In the wild type hermaphrodite all the V cells divide to generate epidermal alae; however, the V5.pa cell also makes a neuronal structure called a postdeirid (consisting of 2 neurons and 2 support cells) during L2. In *lin-22* animals, two of the anterior V cells can also be seen generating postdeirids during the L2 stage. Ectopic postdeirids can be generated by V1-V4, but the frequency of postdeirid formation by V1 is only 6%, as compared to 79-91% for V2-V4. Interestingly, the V4.pa branch of this lineage divided an extra time in the L3 stage and later produced a single sensory ray during L4. This extra L3 division and the generation of sensory rays normally occur only in the male posterior body region, and these structures are found ectopically in *lin-22* males (see below). This phenotype was not seen in animals lineaged previously (Fixsen, 1985).

b. V cell lineages in wild type and *lin-22* males. In wild type males V1-V4 adopt the same epidermal fates seen in the hermaphrodite. The male V5 cell also produces a postdeirid in L2. Unlike hermaphrodites, however, wild type males generate additional sensory rays from V5 and V6 during L4. The generation of these rays is preceded by an extra cell division during the L3 stage. Males mutant for *lin-22* generate ectopic postdeirids in V2-V4 at the

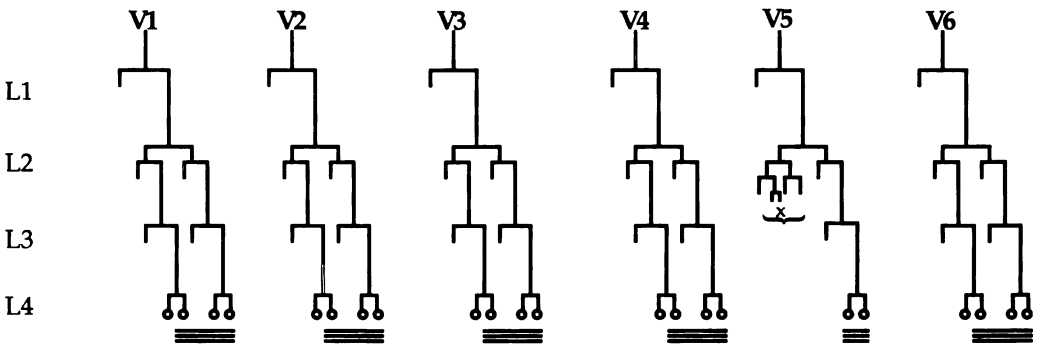
same frequency as hermaphrodites, but the frequency of postdeirid production by V1 is significantly higher (43%). In addition, the anterior V cells undergo an extra round of cell division in L3, and can also generate ectopic sensory rays. Polarity reversals often occur during the L3 proliferative and L4 ray precursor cell divisions, but only in anterior V1-V4 cells (polarity reversals were seen in 46% of these divisions in a sample of 6 animals lineaged). These reversals appear to occur in the ray neuroblast divisions as well.

c. V cell lineages in wild type, *mab-5*, and *mab-5; lin-22* males (Kenyon, 1986). Loss of *mab-5* function leads to the loss of the L3 proliferative divisions in V5 and V6 and blocks the formation of sensory rays. Loss of *mab-5* function in a *lin-22* mutant, however, does not lead to the complete loss of the L3 proliferative divisions in any of the V cell lineages.

METHODS. Lineages were carried out at 20°C as described in (Sulston and Horvitz, 1977). All strains scored were mutant for *him-5(e1490)*, which increases the number of male progeny. The *lin-22* allele used was *n372*. *lin-22* male #1 is a composite lineage of V1-V4 from one male and V5-V6 from a second male. The *mab-5(e1239)* and *mab-5; lin-22* lineages were from Kenyon (1986), but an additional 12 animals were scored for the presence of L3 proliferative divisions in the V cells.

Figure 3-1

a. wild type hermaphrodite



lin-22 hermaphrodite

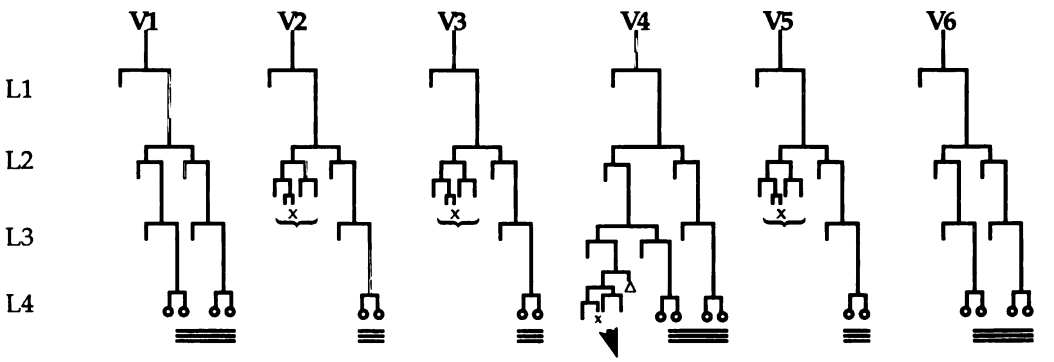
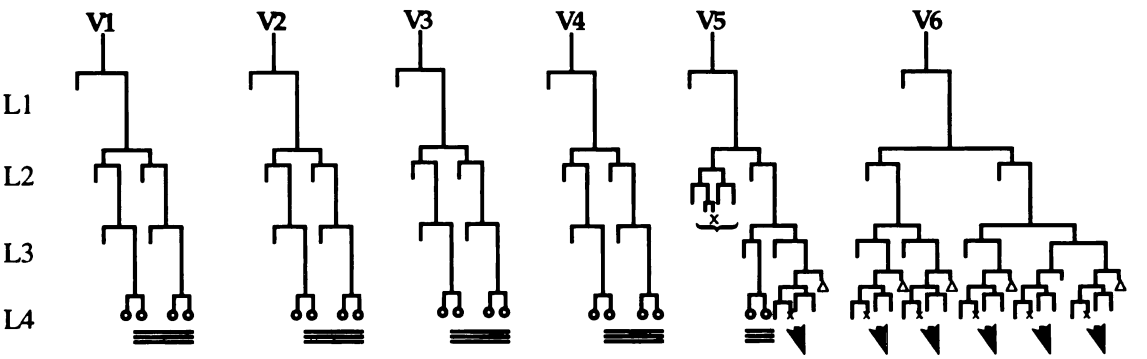
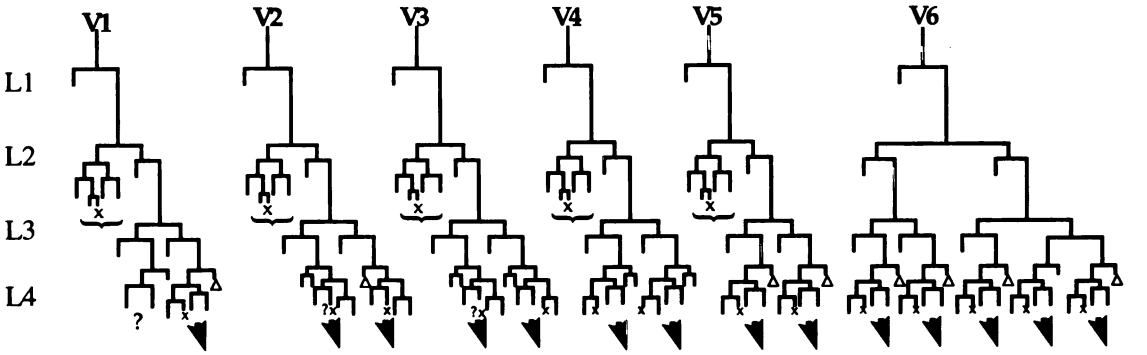


Figure 3-1

b. wild type male



lin-22 male#1



lin-22 male#2

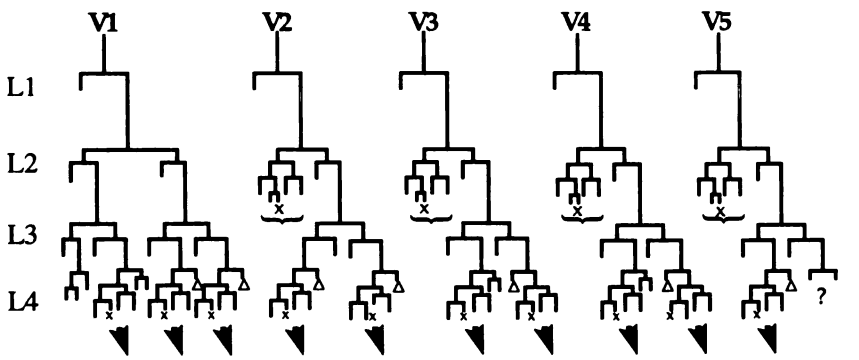
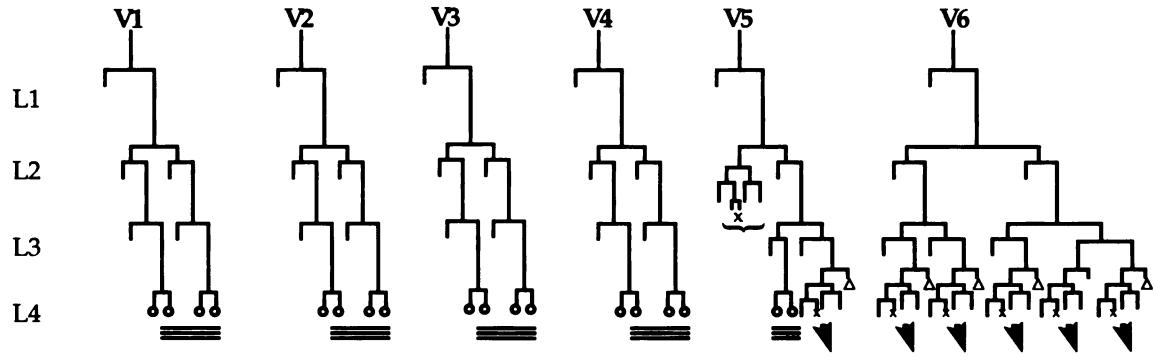
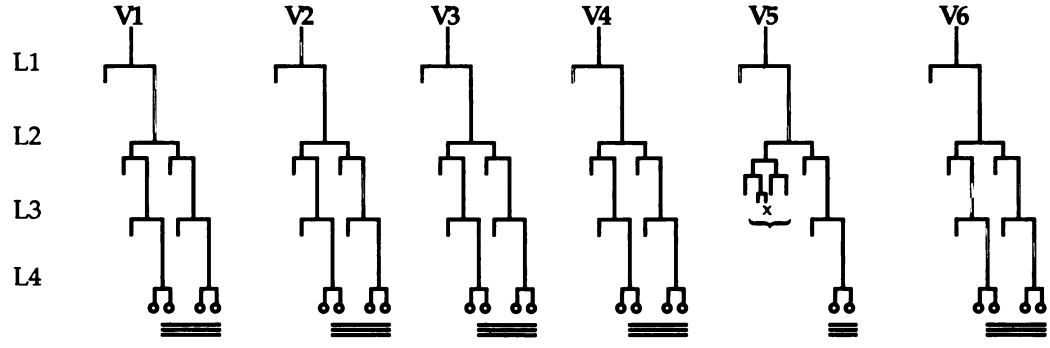


Figure 3-1

c. wild type male



mab-5 male



mab-5 lin-22 male

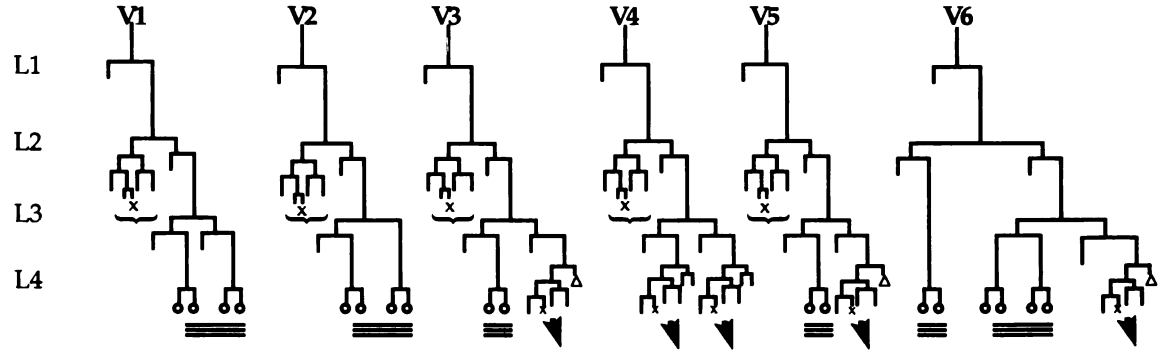


Figure 3-2. *lin-22*, a basic helix-loop-helix protein, is homologous to the *hairy* gene of *Drosophila*

a. The known *lin-22* cDNA sequence. The DNA sequence is listed on top of the predicted protein sequence. Boxed DNA contains the SL1 splice leader sequence. Arrows denote splice junctions. The conserved basic helix-loop-helix domain is underlined. That this gene encodes *lin-22* has been demonstrated by identifying the DNA lesion in the *n372* allele. *n372* contains a missense mutation that alters a conserved glutamate residue to a lysine residue in the basic region of the protein (marked with an "*"). Interestingly, a mutation of this identical glutamate to a lysine is also found in the strongest allele (*u282*) of *lin-32* (Zhao and Emmons, 1995).

b. Comparison of predicted *lin-22* protein with closely related sequences. *lin-22* is displayed on top followed by the top four homologous proteins (excluding mouse *HES-1*) as predicted using BLAST: *Tribolium hairy* (Sommer and Tautz, 1993), the human *hairy*-like protein (Feder, *et al.*, 1994), *HES-1* protein from rat (Sasai, *et al.*, 1992), and *deadpan*, (Bier, *et al.*, 1992). Also included are the sequences of *hairy* and the E*(spl) m7* transcript from *Drosophila* (Klambt, *et al.*, 1989; Rushlow, *et al.*, 1989). The basic helix-loop-helix domains are indicated above the sequences and conserved amino acids are boxed. The glutamate residue altered in the *n372* allele is marked with an "*" below the E*(spl) m7* sequence. This glutamate is 100% conserved among all other members of this family, and in fact the *h5* allele of *hairy* changes this glutamate to a valine (Wainwright and Ish, 1992). The actual C-terminus of the cDNA has not yet been identified. All of the DNA C-terminal to the second helix contained on the 15 kb rescuing genomic fragment (3.5 kb in all) has been sequenced and does not contain the canonical trp-arg-pro-trp

sequence found in virtually all other *hairy* and *E(spl)* homologs. A potential coding sequence containing trp-lys-ile-trp-val-trp has been identified, and may serve as the C-terminus of *lin-22*. This is interesting because in *Drosophila* two pro-to-leu mutations found in this C-terminal tetrapeptide cause loss of *hairy* function.

METHODS. A *Tc1* transposon genetically linked to the *lin-22* gene was identified and cloned (Costa, *et al.*, 1988). The genomic DNA flanking this *Tc1* was subcloned and used to identify 8 Yeast Artificial Chromosomes (YACs) generated by the *C. elegans* genome center in St. Louis. These 8 YACs were gel isolated, purified and injected independently into the germline of *lin-22* hermaphrodites, along with the dominant cotransformation marker *rol-6* (Mello, *et al.*, 1991). Marker rescue was only seen after two generations in animals containing stable arrays (lines) of the injected DNA, and rescue was observed for only a single YAC, Y68H6, at a frequency of 14-25% transformed lines. The entire YAC (240 kb) was used as a radiolabeled probe against an unamplified *C. elegans* genomic lambda library after being hybridized for two hours with unlabeled total genomic DNA to "remove" labeled repetitive DNA (Church and Gilbert, 1984; Sambrook, *et al.*, 1989; Frazer, *et al.*, 1992). 28 clones were identified, put into 6 pools, and injected into *lin-22* hermaphrodites. A single clone containing 15 kb of genomic DNA was found to rescue the *lin-22* phenotype. Lambda subclones were sequenced (Sequenase, USB) and a region homologous to members of the *hairy* family of helix-loop-helix proteins was identified. Using primers to the bHLH domain, we confirmed that this gene was *lin-22* by PCR-sequencing reverse-transcribed total RNA from wild type and the three *lin-22* mutant alleles (Superscript II; dsDNA cycle sequencing system, GIBCO BRL). The structure and sequence of the *lin-22* cDNA was confirmed with the genomic DNA sequence.

Figure 3-2

a.

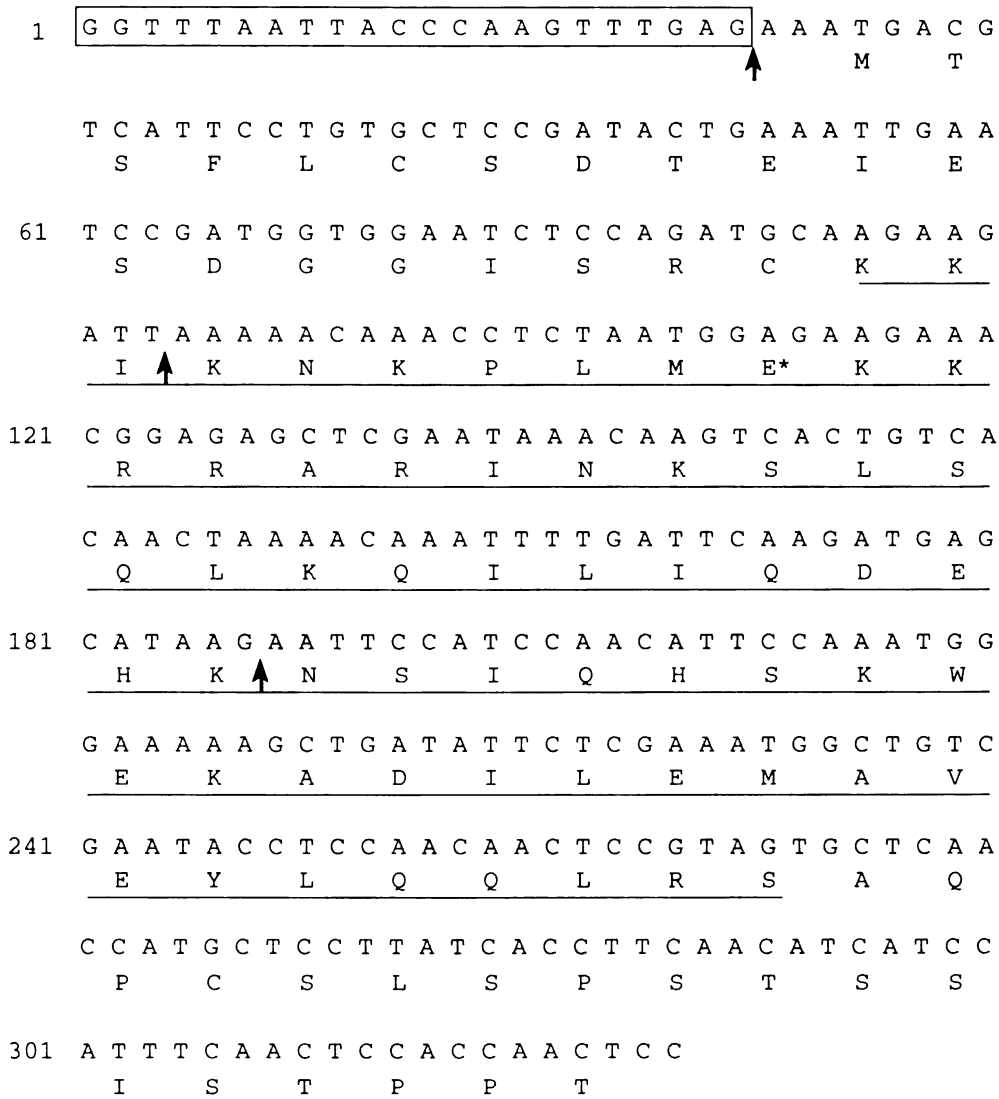

1 G G T T T A A T T A C C C A A G T T T G A G A A A T G A C G
M T
T C A T T C C T G T G C T C C G A T A C T G A A A T T G A A
S F L C S D T E I E
61 T C C G A T G G T G G A A T C T C C A G A T G C A A G A A G
S D G G I S R C K K
A T T A A A A A C A A A C C T C T A A T G G A G A A G A A A
I K N K P L M E* K K
121 C G G A G A G C T C G A A T A A A C A A G T C A C T G T C A
R R A R I N K S L S
C A A C T A A A A C A A A T T T T G A T T C A A G A T G A G
Q L K Q I L I Q D E
181 C A T A A G A A T T C C A T C C A A C A T T C C A A A T G G
H K N S I Q H S K W
G A A A A A G C T G A T A T T C T C G A A A T G G C T G T C
E K A D I L E M A V
241 G A A T A C C T C C A A C A A C T C C G T A G T G C T C A A
E Y L Q Q L R S A Q
C C A T G C T C C T T A T C A C C T T C A A C A T C A T C C
P C S L S P S T S S
301 A T T T C A A C T C C A C C A A C T C C
I S T P P T

Figure 3-2

b.

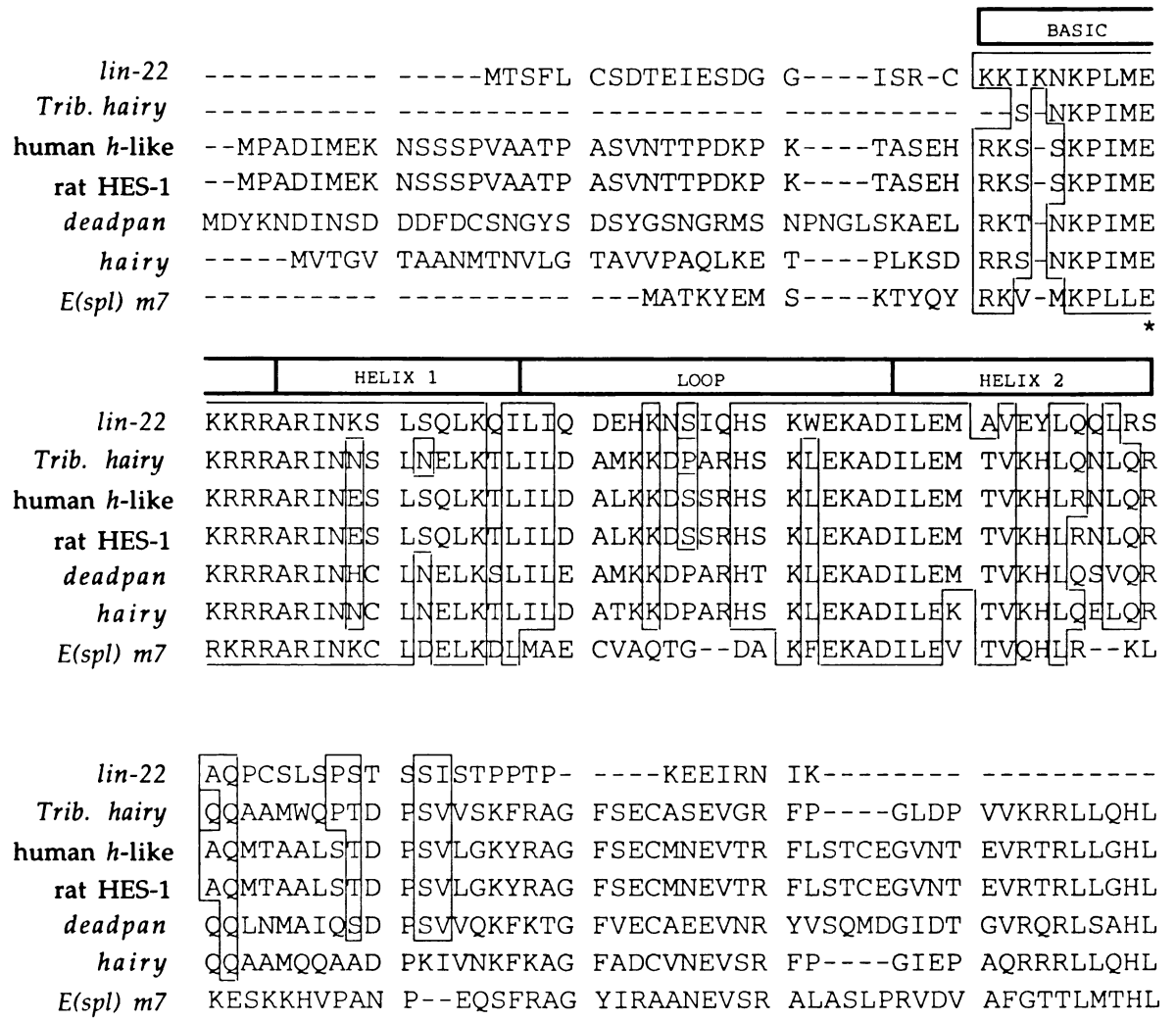

BASIC
HELIX 1
LOOP
HELIX 2
lin-22
Trib. hairy
human h-like
rat HES-1
deadpan
hairy
E(spl) m7

Figure 3-3. Efects of the *lin-32* mutation on wild type and *lin-22* males.

○ = epidermal cell fate. △ = epidermal tail seam cell fate.  = the postdeirid lineage. ■ = a ray neuroblast lineage (). "X" = cell death. L3 proliferative divisions are shown as bold lines. Mutations in *lin-32* prevent the formation of larval neuroblasts (Zhao and Emmons, 1995). As a consequence, in *lin-32* mutants the V5 postdeirid is not generated, nor are the V5 and V6 sensory rays, and epidermal cells are made instead. However, the extra L3 proliferative divisions seen in V5.ppp and the V6 descendants still occur in a *lin-32* mutant. In addition, although the ray neuroblast adopts an epidermal fate, its parent, the ray precursor cell, still divides to generate what would have been the ray "neuroblast" and its sister, the tail seam cell (Zhao and Emmons, 1995). Males with mutations in both *lin-22* and *lin-32* still fail to generate either postdeirid or ray neuroblasts, but the extra L3 proliferative divisions and the generation of ectopic ray precursors (as is seen by the formation of ectopic tail seam cells, albeit without the associated ray neuroblast) still occurs in the anterior V cells.

METHODS. C. K. lineaged(n=3)*lin-32(e1926)* males for the L3 proliferative divisions and (n=12) males for the ray precursor cell fate, and also lineaged (n=2) *n372; e1926* males for both. *n372; e1926* (n=61) was scored for postdeirid production in L2 as described in (Waring, *et al.*, 1992). L3 proliferative divisions were specifically lineaged for *u282* (n=3) and *n372; u282* (n=2). The formation and location of tail seam cells was analyzed during L4 lethargus for both *u282* (n=14) and *n372; u282* (n=7). Tail seam cells can be identified by the formation of characteristic syncytial tail seam bags (Sulston, *et al.*, 1980).

Figure 3-3

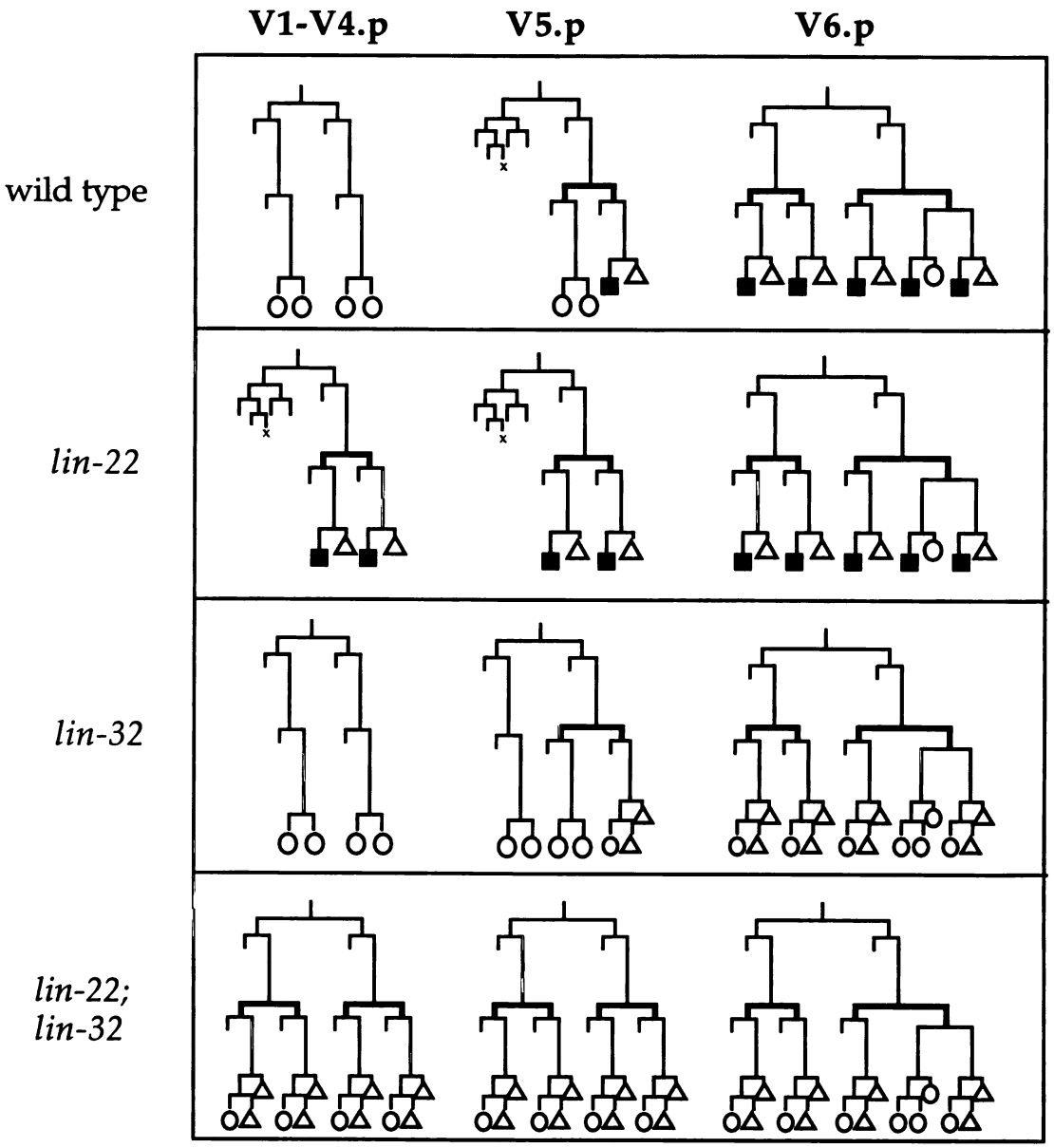


Figure 3-4. Mab-5 protein expression in wild type and *lin*-22 males.

a-b. anti-Mab-5 antibody staining in the anterior (**a**) and posterior (**b**) of a wild-type male during the beginning of the L3 stage (approx. 25 hours post-hatching at 20°C). No anterior staining is present. In contrast, all the V6 daughters express Mab-5. Only the two posterior daughters of V5 (V5.ppppa and V5. ppppp) that will make a sensory ray are found to express Mab-5 in the V5 cell. The location of the Vn.pap and Vn.ppp daughters are indicated by the lineage drawings.

c-d. Mab-5 protein expression ina *lin*-22(-) male. All the daughters of V6 stain as in wild type, but now the daughter cells from both branches of the V5 lineage express Mab-5 (**d**). Interestingly, the expression in the anterior branch is consistently weaker than that seen in the posterior branch. In addition, Mab-5 expression is now seen in the anterior V cell descendants of V1 and V2 (**c**), and V4 (**d**). Expression in the anterior cells mimics the pattern seen in V5, with anterior the anterior daughters staining more weakly.

e-f. Schematic of Mab-5 expression seen in V1-V6 in wild type (**e**) and *lin*-22 (**f**) males. Time post-hatching is given at the right. The wild type pattern is that seen by S. Salser (pers. comm.). In *lin*-22, the V6 pattern of expression is identical to that seen in wild type. Early V5 staining is also identical: initially mab-5 is not expressed during the first larval stage (≈ 0-16 h post hatching) but subsequently comes on during the second larval stage (L2; 16-25 h post hatching). This staining persists throughout the L2 stage until just before the proliferative L3 division (at ≈ 27-28 h). Staining appears after the first L3 division in only the posterior daughter, but then comes on in both branches after the next division. This identical pattern of expression is displayed by the anterior V cells (including the H2 cell of the head region) in a *lin*-22 mutant.

The numbers of *lin-22* animals examined at each stage was ≥ 8 for the following time points: 4-6h, 8-10h, 15-17h, 19-21h, 21-23h, and 25+ hours. The pattern of mab-5 expression appears to be identical for *lin-22* hermaphrodites and males through the L2 stage. After the L3 divisions, the number of hermaphrodites expressing ectopic *mab-5* drops to only 11/25 as opposed to 25/27 in males. Generally, if a hermaphrodite had anterior staining, it was weak and seen in fewer cell than found in the male.

METHODS. Animals were staged and stained for *mab-5* expression using a polyclonal antibody following the protocol of S. Salser, pers. comm. Animals were scored using fluorescence microscopy at 100x magnification, and those with unambiguous morphology and obvious staining were scored. Animals from timepoints with weak or nonexistent anterior staining (i.e.. 21-23 h or later hermaphrodites) were first selected on the basis of good morphology and clear V6 staining. DAPI staining was used to locate and identify the nuclei of V cell daughters. L3 males and hermaphrodites were distinguished on the basis of their tail morphology and the number of cells in the tail as assessed using DAPI.

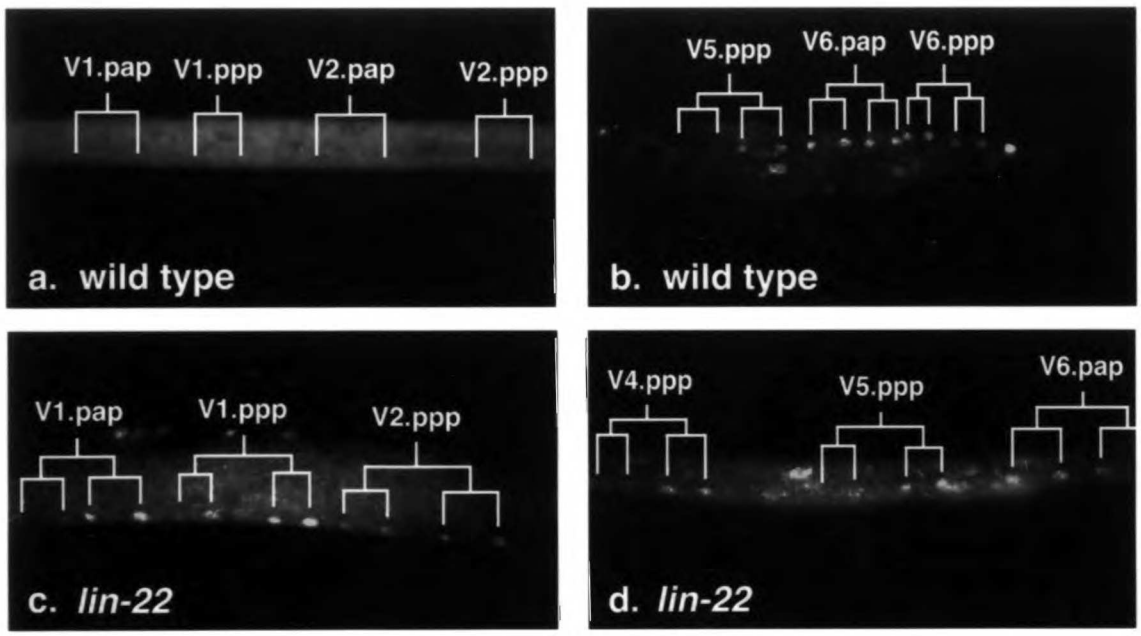
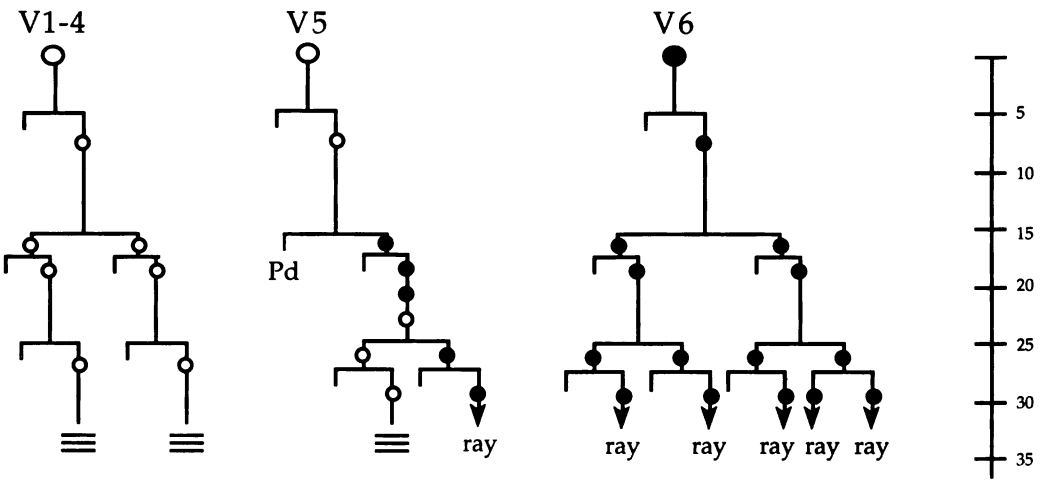
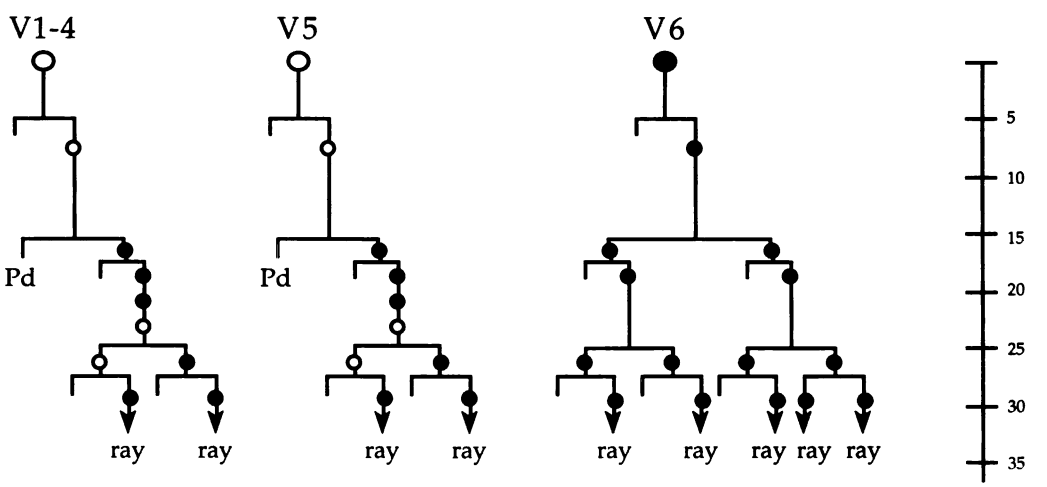

V1.pap
V1.ppp
V2.pap
V2.ppp
a. wild type
V5.ppp
V6.pap
V6.ppp
b. wild type
V1.pap
V1.ppp
V2.ppp
c. lin-22
V4.ppp
V5.ppp
V6.pap
d. lin-22

Figure 3-4

e. wild-type



f. *lin-22*



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CHAPTER 4

Further genetic and molecular characterization of *lin-22* function

Summary of Genetic Data

Additional analysis and comparison of the three *lin-22* alleles

Figures 4-1 through 4-3 display early larval lineages of lateral ectodermal cells in *lin-22(n372)* mutant animals. The effects of the *lin-22* mutation are first seen during the second larval stage (L2), when the cells V1-V4 adopt the V5 cell fate and generate postdeirids in both hermaphrodites and males. During the L3, the anterior V cells of the male also undergo the posterior-specific proliferative division that occurs at this time. Interestingly, we discovered that the H2 cell of the head ectoderm, the cell just anterior to V1, was also affected in a *lin-22* mutant. In the wild type, the H2 cell simply undergoes a single division during the L2 stage. However, in *lin-22* mutants H2 adopted a V cell fate, and was seen to undergo a second round of cell division at the beginning of L2. H2 never appeared to generate an ectopic postdeirid during L2, but the H2 daughters in males were capable of undergoing the L3 proliferative division (and of making sensory rays). *lin-22* was previously thought to only affect the V cells (Fixsen, 1985), but now we know its domain of activity extends more anteriorly.

Figure 4-3 indicates the extent to which polarity reversals could be found in the ectopic L3 divisions seen in the *lin-22(-)* male V cell lineages. In the initial characterization of *lin-22*, occasional polarity reversals were mentioned but not described (Fixsen, 1985). We find that these reversals occur with a frequency of 39% for the L3 divisions shown in Figure 4-3. An analysis of the L3 proliferative and ray precursor divisions (to make a tail seam and a ray neuroblast cell; see Figure 3-1b) from all animals lineaged show that up to 46% of these divisions exhibit a polarity reversal. Reversals

also can occur in the ray neuroblast lineage itself (Figure 3-1b). Polarity reversals can occur in the V5 and V6 lineages after ablation of neighboring cells (Sulston and White, 1980), so mutations in *lin-22* appear to cause the same effects as removing cell signaling. More evidence for the importance of signals in regulating cell polarity comes from the analysis of *lin-44* and *lin-17*. These two mutants cause cellular polarity reversals (Sternberg and Horvitz, 1988; Herman and Horvitz, 1994) and are homologs of *wingless* (*lin-44*; M. Herman, pers. comm.) and *frizzled* (*lin-17*; Sawa and Horvitz, pers. comm.). These two genes are implicated in cell signaling in *Drosophila* (Krasnow and Adler, 1994; Park, *et al.*, 1994a; Park, *et al.*, 1994b; Siegfried, *et al.*, 1994; Vincent and Lawrence, 1994).

Figure 4-4 examines the differences in alae production by the three *lin-22* alleles at 20°C. Failure to produce alae in a *lin-22* male is a reflection of the extent to which the V cells have adopted ectopic neuronal fates: the greater the anterior-to-posterior transformation, the fewer the number of epidermal seam cells made, which results in the loss of alae. Figure 4-4 suggests that the alleles *n372* and *mu2* are phenotypically very similar. However, it is clear that *mu5* generates much more alae than either *mu2* or *n372*, and could therefore be a weaker allele of *lin-22*. This is also supported by the observation that *mu5* males appear to mate more efficiently than either *mu2* or *n372*.

All three alleles of *lin-22* were tested for temperature-sensitivity of alae production (Figure 4-5). The data shows that for all three alleles, alae production was greatly reduced at 25°C relative to 16°C or 20°C (which appeared similar to one another). This could indicate that all three alleles of *lin-22* produce a temperature sensitive gene product. However, the temperature sensitivity of ray production was also observed for the *pal-*

1(e2091) mutation (D. Waring, pers.comm.), so it appears that the process of sensory ray formation itself may be inherently temperature sensitive.

To further examine the potential temperature-sensitive nature of the *lin-22* alleles, we analyzed the effects of temperature on the formation of ectopic postdeirids in *lin-22(n372)* (Figure 4-6). There was a subtle increase in the number of postdeirids formed at higher temperatures, consistent with either the *lin-22* gene products being temperature-sensitive or with a general temperature-sensitivity for neuronal production.

Ectopic postdeirid production by the V cells in *lin-22*(-) is not uniform, and V1 generates postdeirids at a much lower frequency than V2-V4 (Fixsen, 1985; Waring, *et al.*, 1992). However, when examined more fully (Figure 4-7), we found that the production of postdeirids by V1 is strongly dependent on the sex of the animal. Hermaphrodites were found to seldom generate postdeirids from V1 (6%), while males made V1 postdeirids over 40% of the time. In both cases V2-V4 made postdeirids at a roughly equal frequency of 79-91% for hermaphrodites and 76-94% for males.

lin-22 mutations do not have dramatic, if any, affects on the fates of the Q cells and their descendants. However, when analyzing a strain containing 3 copies of the *lin-22* gene (using the free duplication *mDp1*), we saw that 19% of the left sides had Q cells anterior to their wild type positions (Figure 4-8). Since *mab-5* regulates the position of the QL daughters (Kenyon, 1986; Salser and Kenyon, 1992), and since *lin-22* appears to regulate *mab-5*, it is likely that this Q cell phenotype is due to extra copies of *lin-22* inhibiting *mab-5* activity in the QL cells.

Effects of the Hox genes on the *lin-22(n372)* postdeirid and sensory ray phenotypes

Mutations in the Hox gene *mab-5* lead to a V cell phenotype that is roughly opposite to that seen in *lin-22* mutants: the posterior V cells fail to undergo their L3 proliferative divisions, and adopt epidermal, instead of neuronal, cell fates. Based on the phenotypes, it might be expected that one gene would be found to be genetically epistatic to the other in the formation of sensory rays vs. alae. The *mab-5; lin-22* double mutant was first analyzed by Kenyon (1986), who showed that no clear epistasis exists between the two mutations: the generation of rays was reduced, particularly in the anterior, but not eliminated or unaffected in the double mutant (see Figure 4-9). Kenyon (1986) altered the dosage of *mab-5* in a *lin-22* mutant background and found that as the number of copies of *mab-5* increased, the number of rays generated also increased (see also Figure 4-10). I found that when the copy number of *lin-22* is decreased in a *mab-5* mutant background, the number of rays generated also increases. This data supports the original *mab-5* dosage data (Kenyon, 1986), and further suggests that the *mab-5* and *lin-22* genes are functionally antagonistic and might act to negatively regulate each other.

The ectopic rays produced in the *mab-5; lin-22* double mutant are in part due to the activities of the remaining Hox genes *lin-39* and *egl-5* (Figure 4-11 and 4-12). *lin-39* is a *Deformed/Sex-combs-reduced* homolog that affects the fates of cells in the central body region (Clark, *et al.*, 1993; Wang, *et al.*, 1993). *egl-5* is homologous to *Abdominal-B*, and affects the fates of numerous cells in the tail region (Chisholm, 1991). The removal of either gene activity from the *mab-5; lin-22* double mutant leads to the reduction of rays from the particular body region in which these genes normally function: loss of *lin-39*

leads to fewer rays made in the central body region, while loss of *egl-5* removes rays from the tail. However, removing the activities of all three Hox genes from a *lin-22* male does not abolish ray formation. In fact, it appears from the graph that rays are made at a fairly high frequency in the triple Hox mutant. Unfortunately, all we can really say from this data is that the Hox genes *lin-39*, *mab-5*, and *egl-5* are not strictly required for ray formation. The actual numbers are likely to be biased due to the general inviability of the strain.

Because Hox gene activity appears important for the generation of ectopic rays in *lin-22* mutants, I examined the effects of the Hox mutations on *lin-22* postdeirid production (Figure 4-13). Mutations in *mab-5*, *lin-39*, and *egl-5* have no effect on the generation of wild type postdeirids, and *mab-5* and *egl-5* do not affect the ectopic postdeirids made in *lin-22*(-). Therefore, it was surprising to find that a mutation in *lin-39* does affect postdeirid production in *lin-22*. As seen in Figure 4-13 loss of *lin-39* function results in the drastic reduction of postdeirids generated by V2-V4 in both hermaphrodites and males. Because of the difference in postdeirid production between V1 and V2-V4 in males and hermaphrodites, we see that the *lin-39* mutation effectively causes V2-V4 to adopt the V1 cell fate, and make postdeirids at the V1 frequency. This transformation is also seen in *lin-39 mab-5; lin-22* as well. While *lin-39* is expressed in V2-V5, there was no V cell phenotype associated with mutations in *lin-39*. Thus, this is the first example of an effect of *lin-39* on V cell development, and suggests that *lin-39* is playing a role in making the V cells of the central body region (V1-V5) different from the most anterior cell, V1.

Effects of the Hox genes on the ectopic L3 proliferative divisions in *lin-22(n372)*

In wild type animals, *mab-5* activity is required for the generation of rays, and for the L3 proliferative divisions that precede them, in both V5 and V6. Since *mab-5* expression is expanded in a *lin-22* mutant, and this ectopic activity affects the number of ectopic rays made, we wondered to what extent the *mab-5* mutation affects the ectopic *lin-22* L3 proliferative divisions. There was only limited lineage data from a *mab-5; lin-22* males (Kenyon, 1986), so we extended this analysis and confirmed that *mab-5*(+) activity is not strictly required for these L3 divisions in *lin-22* (Figure 4-14).

Because of the observation that both *lin-39* and *egl-5* acquire novel ray promoting activity in the *mab-5; lin-22* double mutant (Figure 4-12), we lineaged *lin-39; lin-22* and *lin-39 mab-5; lin-22* animals in order to assess whether *lin-39* was partly or fully responsible for the ectopic L3 proliferative divisions seen in *lin-22* and *mab-5; lin-22* mutant animals. Analysis of the *lin-39; lin-22* double mutant shows that *lin-39*(+) activity is also not required for these L3 divisions (Figure 4-15). When both *mab-5* and *lin-39* activities are removed from a *lin-22* male, the number of L3 proliferative divisions appears reduced, but is clearly not eliminated (Figure 4-16). Mutations in *egl-5* have no effect on these L3 divisions in the wild type, and it was not examined. These data suggest that the known Hox genes are not responsible for the ectopic L3 divisions in *lin-22* mutant males.

Analysis of the *lin-32* mutation and its effects on *lin-22*

An extensive analysis of *lin-32*(e1926) and *lin-22(n372); lin-32*(e1926) was carried out by Kenyon (Figure 3-3). This allele of *lin-32* blocked the formation of ectopic postdeirids, but affected neither the L3 proliferative divisions or the formation of ray precursor cells seen in *lin-22* males. However, this is not the strongest known *lin-32* allele (Zhao and Emmons, 1995). Figure 4-17 is an analysis of the effects of the strongest *lin-32* allele, *u282*, on the wild type V5 and V6 L3 proliferative divisions. Loss of *lin-32* function does not interfere with the normal doubling divisions generated by V6.pap, V6.ppp, or V5.ppp. However, it was seen that the V5.pa branch of the lineage that would have generated the postdeirid did not undergo an L3 proliferative division in a *u282* mutant (this is also true for the 3 *e1926* animals analyzed as well). So what happens to the ectopic L3 division in a *lin-22; lin-32* double mutant? Lineaging this division shows that all the Vn.pap and Vn.ppp cells, including the V5.pap branch, undergo the L3 proliferative divisions in *lin-22; lin-32* males (Figure 4-18). L3 lineages of *lin-22* males with mutations in both *lin-32* and *mab-5* indicate that the L3 proliferative divisions still occur in this background as well (Figure 4-19).

Ectopic L3 proliferative divisions occur in *lin-22* mutant males even in the absence of: 1) *mab-5*, 2) *lin-39*, 3) *mab-5* and *lin-39*, 4) *lin-32*, and 5) *mab-5* and *lin-32*. Taken together, these data intimate the existence of an additional gene activity that is regulated by *lin-22*(+) to block L3 proliferative divisions in the anterior V cells.

Finally, Figure 4-20 outlines the strategy for mosaic analysis of the *lin-22* gene. This strategy was never successful, probably because the free duplication *mDp1* was lost from the soma at a very low frequency.

Summary of Antibody Staining Data

In addition to an analysis of the Mab-5 expression pattern in *lin-22* animals during the L3 stage (Figure 3-4), the L2 stage was also intensively examined. Figure 4-21 shows the L2 staining pattern of *mab-5* in a *lin-22* mutant at 15-17 hours post-hatching. The ectopic staining of *mab-5* can be seen in the posterior daughter(s) of V1.p, V2.p, and V4.p, as well as in H2.p. Table 4-1 outlines the *mab-5* expression pattern at various timepoints from early L1 until the L3 proliferative divisions. The results indicate that the ectopic staining is found in a pattern identical to that seen for *mab-5* expression in the wild type V5 cell (S. Salser pers. comm.).

Because a mutation in *lin-22* alters the protein expression pattern of the Hox gene *mab-5*, I decided to examine the expression pattern of *lin-39* in a *lin-22* mutant. An analysis of *lin-39* RNA (Table 4-2) and protein (Table 4-3) expression in *lin-22* shows that *lin-22* mutations do not cause the expansion of the *lin-39* domain of expression, not even at a time (15-17 hours) in which *lin-22* clearly alters *mab-5* expression. (The levels of *lin-39* transcript appeared to be slightly elevated in the *lin-22* mutant, but since there can be a significant amount of slide to slide variation in the staining protocol, this experiment needs to be repeated before it is believed).

In an attempt to distinguish between the formation of postdeirid vs. ray neurons in various *lin-22* mutants, I modified a protocol in which the nucleotide analog 5'-bromo-2'deoxyuridine (BrdU) could be used to selectively label the nuclei of cells born after the L3 proliferative divisions (Figure 4-22). The standard staining protocol for MH27 (Francis and

Waterston, 1991) was then used to visualize the seam cell outlines in conjunction with the BrdU-labeled nuclei. This protocol was perfected but never utilized.

lin-22 Cloning Data

This is the complete strategy of the cloning of *lin-22*, encompassing Figures 4-22 to 4-24, and Table 4-4. Strain maintenance and construction carried out according to S. Brenner (Brenner, 1974). Southern hybridization analysis and the maintenance and screening of λ libraries was carried using standard techniques (Church and Gilbert, 1984; Feinberg and Vogelstein, 1984; Sambrook, *et al.*, 1989). Transformations were carried out according to (Mello, *et al.*, 1991). YAC preparation and maintenance was carried out according to (Burmeister, *et al.*, 1991; Gaensler, *et al.*, 1991) and the use of cold DNA to "swamp" out repetitive sequence was a modification of Frazer (Frazer, 1993).

1. The *lin-22* mutation mapped genetically to the left arm of chromosome IV between the genes *lin-1* on the left and *unc-33* on the right, a span of approximately 8 genetic map units. At the time *unc-33* was cloned, but *lin-1* was not, and the next DNA contig was associated with the gene *daf-1*, many map units to the left of *lin-1*.

2. We attempted to clone *lin-22* by first isolating a physically linked polymorphism, in this case a *Tc1* transposable element (Figure 4-23;(Costa, *et al.*, 1988; Ruvkun, *et al.*, 1989)). The strategy was to cross a strain containing *lin-1* and *unc-33* mutations, which only contains about 30 *Tc1* elements, to a

different *C. elegans* strain containing upwards of 300 *Tc1* elements, and then selectively maintain only those extra *Tc1* elements contained within the region between *lin-1* and *unc-33*. *lin-1(e1275) unc-33(e204)* were crossed to *him-5(e1490)* males and the F1 males (*e1275 e204/ +*) were crossed to N62 and TR403 hermaphrodites. The hermaphrodite progeny from this cross were cloned out and their *e1275 e204/* N62 male progeny were identified and mated to *lin-1(e1275) lin-22(n372) unc-33(e204)* hermaphrodites in single male matings. The resultant *e1275 n372 e204*/N62 male cross progeny were backcrossed to *e1275 n372 e204* in single male matings, making sure that no recombination had occurred between *lin-1* and *unc-33*. This process was repeated for a total of eight backcrosses.

The TR403 derived congenic strain, 3C7, was made by Elly Tanaka, but was subsequently found to contain no extra transposable elements from this region. Three resulting congenic strains were generated from N62 (C3, P3, and N22) that contained variable numbers of extra *Tc1* elements potentially in the *lin-1* to *unc-33* vicinity based on Southern blot analysis of XbaI digested congenic DNA probed with 32P-labeled *Tc1* DNA. Thirty-one recombinants were generated between *lin-1 lin-22 unc-33* and the three congenic strains. These recombinants were made homozygous and DNA was prepared from them. Southern blot analysis identified a single *Tc1* element that appeared to be closely linked to *lin-22*. An additional 17 recombinants were later generated between *lin-1 lin-22 unc-33* and congenic strain C3, which contained only 2 extra *Tc1* elements from this region. This showed unequivocally that this *Tc1* (called *Tc1* Band#4) was approximately 0.25 genetic map units to the left of *lin-22* (Figure 4-23).

3. The XbaI DNA fragment containing this *Tc1* Band #4 and the flanking genomic DNA was cloned into pIBI31 (IBI). Several attempts to use this genomic DNA to isolate clones from a genomic lambda library were unsuccessful. From this fragment a 450 bp subclone of genomic DNA was isolated that was free of repetitive DNA. When this fragment was used to probe a polytene YAC blot containing the entire known C. elegans genome, no positives were identified.

4. The 450 bp subclone was then sent to the lab of Bob Waterston at Washington University at St. Louis. Bob Waterston is in charge of one of the C. elegans genome centers, and it is here that all the C. elegans YAC clones are generated. The center had generated many "orphan" YACs that were not affiliated with any particular chromosome, so they were very interested in using my DNA to help identify YACs from this region of chromosome IV (which would of course help me to get DNA from the *lin-22* region. DNA from the 450 bp subclone was sent to St. Louis along with DNA sequence of the subclone that would allow them to make PCR primers. Using the 450 bp *lin-22*-linked DNA as a probe, Bart Lutterbach in the Waterston lab identified eight YACs and one cosmid from this region. He then confirmed the result using PCR with the primers against the 450 bp subclone.

5. The eight YACs were prepared in agarose blocks as per (Burmeister, *et al.*, 1991; Frazer, 1993) and run on Pulse-field electrophoresis gels (PFGE). The bands containing the YACs were identified by Southern analysis using the pYAC4 vector as probe. Subsequently, PFGE gels were run for each YAC, and the YAC bands were cut out of the gel. The gel slices were treated overnight with agarase (NEB protocol), placed on ice 5 minutes, spun 5 minutes in

Eppendorf microfuge to pellet undissolved agarose, phenol/$CHCl_3$ extracted twice, and then precipitated with 1/10 volume 3M NaOAc + 2.5 volumes EtOH. DNA was also made from the single cosmid from the region, K08D11.

6. DNA from all eight YACs + K08D11 were independently microinjected into the gonads of *lin-22* hermaphrodites at an approximate concentration of 3-10 ng/uL, along with pRF4 (a *rol-6* dominant coinjection marker) at a concentration of 50 ng/uL. No rescue of the *lin-22* phenotype was ever seen in the F1 generation, so established lines able to transmit the injected DNA to their progeny had to be generated for each YAC tested (see Table 4-4). A single YAC (Y68H6) was found to rescue the *lin-22* phenotype.

7. Y68H6 was used as a probe against an unamplified *C. elegans* genomic library and found to hybridize to hundreds of clones, indicating the presence of repetitive DNA. Y68H6 was then used to probe the unamplified library using cold genomic DNA to "swamp out" the repetitive DNA contained in the YAC (see Frazer, 1993). The protocol involves mixing radiolabeled YAC probe with 10-20 (up to 80) ug of sheared total worm DNA and pYAC4 vector DNA. This mix is boiled together for 5' and allowed to cool for 2 hours at 65°C before being used. In theory, this helps remove radiolabeled repetitive DNA from the pool of probe. Using this technique 41 positives were identified, and DNA was made from 28 clones. These clones were hybridized to one another to look for overlap and subsequently placed into pools of 4-6 based on the overlap data (see Table 4-4).

8. Two pools of l clones were found to rescue the *lin-22* alae defect. Only a single 15kb clone, λ10, was capable of completely rescuing the *lin-22*

phenotype alone (see Table 4-4). Lambda DNA from this clone was subcloned into into ≈6kb (RV6) and ≈9 kb (8.5#6) fragments, neither of which was able to rescue the *lin-22* phenotype (Figure 4-24). The plasmids could only be maintained intact using SURE cells (Stratagene; cells particularly suitable for growing DNA with odd repeat sequences). A number of restriction fragments from this l clone were also generated and used in rescuing attempts. Only a single 12 kb fragment showed rescuing activity (Figure 4-24). 10^6 clones from the Martin 1-2kb cDNA library were screened with the 15 kb λ10 insert. A single positive was identified, and which hybridized to the righthand 3 kb SpeI-XhoI fragment of lambda 10. When sequenced, this cDNA did not contain any large open reading frames, so it is unclear if any of this sequence is part of an actual gene. (It is most likely that this sequence contains some repetitive DNA that is found in this region of λ10, as the sequence never matched any of the genomic sequence from this region).

9. We started to sequence the genomic DNA in an attempt to discover an open reading frame that could encode the *lin-22* gene. Clone 8.5#6 was subcloned into 3 smaller fragments in pBluescript KS- : 8.5L, 8.5C, and 8.5R (Figure 4-24). Sequencing primers homologous to pBluescript were used to generate the initial sequence, and subsequently a Millipore Cyclone Plus Oligonucleotide Synthesizer was used to make primers to sequence walk. The obtained genomic DNA and the primers used are listed in Figure 4-25. We started sequencing all three of these subclones until the rescue data indicated that the 8.5C region was important. It was within this clone that the bHLH homology was discovered. The large ≈ 1.5 kb intron contained between helix 1 and helix 2 was not sequenced because of strong sequence stops. All

the available genomic sequence 3' to helix 2 was subsequently sequenced in an attempt to locate the C-terminus of the gene (Figure 4-25e-i).

10. We used RT-PCR in an attempt to identify the *lin-22* cDNA. Total RNA from a *him-5(e1490)* strain was reverse transcribed using an oligo dT primer. Using a primer homologous to the C. elegans SL1 splice leader sequence and a second primer that hybridized to helix 2 of the basic helix-loop-helix domain, a total of 40 rounds of PCR were carried out (hot start at 94°C and then each round at 1' 94°C, 1' 55°C, and 3' 72°C) on the reverse transcribed RNA to generate the 5' end of the *lin-22* sequence. This was followed by an additional 30 rounds of PCR on the product using a nested internal primer. The identical strategy was followed using oligo dT and a primer homologous to helix 1 to identify the 3' end of the gene. This strategy worked extremely well for identifying a putative 5' end; however, I could never conclusively locate the actual 3' end of the gene. In one instance the use of the helix 1+ oligo dT primers led to the identification of real cDNA sequence (containing helix 2), but it was prematurely terminated because the oligo dT primer had hybridized to an A-rich region of what appeared to be coding sequence (see the region labeled SHR in Figure 4-25e). A second set of nested primers was built in this region, and these primers were again used with oligo dT in PCR reactions to find the 3' end. Again, a band was generated in which the oligo dT had hybridized to another A-rich stretch that was in frame with the last sequence generated. This sequence may be part of the *lin-22* gene, but since there was no intron between the primers, it is unclear if this sequence is really cDNA or is due to genomic contamination. I made yet another primer homologous to the end of this sequence (Figure 4-24e), but could never get a PCR band using this primer in conjunction with oligo dT. It is possible that

these sites of spurious dT hybridization arose in the reverse transcription reaction, so I tried a number of methods to eliminate this potential problem, such as lowering the concentration of oligo dT, and raising the temperature. However, neither of these attempts were successful.

11. This basic HLH gene was shown to be *lin-22* because RT-PCR of the three *lin-22* mutants led to the identification of a mutation in glutamate-30 of the cDNA sequence of the *n372* allele. The presence of this mutation was confirmed by PCR sequence analysis of this portion of *n372* genomic DNA.

12. Over 4 million clones from 3 different cDNA libraries were screened using the PCR-derived *lin-22* cDNA as probe, and no positives were identified. This *lin-22* cDNA sequence was also used to probe a Northern blot of worm RNA made by S. Shivakumar. A single band was identified in a lane containing 2 ug of polyA+ RNA after exposure for 2 weeks using 2 ^{32}P-enhancing screens. The size of the band was ≈ 400-450 bp.

Figure 4-1. LI-L2 lineages of the lateral ectoderm of *lin-22(n372); him-5(e1490)* animals.

The vertical axis represents time and the horizontal axis represents the direction of cell division, with anterior to the left. The approximate stage of the animal is shown to the right of each set of lineages. Dashed lines indicate that a particular cell or branch of a lineage was not observed. Cell deaths are indicated by "X". Complete and partial postdeirid lineages are indicated by a bracket. The wild type lineage for both males and hermaphrodites is shown above the mutant lineages for reference. The L1 lineages of H1-V6 are not obviously altered by a mutation in *lin-22*. However, during the L2 stage the anterior V cells V1-V4 adopt the fate of the V5 cell and divide to generate ectopic postdeirids (or the beginning of the postdeirid lineage). In addition to transforming V1-V4 into a V5-like fate, the *lin-22* mutation can also cause the posterior branch of H2 to adopt the V cell fate (at a frequency of 2/6) and undergo a doubling division during the L2 stage. However, H2 was not observed to generate a postdeirid (0/6 animals).

METHODS. Lineages were carried out at 20°C as per Sulston and Horvitz (1977).

Figure 4-1

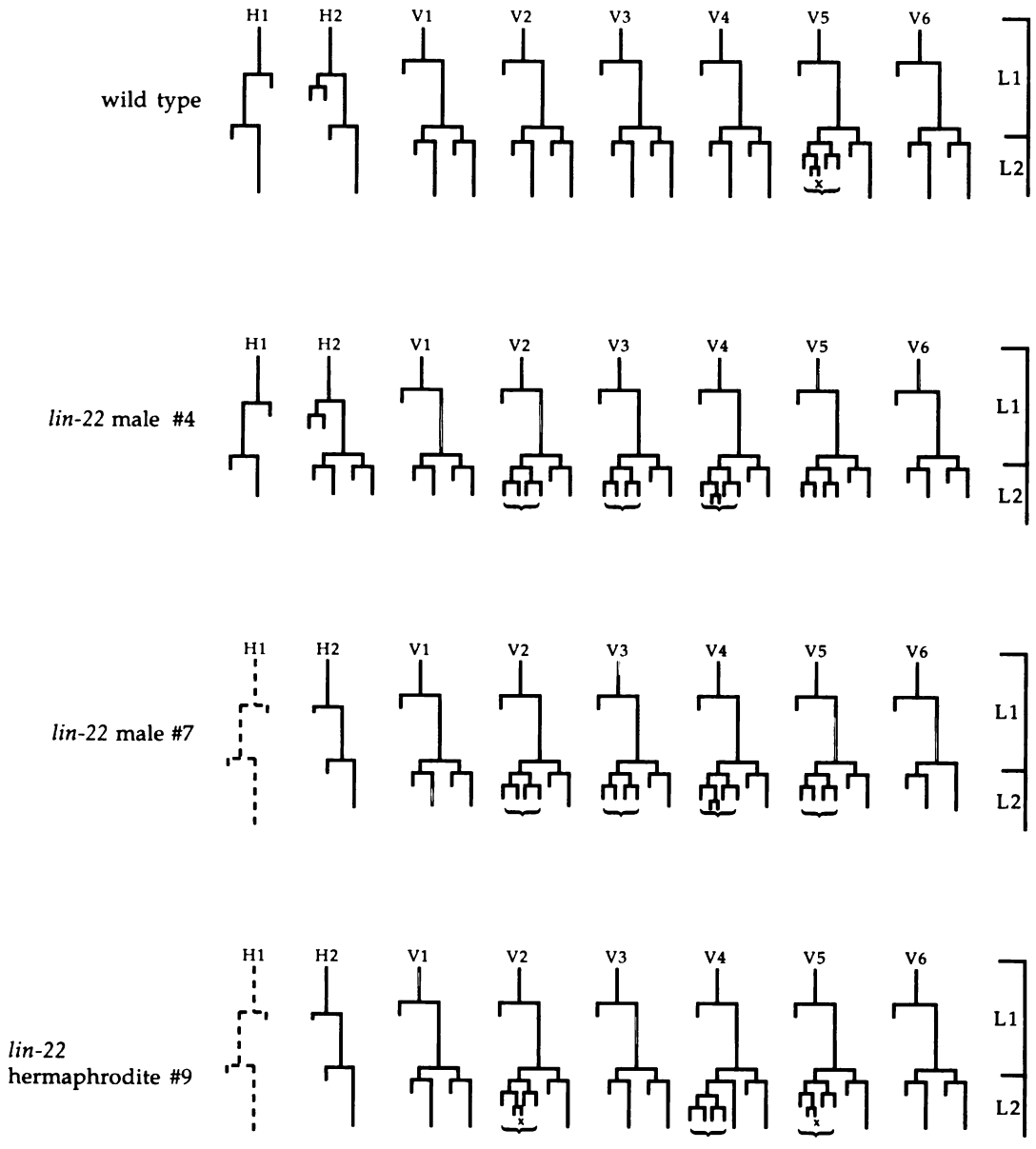

H1
H2
V1
V2
V3
V4
V5
V6
L1
L2
wild type
lin-22 male #4
lin-22 male #7
lin-22
hermaphrodite #9

Figure 4-1, cont.

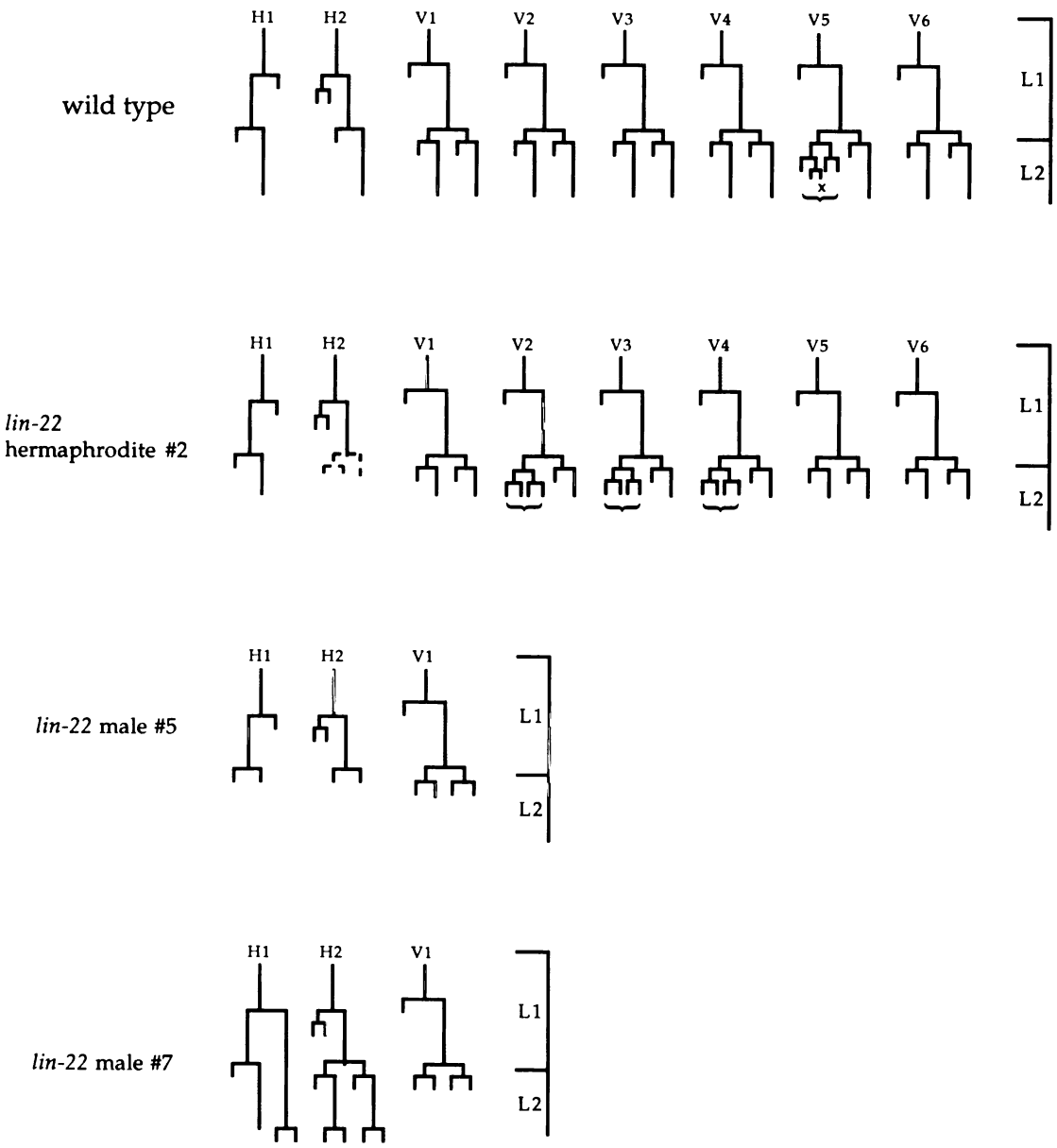


Figure 4-2. L2 larval lineages of cells of the lateral ectoderm in *lin-22(n372); him-5(e1490)* males.

The vertical axis represents time and the horizontal axis represents the direction of cell division, with anterior to the left. The approximate stage of the animal is shown to the right of each set of lineages. Dashed lines indicate that a particular cell or branch of a lineage was not observed. A "?" indicates that the fate of the particular cells indicated was not observed.  = postdeirid lineage. Cell deaths = "X". The "L" or "R" designation following the number of each animal indicates that either the left (L) or right (R) side of the animal was lineaged. The L1 lineages were not observed for these animals, but the pattern was inferred by the number and types of cells present. These data again indicate the ability of V1-V4 to adopt a V5 fate in the *lin-22* mutant and to generate postdeirid neuroblasts from the Vn.pa branches of their lineages. A fraction of the V(1-4).pa cells also reiterate the Vn.p lineage to produce a postdeirid neuroblast and an additional seam cell as well (see the V3.pa lineage from male #3CR). The majority of ectopic postdeirid lineages appear to be wild type (sister cells adopt the same fate in only the V3 postdeirid from male #2CL).

METHODS. Lineages were carried out at 20°C according to Sulston and Horvitz (1977).

Figure 4-2

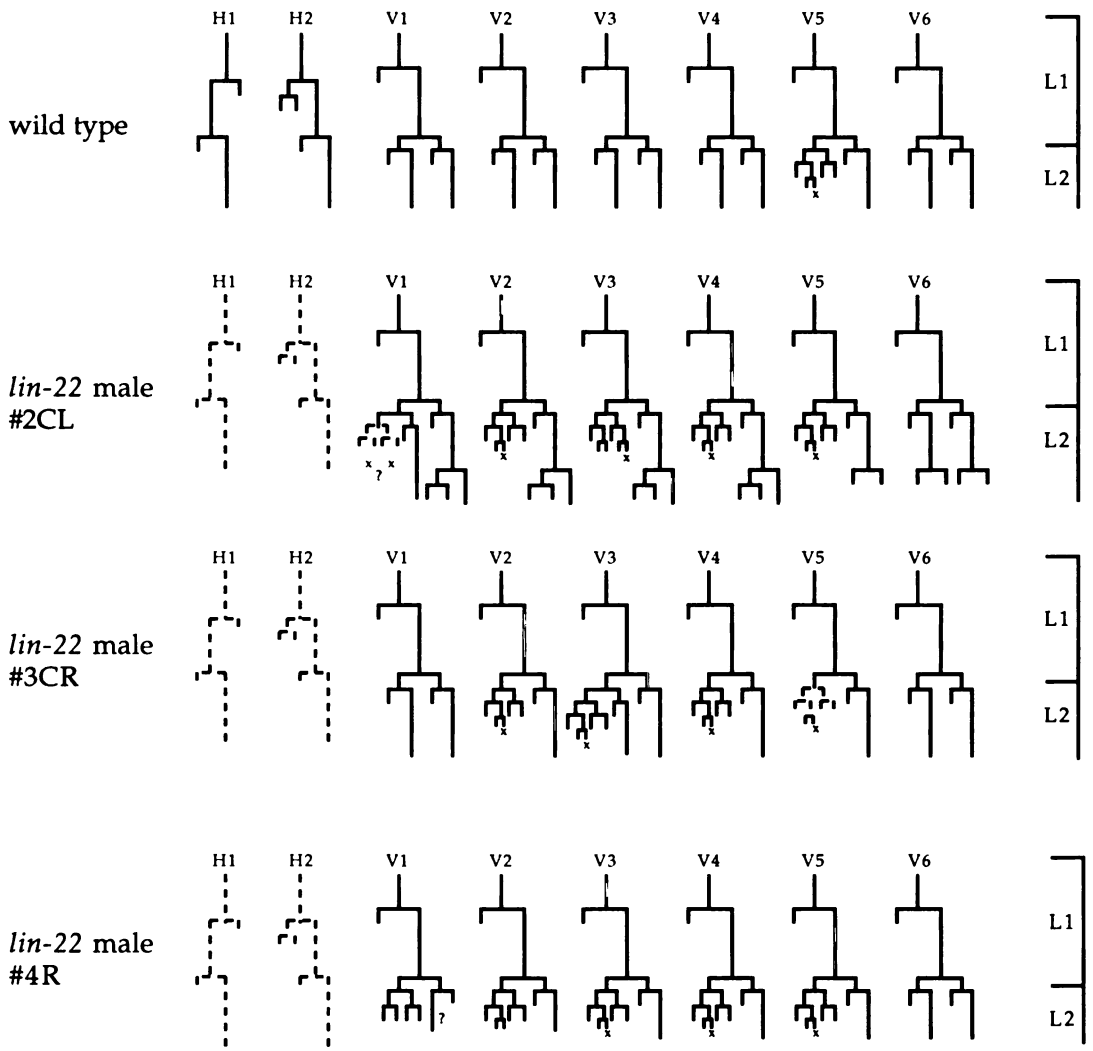

wild type
H1
H2
V1
V2
V3
V4
V5
V6
L1
L2
lin-22 male #2CL
lin-22 male #3CR
lin-22 male #4R

Figure 4-2, cont.

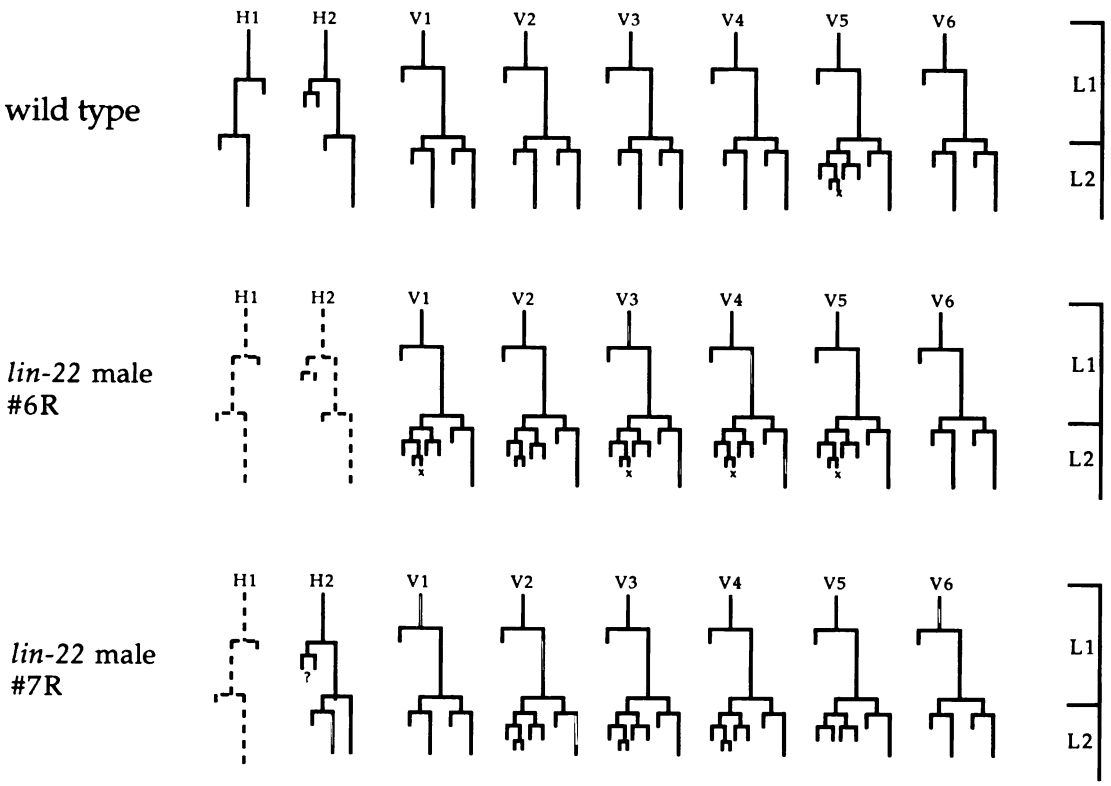


Figure 4-3. L3 lineages of the lateral ectodermal cells in *lin-22(n372); him-5(e1490)* males.

The vertical axis represents time and the horizontal axis represents the direction of cell division, with anterior to the left. Lines of equal length following a division (⊓) indicate that the fates of the daughters were still unknown when the lineage was terminated. The approximate stage of the animal is shown to the right of each set of lineages. Cell deaths are marked with "X". Dashed lines indicate parts of the lineage that were not directly observed, but were inferred from the presence of the correct type and number of cells present when the lineage was initiated. ⊓⊓ = postdeirid lineage. The presence of "L" or "R" following the number of an animal indicates which side of the animal was lineaged. The L3 proliferative division is indicated in **BOLD** lines. The polarity of the granddaughters of the L3 proliferative division is indicated with an arrow (in which an arrow pointing to the right indicates that the posterior daughter was the seam cell). These data show that the majority of the anterior V cells adopt the L3 fate of the V5 daughters and undergo an extra proliferative division at this time. #1AL illustrates that H2 is also capable of adopting the V5 fate and undergoing an extra L3 division. In addition, the extra seam cells produced from reiterations of the Vn.p cell in #2BR fail to undergo the extra L3 division. However, the V4.pappp cell appears to have adopted a ray precursor cell fate. The division of the anterior V4.papppa cell is indicative of this fate. S. Salser has seen that the L3 proliferative division generally precedes and promotes ray precursor formation. However, ray precursors can be generated without this extra division. These lineages also indicate that the ectopic proliferative divisions

seen in the anterior V cells contain polarity reversals at a high frequency (12/31 scorable L3 divisions).

METHODS. Lineages carried out at 20°C according to Sulston and Horvitz (1977).

Figure 4-3

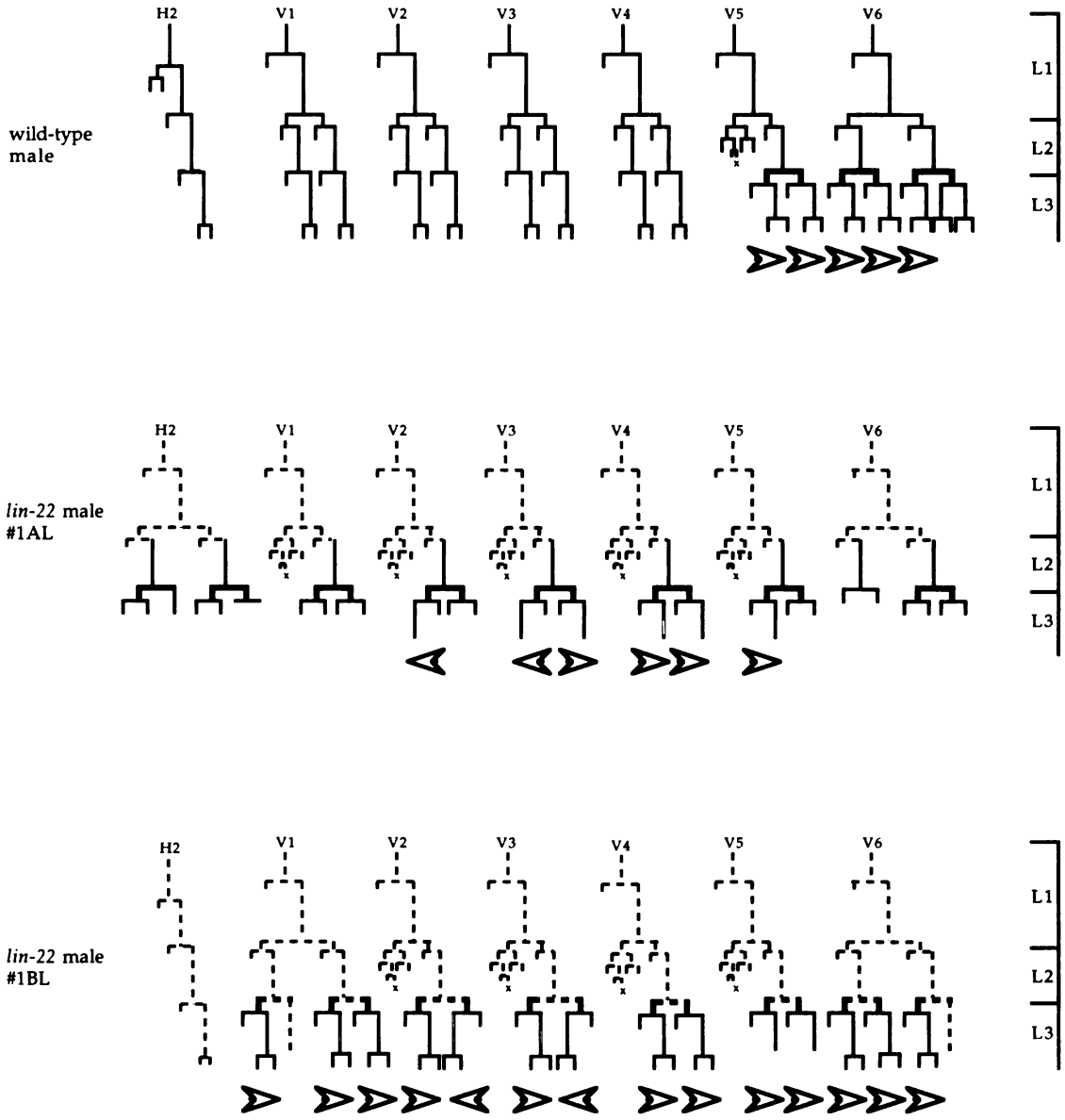

wild-type male
H2
V1
V2
V3
V4
V5
V6
L1
L2
L3
lin-22 male #1AL
lin-22 male #1BL

Figure 4-3, cont.

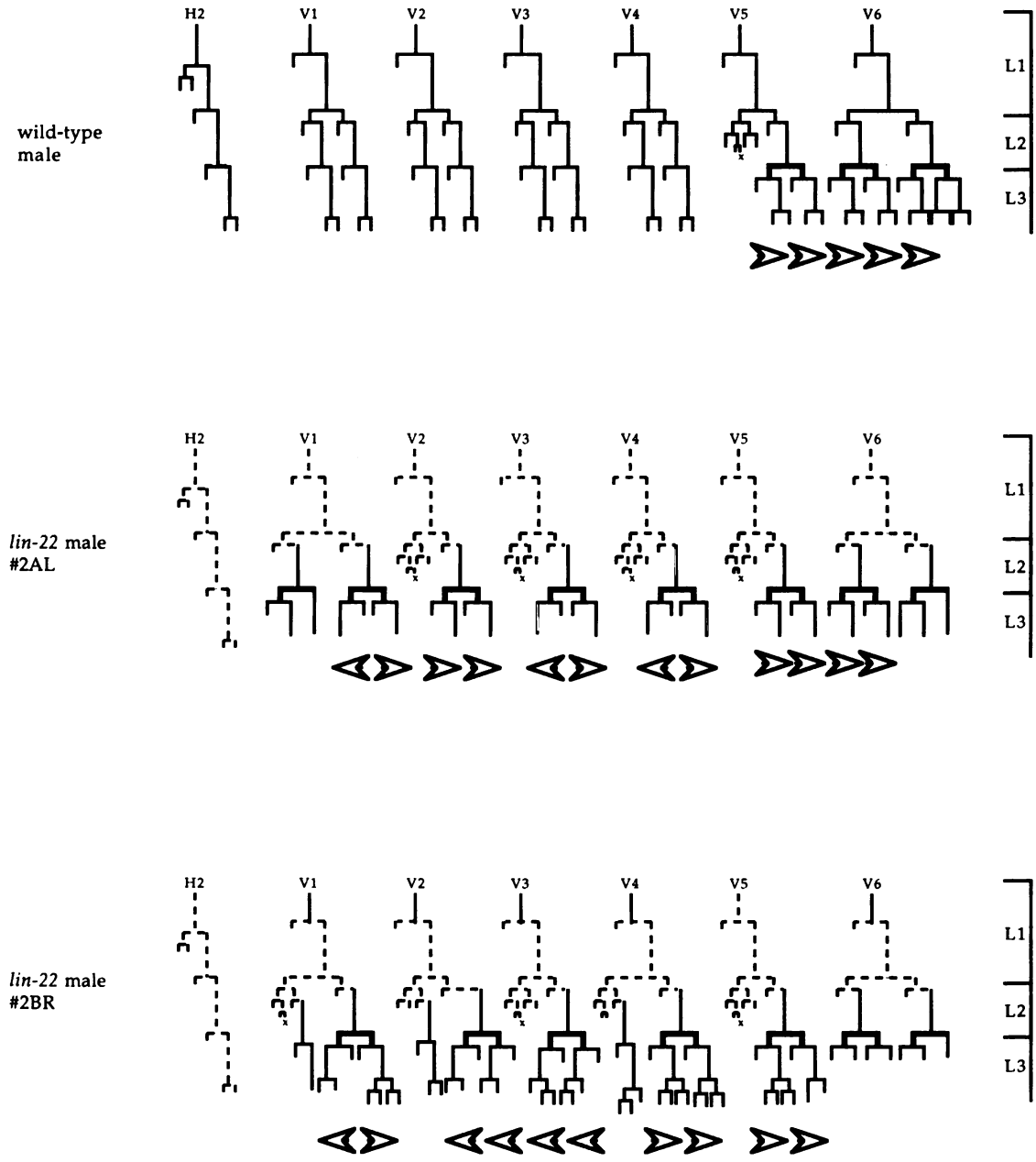


Figure 4-4. Comparison of alae production in the three *lin-22* alleles.

The figure shows the percentage of alae formed in a particular body region, relative to a value of 100% for wild type, graphed against its location along the anterior-posterior axis. The cartoon worm shows (from anterior to posterior): the two pharynx bulbs, the BDU neuron, the anterior bend of the gonad, the ALM neuron, the posterior bend of the gonad, and the tail fan. The number of animals scored was n=35 for *mu5*, n=38 for *mu2*, and n=37 for *n372*. The production of alae is indicative of the formation of epidermal seam cells from the V cell lineages, while the loss of alae reflects the transformation of seam cells into neuronal ray precursor cells seen in *lin-22* mutants. The results of the comparison suggest that *mu2* and *n372* males generated similar amounts of alae, whereas *mu5* makes significantly more alae, especially noticeable in the central body region.

METHODS. Males in L4 lethargus were placed onto 2% agarose pads. The total length of the male and the length and location of each patch of alae was recorded using a reticle under 100x Nomarski optics. All data were then normalized to a single standard length. Wild type male contains virtually 100% alae from the posterior bulb of the pharynx to the start of the tail fan. All alleles were analyzed at 20°C.

Figure 4-4

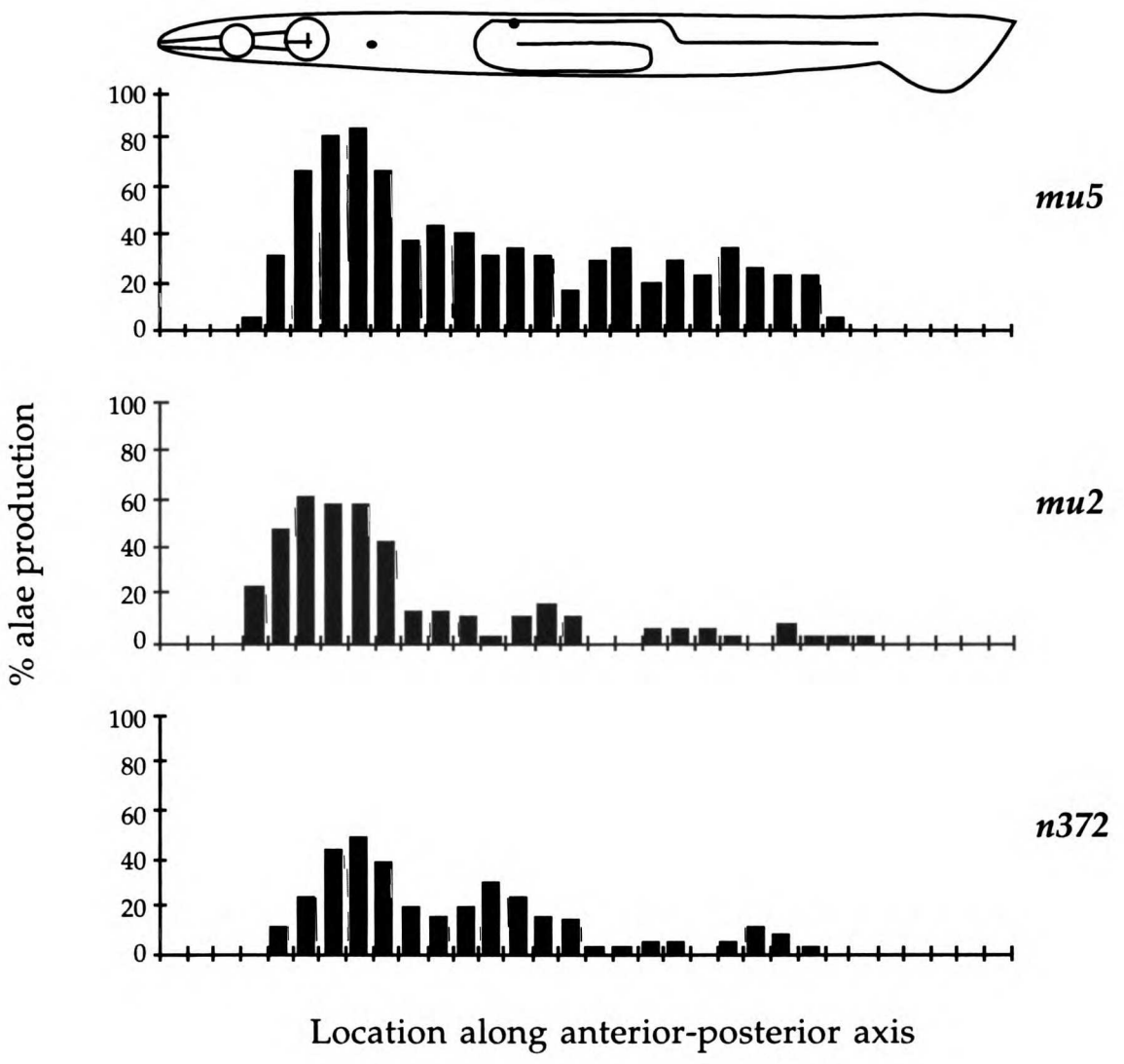

% alae production
100
80
60
40
20
0
mu5
mu2
n372
Location along anterior-posterior axis

Figure 4-5. Temperature sensitivity of alae production in the three alleles of *lin-22*.

The figure shows the percentage of alae formed in a particular body region graphed against its location along the anterior-posterior axis. The production of alae is indicative of the formation of epidermal seam cells from the V cell lineages, while the loss of alae reflects the transformation of seam cells into neuronal ray precursor cells seen in *lin-22* mutants. The cartoon worm shows (from anterior to posterior): the two pharynx bulbs, the BDU neuron, the anterior bend of the gonad, the ALM neuron, the posterior bend of the gonad, and the tail fan. Wild type males make alae at a frequency of ≈100% from the posterior pharynx bulb to the tail fan.

a. Effects of temperature on alae formation in *mu5*. The number of animals scored was : n=37 at 16°C, n=35 at 20°C, and n=39 at 25°C.

b. Effects of temperature on alae formation in *mu2*. The number of animals scored was : n=40 at 16°C, n=38 at 20°C, and n=40 at 25°C.

c. Effects of temperature on alae formation in *n372*. The number of animals scored was : n=39 at 16°C, n=37 at 20°C, and n=34 at 25°C.

Analysis of alae production in **(a)**, **(b)**, and **(c)** indicates that the formation of alae appears to be temperature sensitive for all three alleles, with alae production being favored at lower temperatures while production of neuronal structures is favored at higher temperatures. This is likely to not be the result of a temperature sensitivity in the *lin-22* mutants proteins because similar effects of temperature on ray formation has been seen in other genetic backgrounds. The reasons for why higher temperatures favor neuronal production by the V cells is presently unknown.

METHODS. Males in L4 lethargus were placed onto 2% agarose pads. The total length of the male and the length and location of each patch of alae was recorded using a reticle under 100x Nomarski optics. All data were then normalized to a single standard length. The wild type male contains virtually 100% alae from the posterior bulb of the pharynx to the start of the tail fan.

Figure 4-5

a. *mu5*

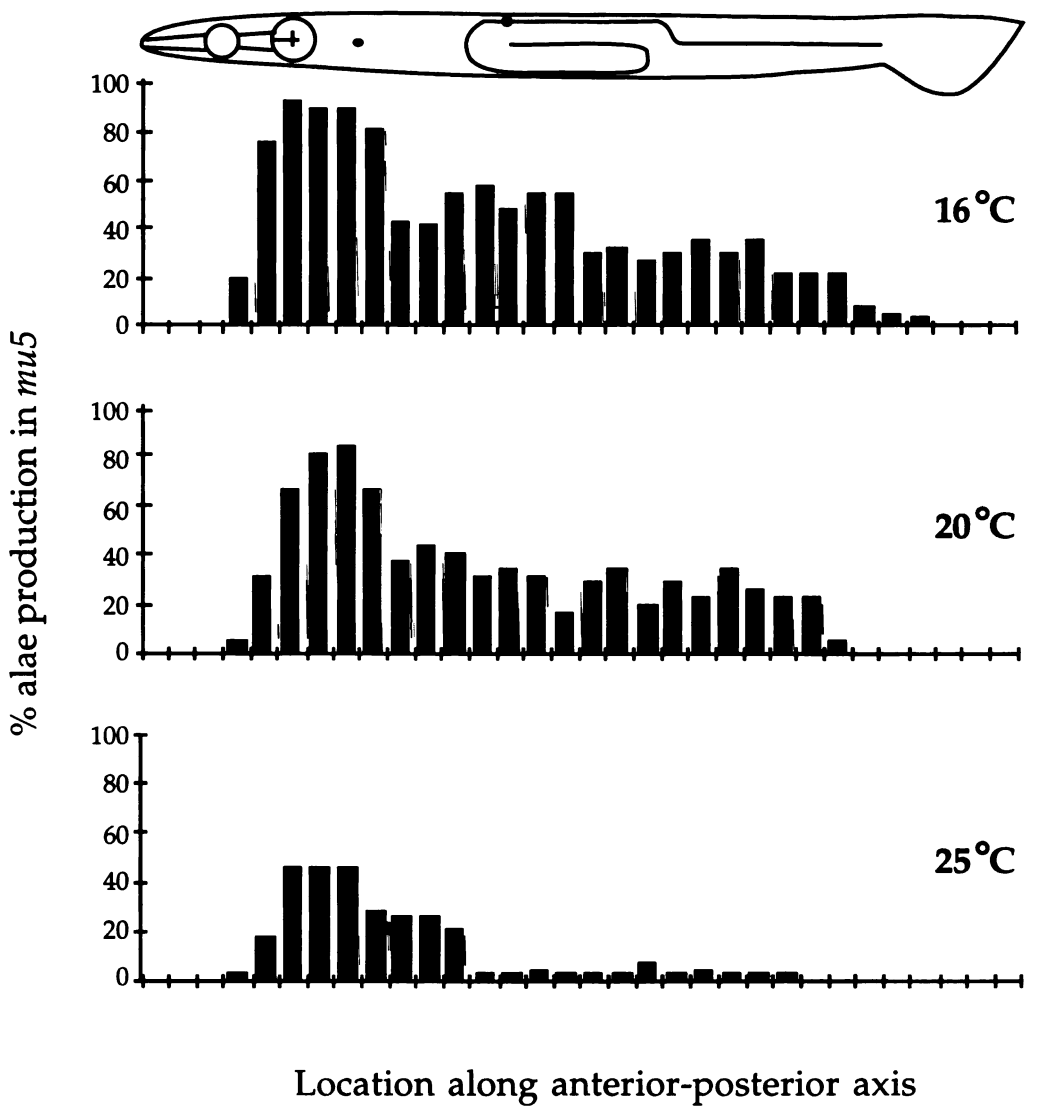


Figure 4-5

b. *mu2*

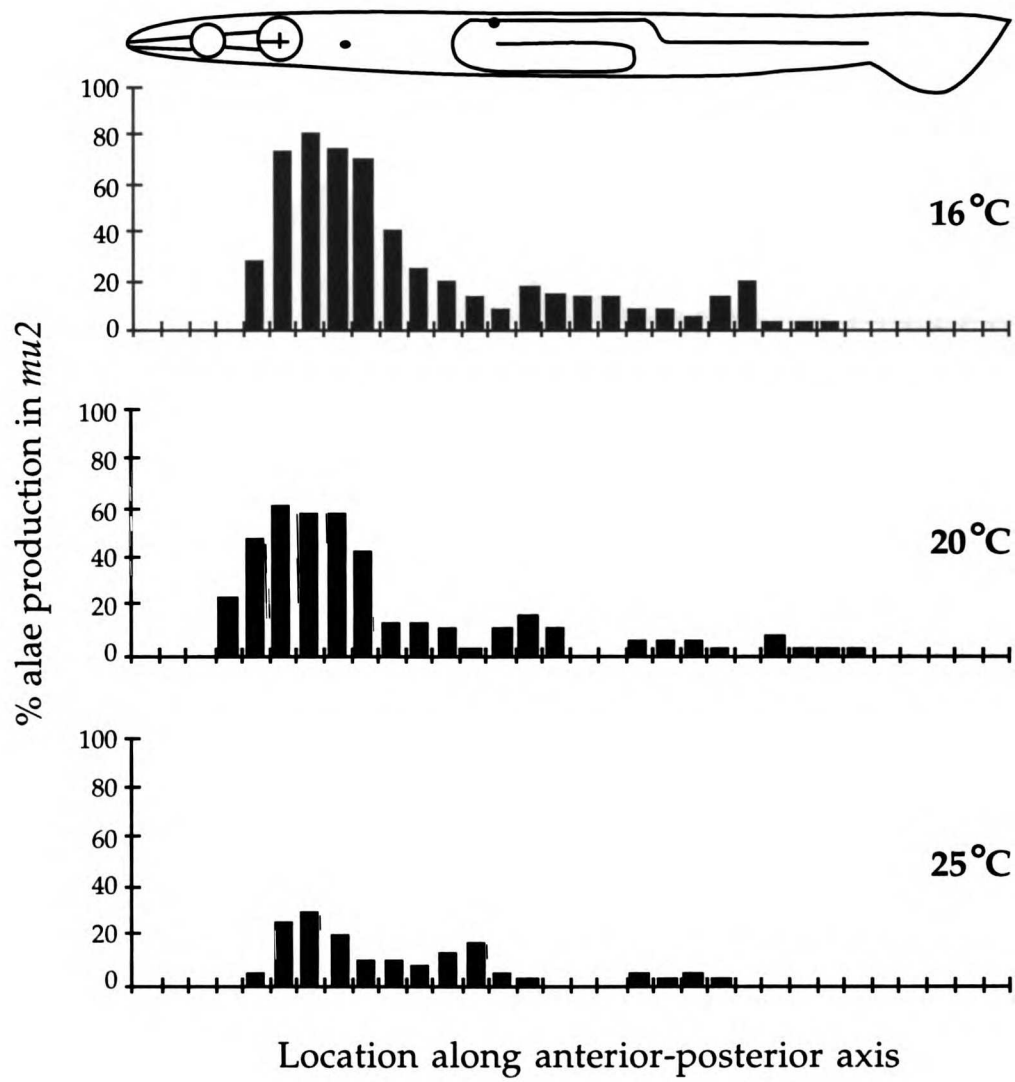


Figure 4-5

c. *n372*

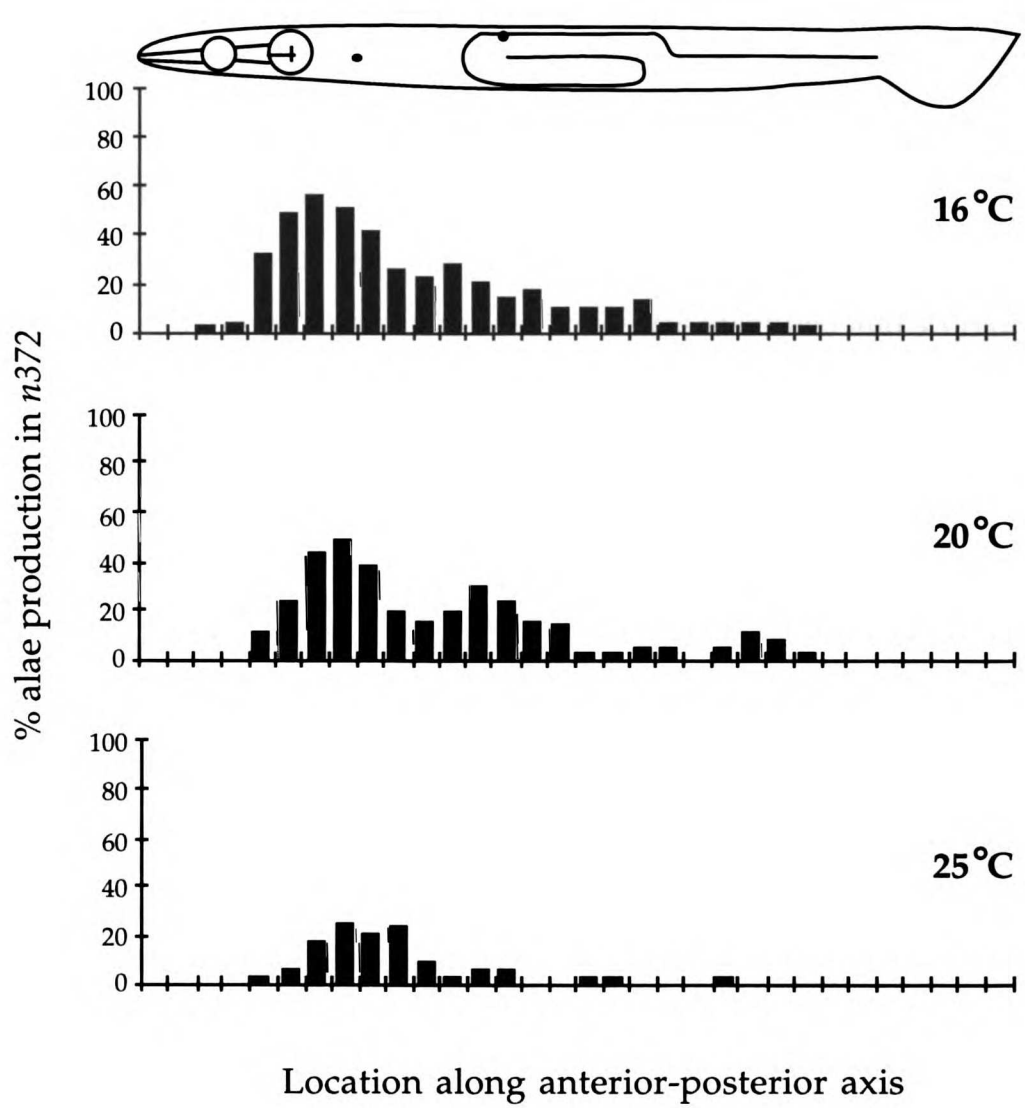


Figure 4-6. Effects of temperature on postdeirid production in *lin-22(n372); him-5(e1490)*animals.

The graph shows the percentage of postdeirid production by each V cell in *lin-22(n372)* mutant animals at three different temperatures. The legend indicates how each temperature is represented. Generation of postdeirids in the *n372* allele of *lin-22* was examined in late L2/early L3 animals at 15°C, 20°C, and 25°C. The number of animals scored at each temperature is given in the legend. There are only slight differences in postdeirid formation by the different V cells at the three temperatures.

METHODS. Postdeirid production by the V cells was identified and scored as per Waring *et al.* (1992).

Figure 4-6

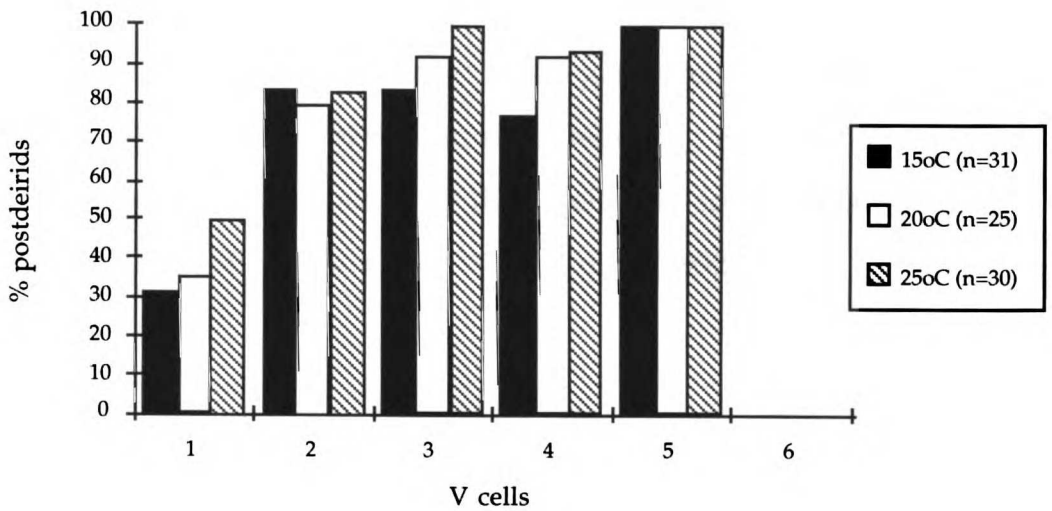

% postdeirids
100
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5
6
V cells
15oC (n=31)
20oC (n=25)
25oC (n=30)

Figure 4-7. Postdeirid production in *lin-22* is dependent on the sex of the animal.

The graph shows the percentage of postdeirid production by each V cell in *lin-22(n372)* hermaphrodites (**a**) and males (**b**). The legend indicates how each sex is represented and lists the number of animals scored. Postdeirid formation by V2-V5 is virtually identical in both males (76-94%) and hermaphrodites (79-91%). However, a statistically significant difference exists in the frequency of postdeirids made by the V1 lineage: males generated V1 postdeirids at a frequency of 43%, while the hermaphrodite frequency was only 6%. METHODS. Postdeirid production by the V cells was scored in late L2/early L3 males and hermaphrodites as per Waring *et al.* (1992).

Figure 4-7

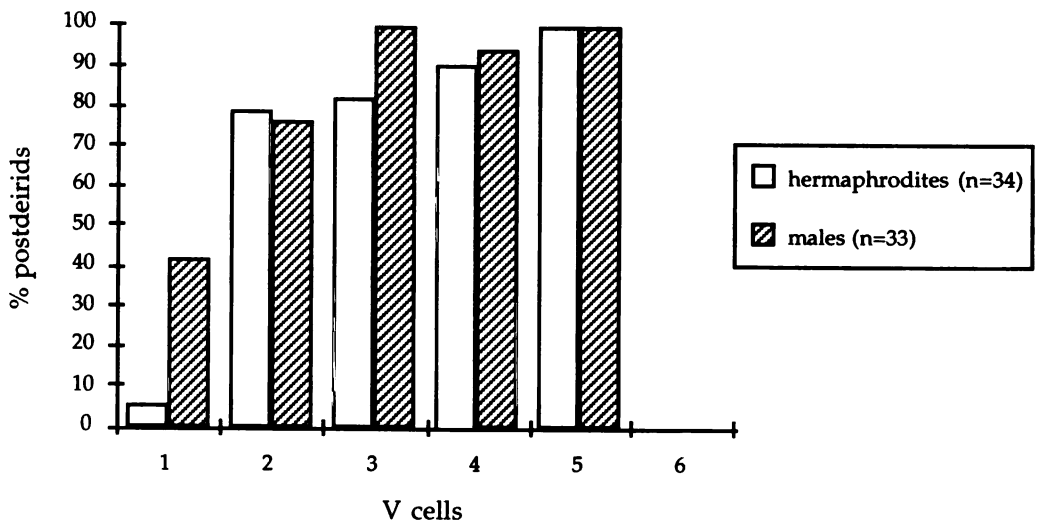
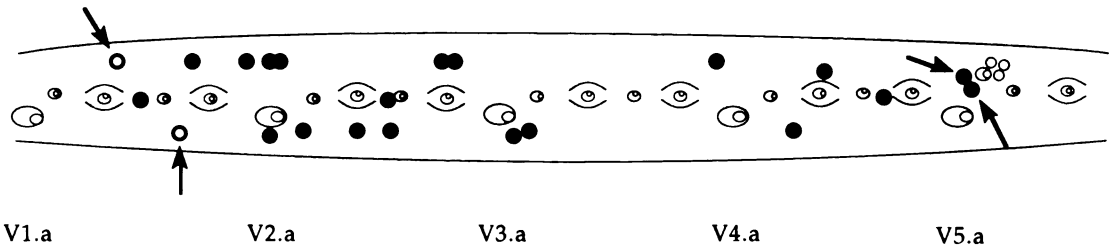

100
90
80
70
60
50
40
30
20
10
0
% postdeirids
1
2
3
4
5
6
V cells
hermaphrodites (n=34)
males (n=33)

Figure 4-8. Three wild type copies of the *lin-22* gene cause the QL descendants to migrate towards to anterior.

The figure shows the left side of C. elegans during late L2, with anterior to the left. The V cell daughters are shown, with the Vn.a cells identified. ● = the wild type QL descendants SDQ and PVM, shown in their proper position next to the V5 postdeirid. ○ = the wild type QR descendants. They are shown here, between V1 and V2, to illustrate the distance that the Q descendants migrate on the right side of the animal. ● = QL descendants SDQ and PVM scored in *lin-22* +/+/+ animals. The approximate locations of each QL descendant is shown. In animals with three wild type copies of *lin-22*, the QL descendants were misplaced anteriorly in 9/47 animals scored (19%). Control animals of the genotype *lin-22*(-)/*lin-22*(-)/+ do not show this phenotype. The *mab-5* gene is necessary and sufficient to direct the keep the QL cells in the posterior (Salser and Kenyon, 1992). *lin-22* can inhibit *mab-5* activity in the V cells, so it is possible that overexpression of *lin-22* repress mab-5 function in the Q cells as well. This phenomenon was never seen in 2 strains carrying extrachromosomal arrays of the *lin-22*-rescuing clone l10. No obvious effects were seen in the QR descendants.

METHODS. 101 animals of the genotype *unc-17(e245) dpy-13(e184); him-5(e1490); mDp1* were scored for the position of QL and QR at 20°C. As a control, 111 animals of the genotype *lin-22(mu2) unc-17(e245) dpy-13(e184); him-5(e1490); mDp1* were scored, and no Q cell defects were observed.

Figure 4-8



● = wild type QL descendants SDQL and PVML

○ = wild type SDQR and PVMR cells

● = SDQL and PVML cells in *lin*-22 +/+/+

Figure 4-9. *mab-5* mutations decrease the number of ectopic rays generated in a *lin-22* mutant male.

Mutations in *lin-22* lead to the generation of ectopic sensory rays in the anterior of the male, resulting in the concomitant loss of alae-producing epidermal cells. The locations of patches of alae are shown relative to the cellular and anatomical markers used to help score their location along the anterior-posterior axis. Alae were scored from approximately the posterior pharynx bulb to the tail fan, using such markers as: the end of the second pharynx bulb, the BDU cell, the anterior bend of the gonad, the ALM cell, the posterior bend of the gonad, and the beginning of the tail fan. Arrowheads represent males with no alae. The loss of *mab-5* activity in a *lin-22* background greatly decreases the formation of ectopic sensory rays, especially in the anterior of the animal, and leads to an increase in alae production. This was first observed by Kenyon (1986).

METHODS. The presence and location of alae was scored in *lin-22(n372)* (n=37), *mab-5(e1239); lin-22(n372)* (n=37), and wild type (n=23) males at 20°C according to the methods in Figure 4-4 and Figure 4-5. All strains contain the mutation *him-5(e1490)*. No sensory rays are produced in *mab-5* mutant animals, and alae extends into the fan of the tail (n=50).

Figure 4-9

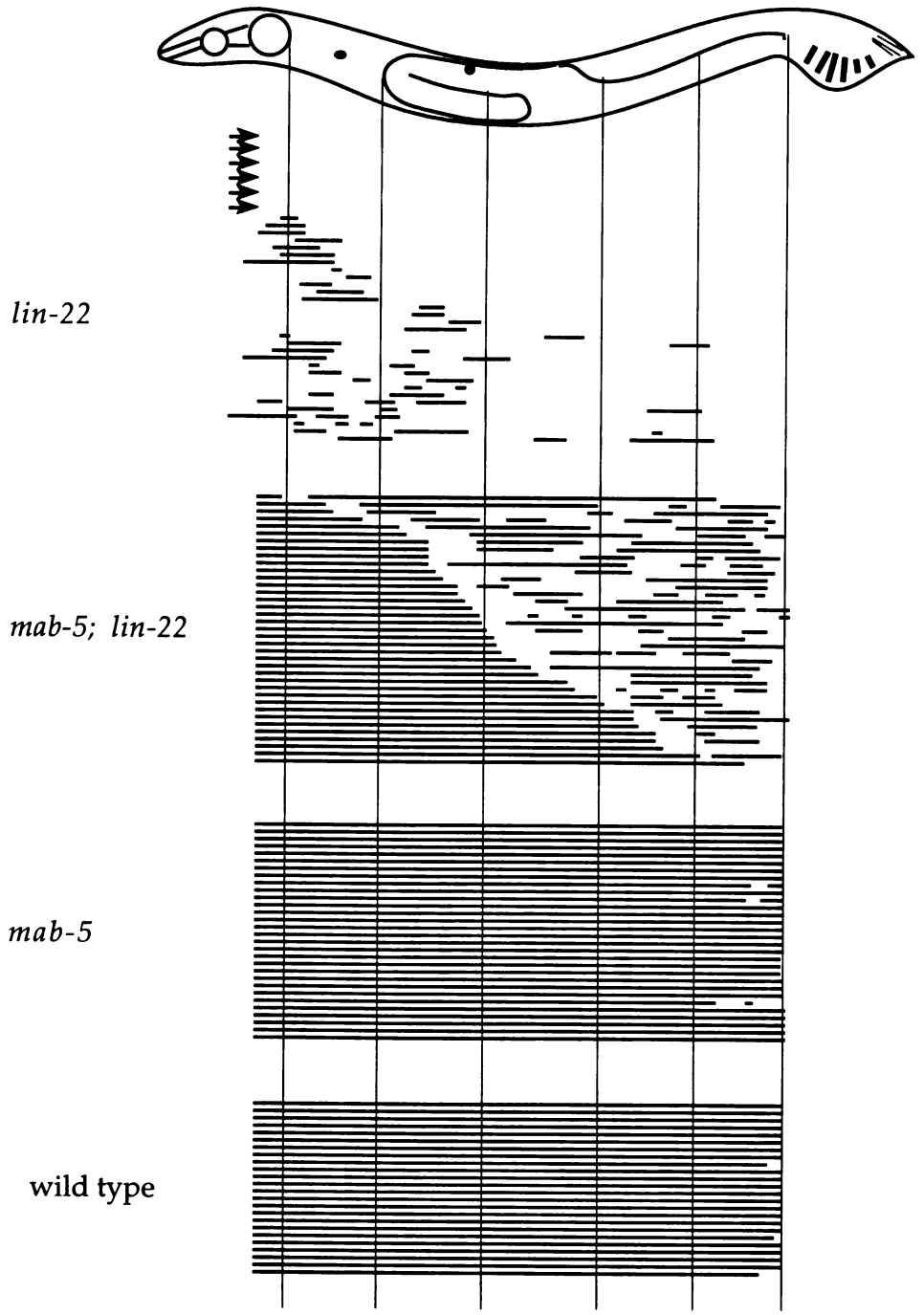


Figure 4-10. Genetic dosage analysis of *mab-5* and *lin-22*.

Schematic showing the phenotypes of animals bearing various copy numbers of the *mab-5* and *lin-22* genes (see also Kenyon, 1986). Genotype is listed at the right with the copy number of each gene shown at the left. Drawings of male worms of the indicated genotype illustrate the phenotype: bold lines indicate the V-derived rays, the triple lines represent cuticular alae, and the four cells of each individual postdeirid are indicated. The wild type male contains alae from the head to the fan and makes six sensory V rays in the tail. In *lin 22* mutants the six V rays are still found in the tail, but addtional rays are generated ectopically in the anterior at the expense of alae. Hemizygosity for *mab-5* leads to an increase in the amount of alae (particularly in the anterior) in a *lin-22* mutant, while two mutant copies of *mab-5* results in even more alae production. In males mutant for just *mab-5* none of the six V rays are produced and the male makes alae into the tail. 26% of *mab-5; lin-22/+* males generate a single V ray, and 2% of these males make two rays. (This was only observed at 25°C, where the production of neuronal structures appears to be favored). Loss of both copies of *lin-22* further increases the average number of V rays in the tail to 1.4 (n=37), with a range of one to four. Thus, these two genes appear to function antagonistically to one another to promote either neuronal (*mab-5*) or epidermal (*lin-22*) cell fates in the V cell lineages.

METHODS. Strain construction and maintenance were performed according to Brenner (1974). The *mab-5* allele used was *e1239*, and the *lin-22* allele examined was *n372*. All strains were *him-5(e1490)*. The following are controls for the generation of single ectopic tail rays at 25°C: *e1239; e1490* =

Figure 4-10

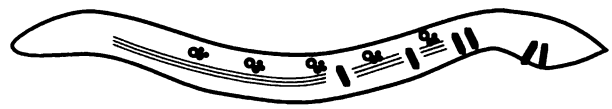
Copies of *mab-5*	Copies of *lin-22*		Genotype
0	2		*mab-5; +/+*
0	1		*mab-5; lin-22/+*
0	0		*mab-5; lin-22*
1	0		*mab-5/+; lin-22*
2	0		*+/+; lin-22*
2	2		wild type

Figure 4-11. *lin-39* and *egl-5* activities play a role in generating the ectopic sensory rays seen in *mab-5; lin-22* mutants.

The locations of patches of alae are shown relative to the cellular and anatomical markers used to help score their location along the anterior-posterior axis (as in Figure 4-9). Alae were scored from the posterior pharynx bulb to the tail fan, using such markers as: the end of the second pharynx bulb, the BDU cell, the anterior bend of the gonad, the ALM cell, the posterior bend of the gonad, and the beginning of the tail fan. When either *lin-39* or *egl-5* activity is removed from the *mab-5; lin-22* double mutant, the formation of ectopic sensory rays is reduced, primarily in those regions in which the particular gene is known to function in the wild type.
METHODS. Late L4 and adult males of each genotype were scored for gaps in alae production along the A-P axis. The location and extent of each gap was estimated by its position relative to numerous body structures and cells along the A-P axis (the end of the pharynx, the BDU cell, the anterior end of the gonad, the ALM cell, the dorsal to ventral bend in the gonad, the start of the fan, and the T rays). All animals grown at 20°C.

Figure 4-11

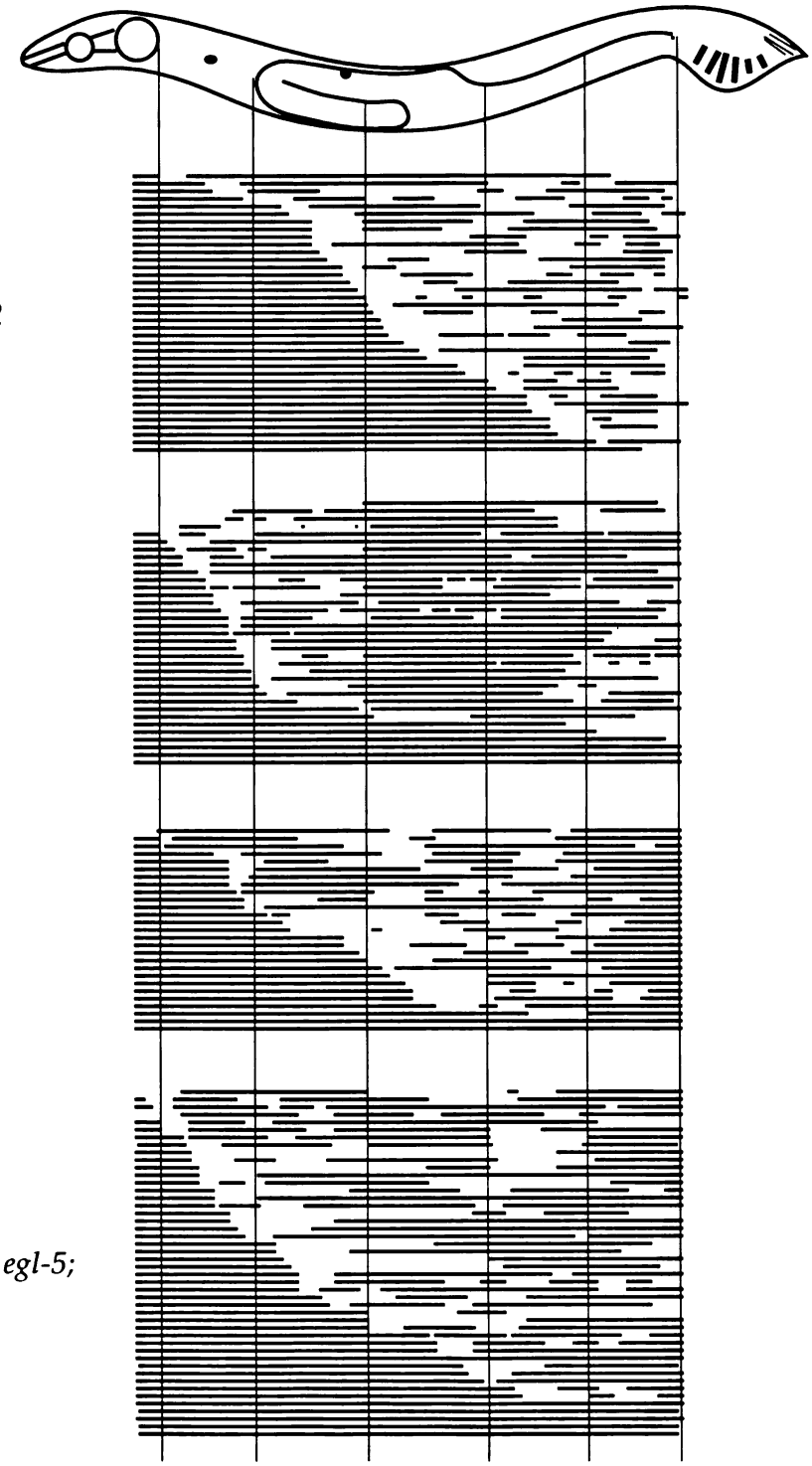

mab-5; lin-22
lin-39 mab-5; lin-22
mab-5 egl-5; lin-22
lin-39 mab-5 egl-5; lin-22

Figure 4-12. Analysis of the ray-promoting activities of the *C. elegans* Hox genes in *lin-22* mutant males.

This figure is a graph version of the data presented in Figures 4-9 and 4-11. The y-axis indicates the position of the various body regions scored (indicated by their relative position to the cartoon worm shown above). The graph at the right, shown with stippled boxes, is the average number of V-derived rays generated in the tail (the x-axis scale is from 0 to 6, 6 being the maximum number of V rays made in the wild type). The graph at the left, shown with solid boxes, gives the average percentage of a region that contained no cuticular alae. Gaps in the alae reflect a transformation of an epidermal lineage into a neuronal lineage. When this takes place, the cells of the lateral epidermis (which generate alae) are replaced by sensory rays and tail seam cells, resulting in gaps in the alae. Loss of *mab-5* function does not eliminate production of sensory rays in a *lin-22* mutant. Removing the activities of the Hox genes *lin-39* (central body region) or *egl-5* (tail) results in decreased ray production in their respective domains of activity. A strain carrying mutations in *mab-5 lin-39 egl-5* and *lin-22* was constructed. Animals of this genotype are stunted, slow growing, and often inviable. However, males which survive are capable of making rays randomly along the A-P axis at a frequency of 18-31% (n=46). Due to male inviability it is unclear how these numbers are biased.

METHODS. Late L4 and adult males of each genotype were scored for gaps in alae production along the A-P axis. The location and extent of each gap was estimated by its position relative to numerous body structures and cells along the A-P axis (the end of the pharynx, the BDU cell, the anterior end of the gonad, the ALM cell, the dorsal to ventral bend in the gonad, the start of the

fan, and the T rays). For each animal, the size of an alae gap was estimated as the % of a particular body region. Given the crudeness of the estimation, the numbers generated are not to be taken as exact. The graph shows the average gap size generated in each body region for a particular mutant strain. The following alleles were used in making the strains: *lin-39(n1760)*, *mab-5(e1239)*, *egl-5(n945)*, and *lin-22(n372)*. All strains contained the mutation *him-5 (e1490)* to increase the number of males generated. The number of animals scored for each strain were as follows: wild type (n=23), *lin-22* (n=37), *mab-5; lin-22* (n=37), *lin-39 mab-5; lin-22* (n=36), and *mab-5 egl-5; lin-22* (n=26).

Figure 4-12

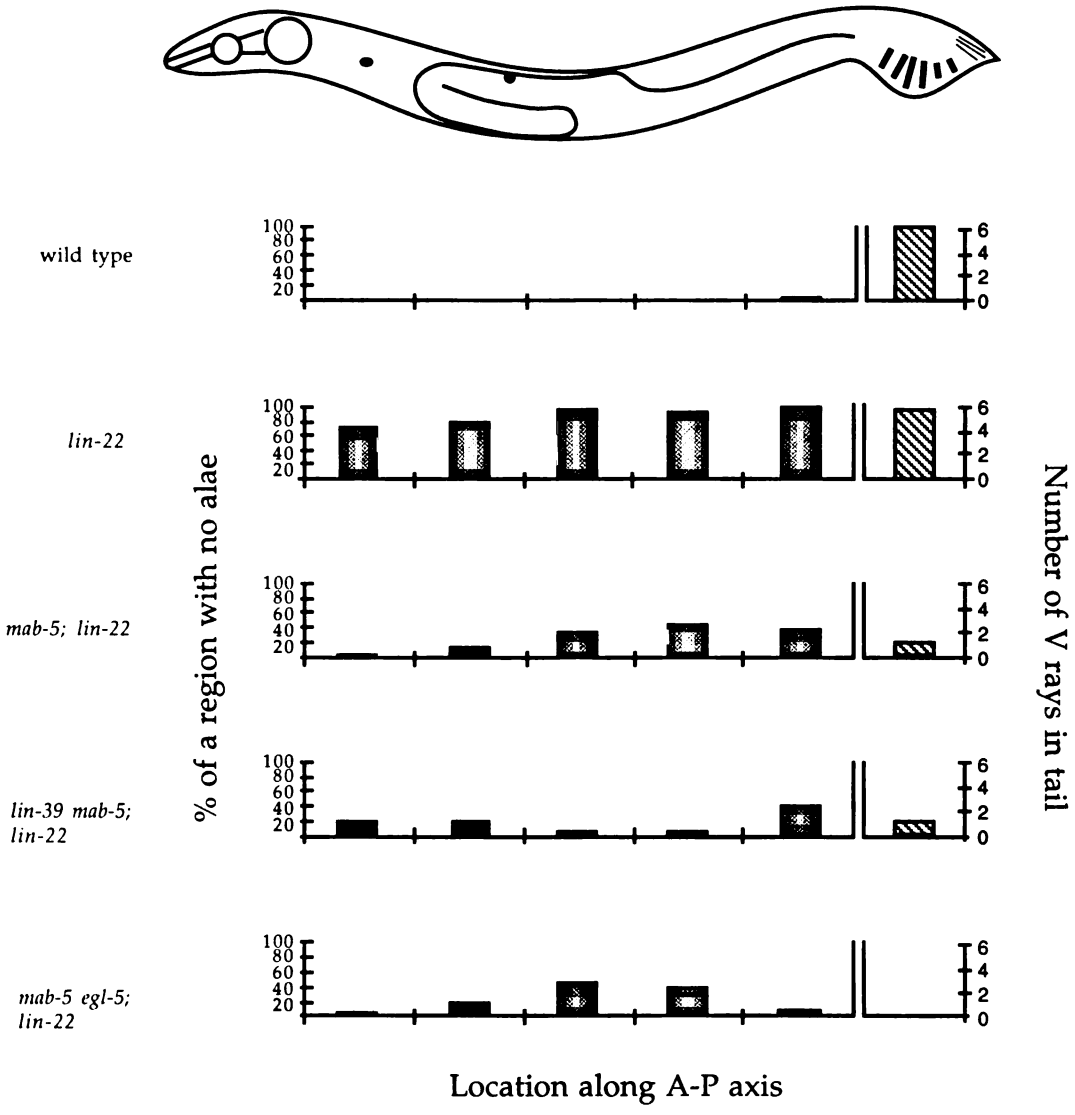

wild type
lin-22
mab-5; lin-22
lin-39 mab-5; lin-22
mab-5 egl-5; lin-22
% of a region with no alae
Number of V rays in tail
100
80
60
40
20
6
4
2
0
Location along A-P axis

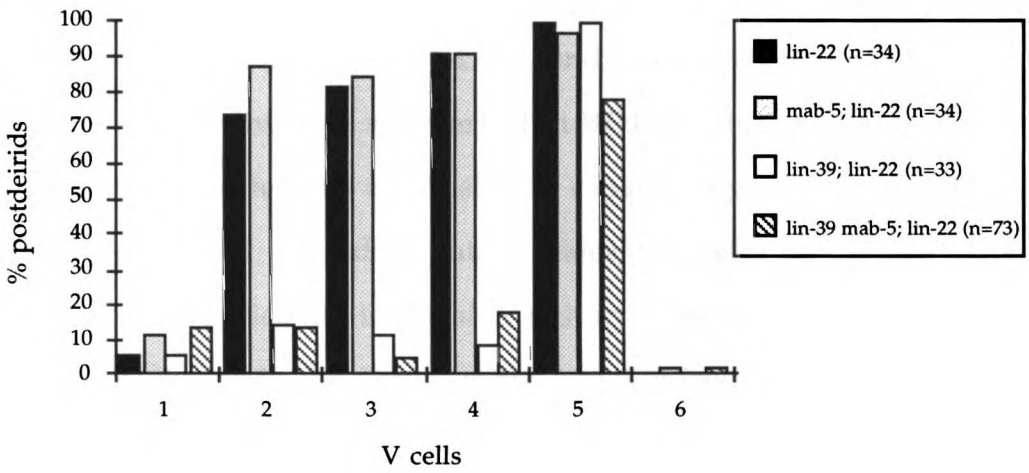
Figure 4-13. Effects of mutations in the Hox genes *mab-5* and *lin-39* on postdeirid production in *lin-22*.

The graphs show the percentage of postdeirid production by each V cell for four different genotypes in both hermaphrodites (**a**) and males (**b**). The legend indicates how each genotype is represented. In both males and hermaphrodites, mutations in *mab-5* had no statistically significant effect on ectopic postdeirid formation in *lin-22*. However, the addition of the *lin-39* mutation to either *lin-22* alone or to *mab-5; lin-22* resulted in lowering the levels of postdeirid production in V2-V4 to that of V1 in both hermaphrodites and males. Although mutations in *lin-39* do not normally affect the V cells, these data suggest a role for *lin-39* in patterning the V cells. *lin-39* expression is seen in V2-V5 (Wang *et al.*, 1993; J. Maloof, pers. comm.), so perhaps *lin-39* functions in these cells to prevent them from adopting a V1 cell fate.

METHODS. Postdeirid production by the V cells was scored as in Waring *et al.* (1992). The following strains were tested: *lin-22(n372), mab-5(e1239); lin-22(n372), lin-39(n1760); lin-22(n372)*, and *lin-39(n1760) mab-5(e1239); lin-22(n372)*. All the strains contained the male-generating mutation *him-5(e1490)*and were grown at 20°C. Animals mutant for *lin-39* were obtained by cutting open gravid hermaphrodites as in Figure 4-15. Postdeirid suppression was also seen for the *mu26* allele of *lin-39* (n=50).

Figure 4-13

a. hermaphrodites



b. males

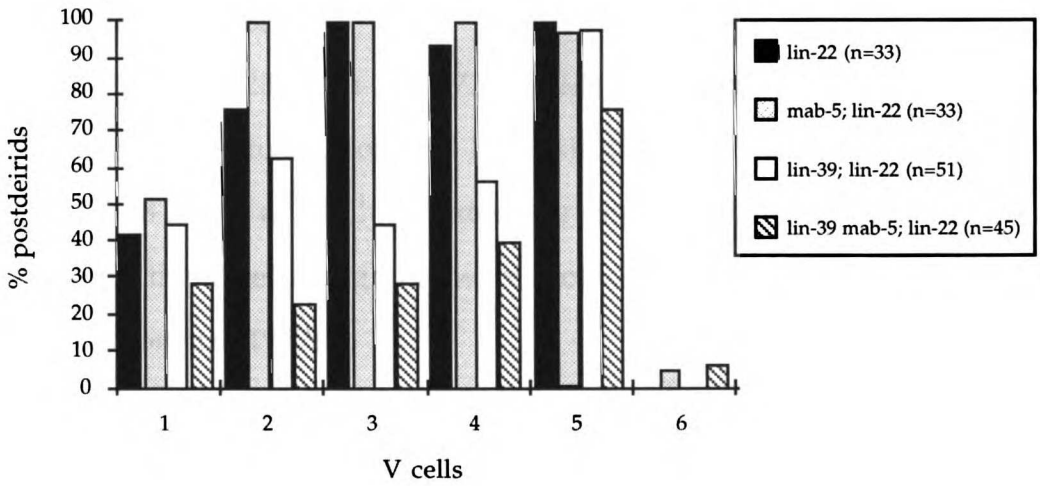


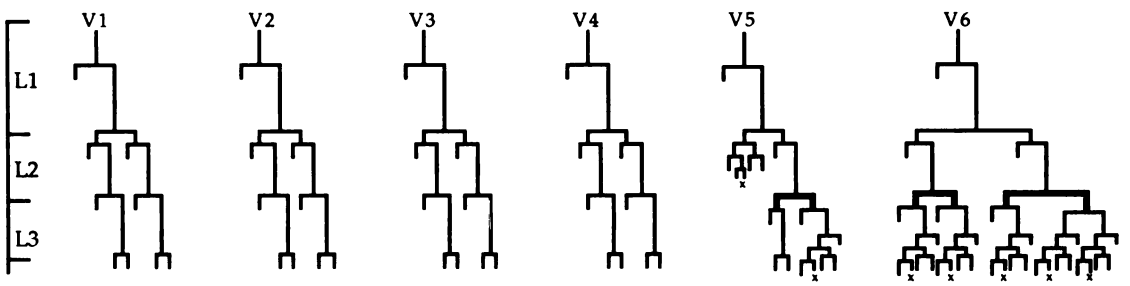
Figure 4-14. Ectopic L3 proliferative divisions still occur in animals mutant for both *mab-5* and *lin-22*.

Representations of the canonical V cell lineages in animals of the genotype *mab-5(e1239); lin-22(n372); him-5(e1490)*. The vertical axis represents time and the horizontal axis represents the direction of cell division, with anterior to the left. The approximate stage of the animal is shown to the right of each set of lineages. The larval stages L1-L3 are indicated at the left of the lineages. The L3 proliferative divisions are marked in **BOLD**. The data is a summary of n=11 males and indicates that all V cells are capable of generating L3 proliferative divisions. In *mab-5* mutant males, the extra L3 divisions do not occur in V5 or V6, indicating that *mab-5* activity is normally required to induce these divisions in the posterior body region (see Figure 3-1c). However, *mab-5* is not required for the proliferative division in *lin-22* mutants: the extra L3 divisions can still occur in anterior V cells and also in V5 and V6 in a *mab-5; lin-22* double mutant [see also a complete *mab-5; lin-22* lineage in Kenyon (1986)].

METHODS. *mab-5(e1239); lin-22(n372); him-5(e1490)* were staged using a Nitex filter according to Sulston and Hodgkin (1988). The animals were grown for 26-28 hours at 20°C (just after the time at which the L3 divisions are occurring) and the V cell daughters were scored using Nomarski optics as per Sulston and Horvitz (1977).

Figure 4-14

wild type



mab-5(e1239); lin-22(n372); him-5(e1490)

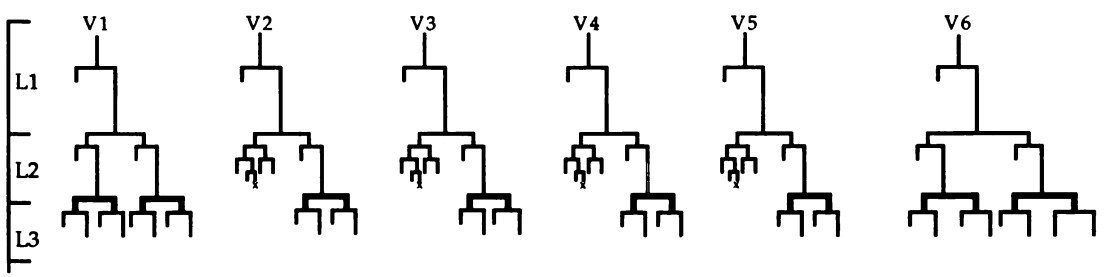


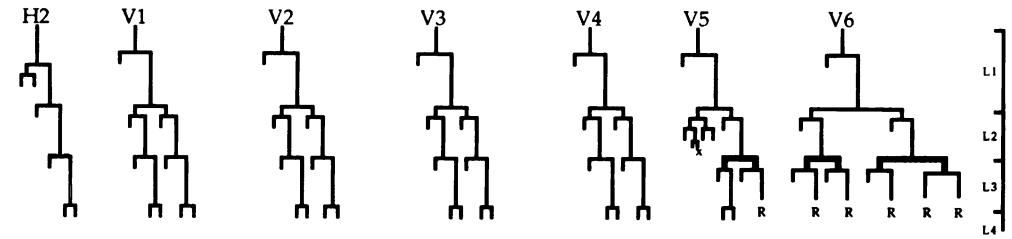
Figure 4-15. Loss of *lin-39* activity does not prevent L3 doubling divisions in *lin-22* males.

The vertical axis represents time and the horizontal axis represents the direction of cell division, with anterior to the left. The approximate stage of the animal is shown to the right of each set of lineages. Lineages indicated by dashed lines were not directly observed. "R" = the formation of a ray cell group from that branch of the lineage. The actual ray lineages were not observed, but the formation of the ray was scored. Larval stages are shown at the right. L3 proliferative divisions are shown in **BOLD**. *lin-39; lin-22* males were lineaged in order to analyze the effects of *lin-39* on the ectopic L3 proliferative divisions seen in *lin-22*. The data show that loss of *lin-39* activity does not prevent the occurrence of normal or ectopic L3 proliferations.

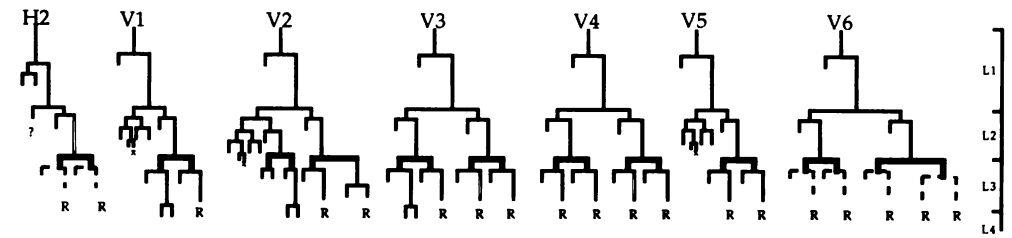
METHODS. Gravid *lin-39(n1760); lin-22(n372); him-5(e1490)* hermaphrodites were placed in M9 + 0.001% Trition X-100 in a depression-well slide and cut open using a scalpel. Eggs were transferred to a seeded plate and allowed to develop 24-36 hours. Late L2 larval stage animals were placed on 2% agarose pads under 22mm coverslips sealed with either wax or vaseline and lineaged under Nomarski optics as per Sulston and Horvitz (1977). Ray cell groups were identified on the basis of their characteristic morphology of three neurons and a nearby tail seam cell.

Figure 4-15

wild-type male



lin-39; lin-22 male #1L



lin-39; lin-22 male #2R

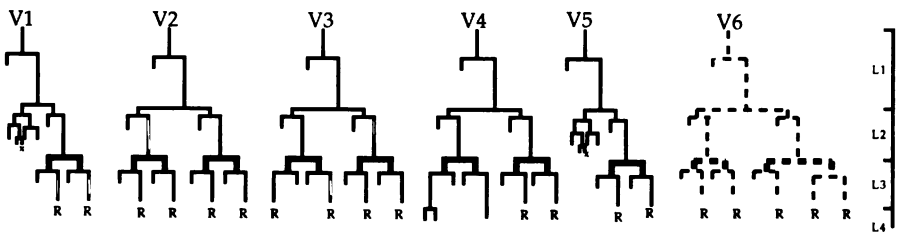


Figure 4-16. Loss of both *lin-39* and *mab-5* function decreases but does not eliminate the ectopic L3 proliferative divisions seen in *lin-22* males.

The vertical axis represents time and the horizontal axis represents the direction of cell division, with anterior to the left. The approximate stage of the animal is shown to the right of each set of lineages. The V cell lineages of this triple mutant were often abnormal enough to prevent an unambiguous determination of the L1-L2 lineages preceding the observed L3 lineages. As a consequence, only a small number of V cell lineages per animal could be determined. The presence of "L" or "R" following the number of an animal indicates which side of the animal was lineaged. Lineages for #3L and #4R were followed to adulthood. The presence of ray cell groups was confirmed (although the cell deaths of the ray lineage were not observed) and the production of a ray by a particular lineage is indicated. The larval stages are indicated to the left of the lineages. The L3 proliferative division is marked in **BOLD.** The data suggest that the loss of both *lin-39* and *mab-5* decreases the frequency with which the L3 proliferative division takes place in the anterior V cells, but does not eliminate it. It is also clear that ray precursor cells do not strictly require the L3 proliferative division in order to make sensory rays.
METHODS. Gravid hermaphrodites of the genotype *lin-39(n1760) mab-5(e1239); lin-22(n372); him-5(e1490)* were placed in M9 + 0.001% triton X-100 in a depression-well slide and cut open using a scalpel. The eggs were collected and allowed to grow into gravid hermaphrodites. Eggs were collected from these hermaphrodites as above and allowed to develop for 24 hours at 20°C. Late L2/early L3 males were placed on agarose pads sealed with wax and lineaged according to Sulston and Horvitz (1977).

Figure 4-16

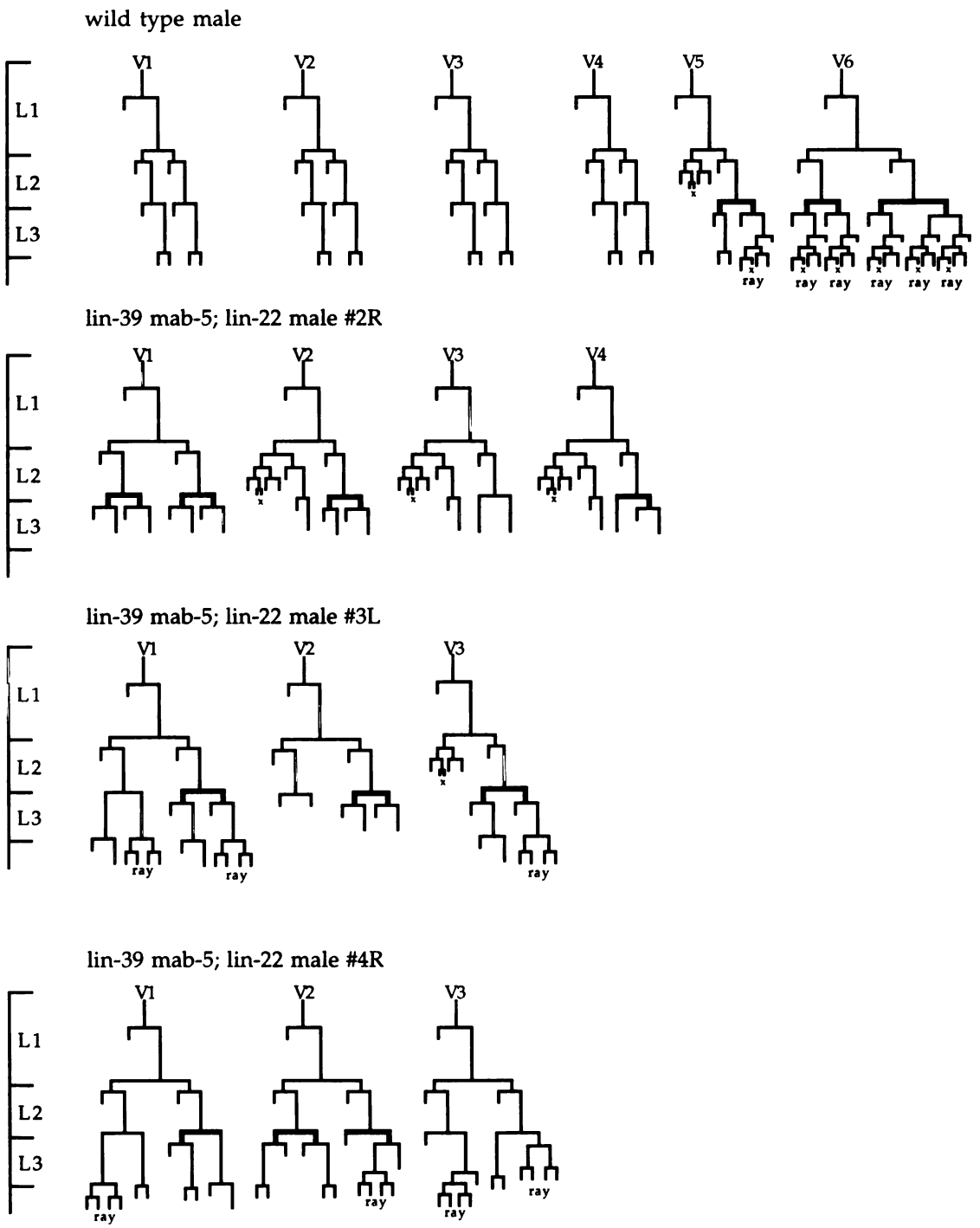

wild type male
L1
L2
L3
V1
V2
V3
V4
V5
V6
ray
lin-39 mab-5; lin-22 male #2R
lin-39 mab-5; lin-22 male #3L
lin-39 mab-5; lin-22 male #4R

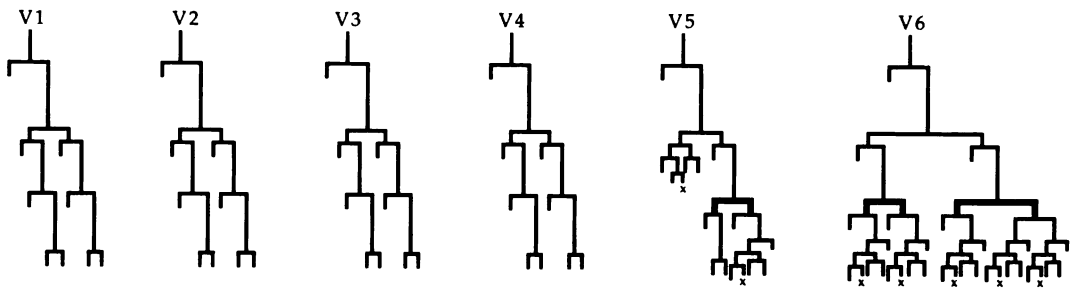
Figure 4-17. Effects of the strongest *lin-32* allele, *u282*, on the L3 proliferative divisions of V5 and V6 in wild type males.

The vertical axis represents time and the horizontal axis represents the direction of cell division, with anterior to the left. The approximate stage of the animal is shown to the right of each set of lineages.  = the postdeirid lineage.  = a ray neuroblast lineage. The L3 proliferative division is in **bold** lines. In an analysis of n=3 males, the normal V6 and V5.ppp L3 proliferative divisions are unaffected by loss of *lin-32* activity. However, the V5.pa branch of the lineage that would normally generate the V5 postdeirid fails to undergo the L3 doubling division in a *lin-32* mutant (indicated with an arrow). This is different from the anterior V cells of *lin-22* mutants, which fail to generate postdeirids. In those lineages, the anterior branch still undergoes the extra L3 division (see Figure 3-1b).

METHODS. Newly hatched *him-5(e1490); lin-32(u282)* animals were staged using a Nitex filter (Sulston and Hodgkin, 1988) and allowed to develop for 25 hours at 20°C. Animals were then mounted on agarose pads and lineaged through the L3 divisions using Nomarski microscopy (Sulston and Horvitz, 1977). V cell patterns were determined based on the morphology, number, and location of specific V cell daughters.

Figure 4-17

wild type male



him-5(e1490); lin-32(u282)

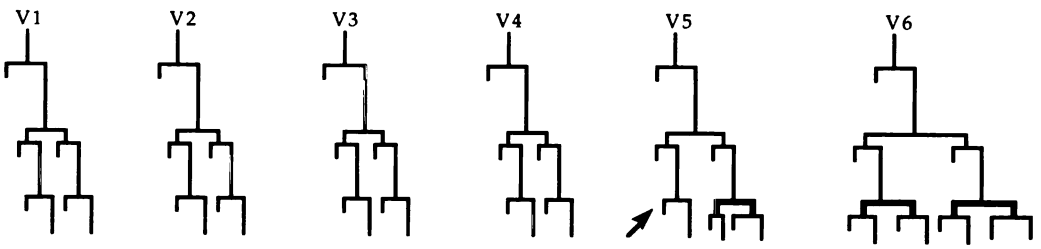


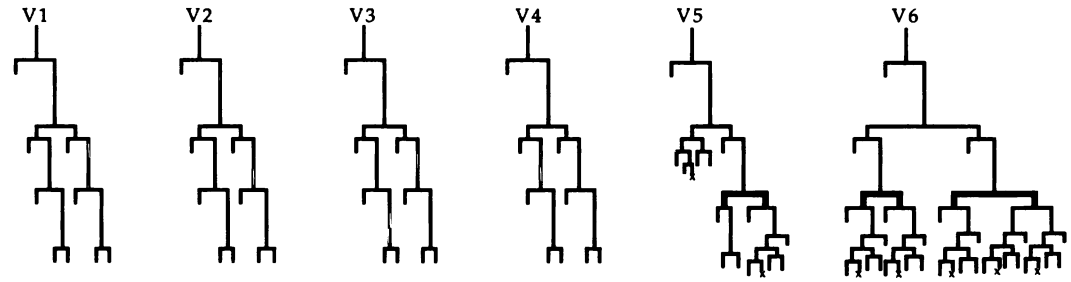
Figure 4-18. Effects of the strongest *lin-32* allele, *u282*, on the L3 proliferative divisions of the V cells in *lin-22* mutant males.

The vertical axis represents time and the horizontal axis represents the direction of cell division, with anterior to the left. The approximate stage of the animal is shown to the right of each set of lineages.  = the postdeirid lineage.  = a ray neuroblast lineage. The L3 proliferative division is in bold. The results show that *lin-32* has no effect on the ectopic L3 proliferative divisions seen in a *lin-22* mutant. Interestingly, the V5.pa branch of the lineage that does not have an extra L3 division in *lin-32* now proliferates as a consequence of *lin-22* activity. This confirms what was seen for *lin-32 (e1926)* (Figure 3-3).

METHODS. Newly hatched *lin-22(n372); him-5(e1490); lin-32(u282)* animals were staged using a 13um Nitex filter (Sulston and Hodgkin, 1988) and allowed to develop for 25 hours at 20°C. Animals were then mounted on agarose pads and analyzed using Nomarski microscopy (Sulston and Horvitz, 1977). V cell patterns were determined based on the morphology, number, and location of specific V cell daughters. The L3 doubling was specifically lineaged in a total of two animals.

Figure 4-18

wild type male



lin-22(n372); him-5(e1490); lin-32(u282)

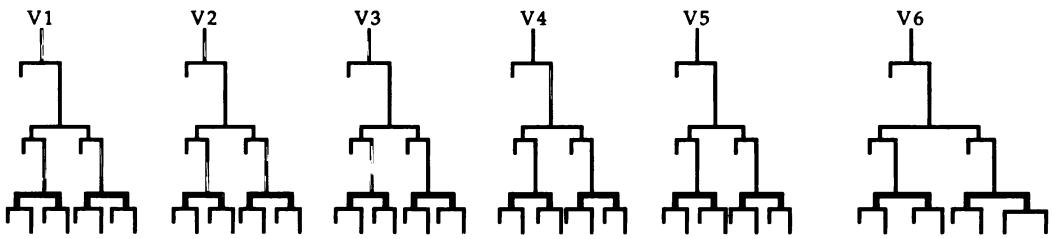


Figure 4-19. Lineages of *mab-5; lin-22; him-5; lin-32* males.

a-c. The vertical axis represents time and the horizontal axis represents the direction of cell division, with anterior to the left. The time of each cell division is given to the left of each set of lineages. The wild type lineages of V1-V4 and V5 are shown to the right of each mutant lineage for reference, with the time of the first L3 division aligned to the first L3 division occurring in the mutants. The wild type cell divisions are shown occurring at their correct developmental time after hatching, with the times indicated to the left. In all cases the cell divisions in the mutant males were delayed as compared to wild type.

a. Lineage of the cells V1, V2, and V3 from the left side of male #1L. The lineage was started at approximately 8 pm and all subsequent divisions occurred at or before the times indicated to the left. The lineage was discontinued at 11 am, but the worm was placed at 8°C overnight and viewed the following day. The animal had clearly undergone further rounds of division, suggesting that this male had experienced delayed development during lineaging, and only the V3.pa branch of the lineage unambiguously underwent the L3 proliferative division.

b. Lineage of V1-V3 from male #3R. Lineage was initiated at approximately 8 pm and continued for 19.5 hours, a which point the lineage was terminated. The lineage suggests that, although delayed, the L3 proliferative division nonetheless can occur in both the anterior and posterior branches of the V cells when both *mab-5* and *lin-32* function is removed from *lin-22* mutants.

c. Lineages of V1-V3 from male #4R. Lineage was started at approx. 8 pm and continued for 17 hours. This animal was also placed at 8°C overnight and viewed the following day. The lineage of this animal again suggests that at

least some V cell daughters are capable of undergoing the L3 proliferative division in the triple mutant. Further rounds of division had also clearly occurred once the animal was removed from the slide, although the exact cell asignments could not be made.

METHODS. Males of the genotype *mab-5(e1239); lin-22(n372); him-5(e1490); lin-32(u282)* were placed on 2% agarose pads under 22 mm coverslips and sealed in with vaseline. Animals were grown and lineaged at 20°C. Animals were lineaged under Nomarski optics as per Sultston and Horvitz (1977).

Figure 4-19

a.

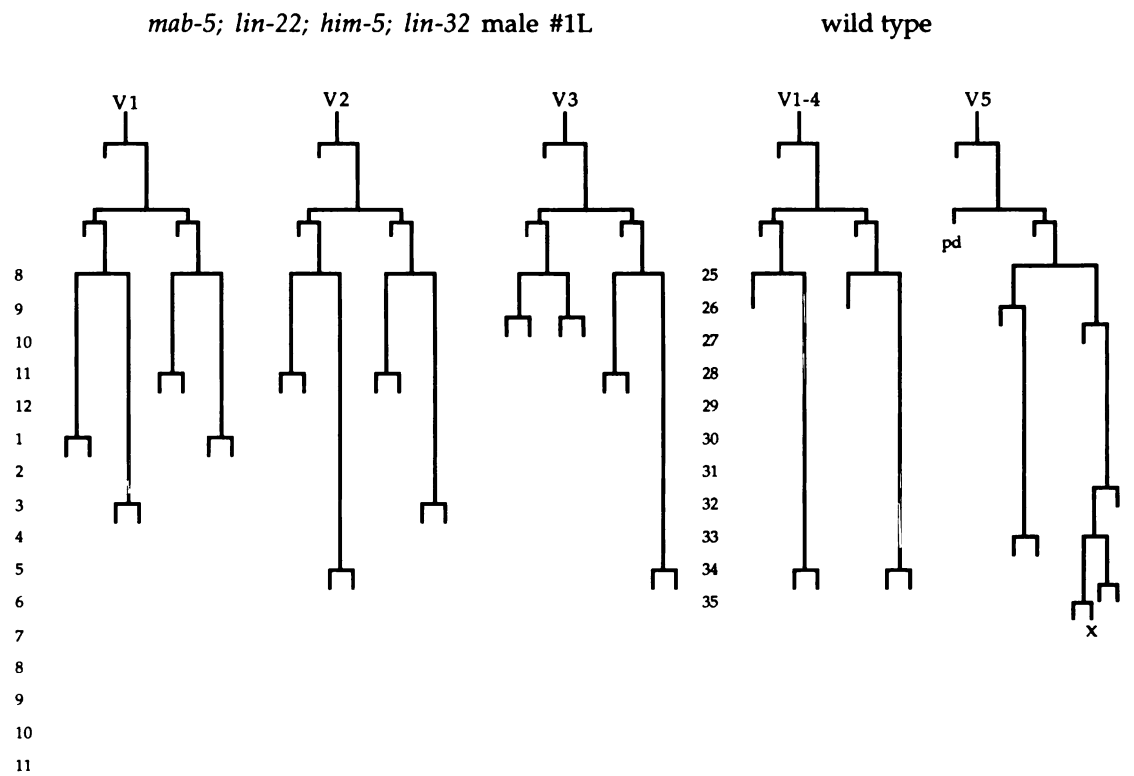

mab-5; lin-22; him-5; lin-32 male #1L
wild type
V1
V2
V3
V1-4
V5
pd
x
8
9
10
11
12
1
2
3
4
5
6
7
8
9
10
11
25
26
27
28
29
30
31
32
33
34
35

Figure 4-19

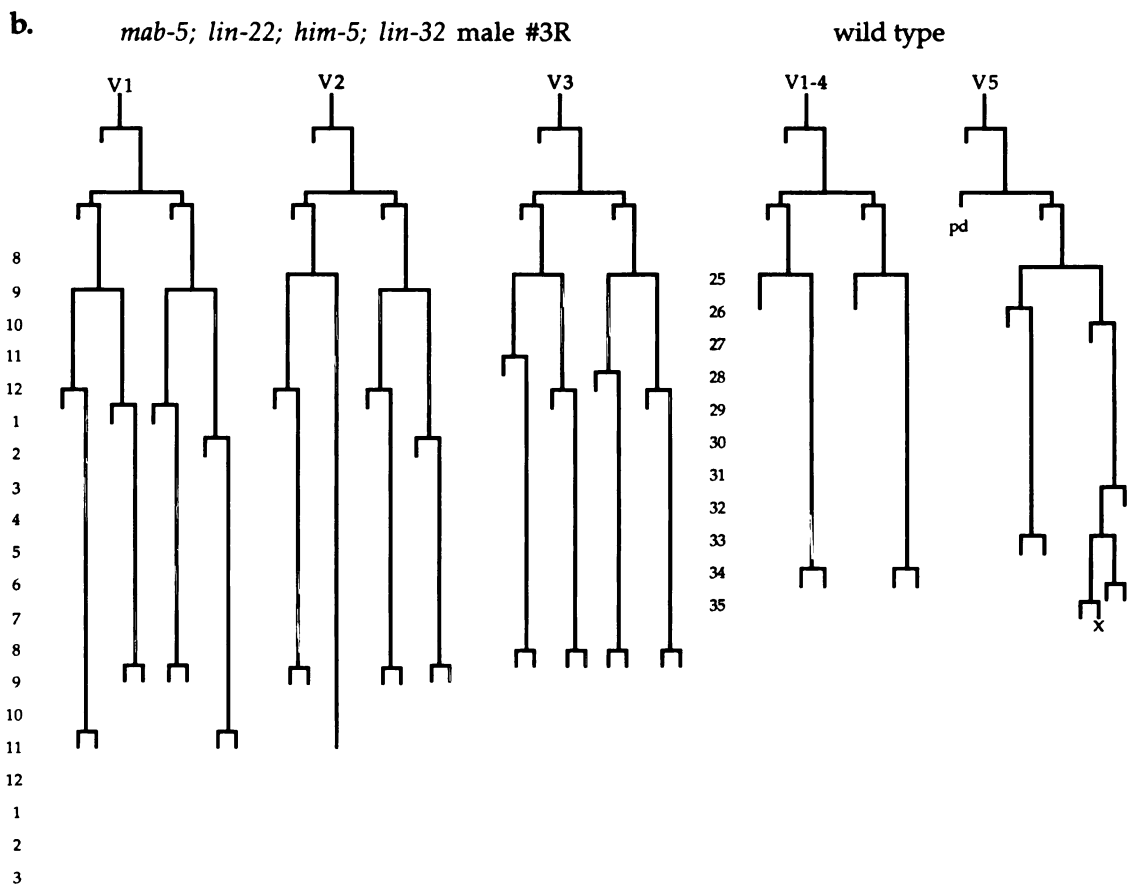

b.
mab-5; lin-22; him-5; lin-32 male #3R
wild type
V1
V2
V3
V1-4
V5
pd
x
8
9
10
11
12
1
2
3
4
5
6
7
8
9
10
11
12
1
2
3
25
26
27
28
29
30
31
32
33
34
35

Figure 4-19

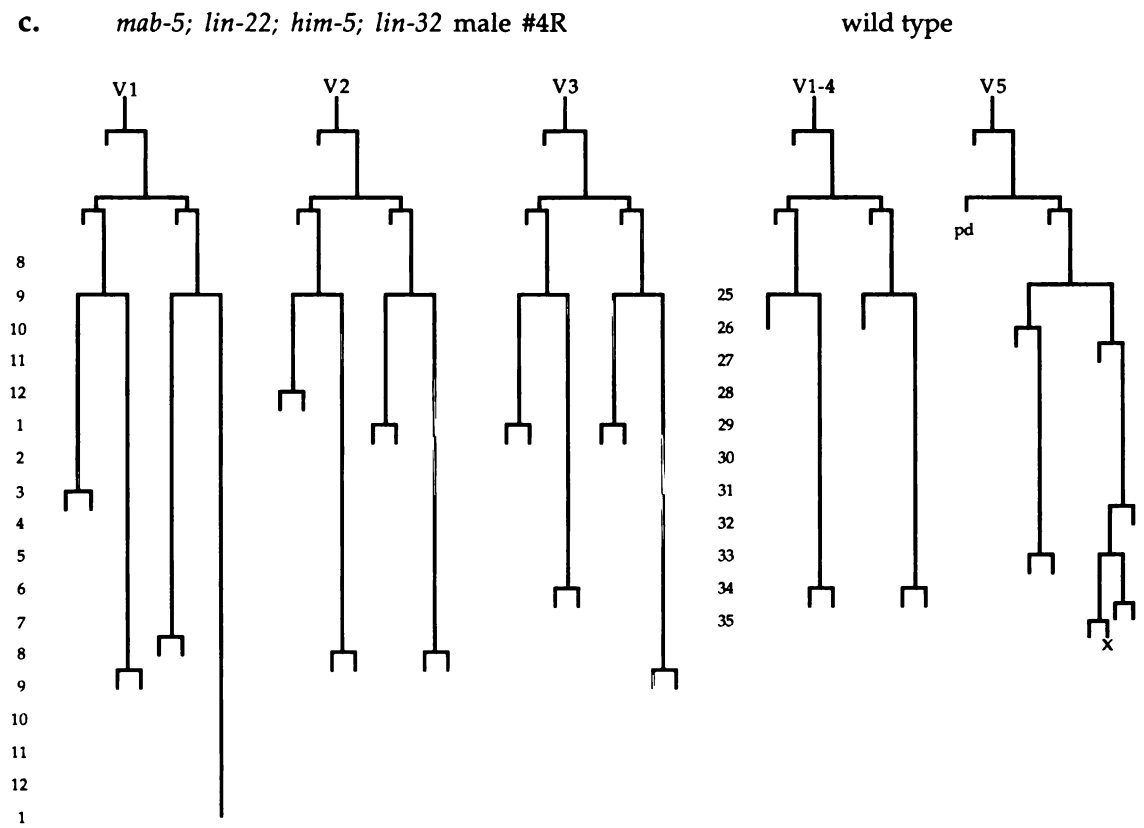

c.
mab-5; lin-22; him-5; lin-32 male #4R
wild type
V1
V2
V3
V1-4
V5
pd
x
8
9
10
11
12
1
2
3
4
5
6
7
8
9
10
11
12
1
25
26
27
28
29
30
31
32
33
34
35

Figure 4-20. Mosaic analysis of the *lin-22* gene.

In an effort to locate the site of action of the *lin-22* gene, mosaic analysis was attempted utilizing the free duplication *mDp1*, a large duplication of the left arm of chromosome IV. Mosaic analysis was carried out using the methods of Herman (1984) and utilizing the potentially cell-autonomous marker *osm-3(p802)*. *osm-3* affects the sensory amphid cells of the head (ADF, ADL, ASH, ASI, ASJ, ASK, and AWB) and the sensory phasmid cells of the tail, of which there is a set on both the right and left sides of the animal. Normally these cells extend processes into the environment which allow them to take up fluorescent dyes such as fluorescein isothiocyanate (FITC) and DiI (Hedgecock *et al.*, 1985). Mutations in several genes, including *osm-3*, block the ability of these cells to take up dye. Many appear to be due to changes in structural genes needed for the correct formation of the external processes. *osm-1* was found to act cell autonomously (Herman). We attempted to use *osm-3* in a similar fashion as a cell autonomous marker, although we did not know if it acted in a cell autonomous manner.

Control L2 and L3 *him-5(e1490)* animals were stained with either FITC or DiI and found to stain reliably in ADF, ADL, ASH, ASI, ASJ, and ASK for FITC, while only staining well in ADL, ASH, ASJ, and ASK (and weakly in ASI and AWB) for DiI. The amphid staining pattern was then examined in both Unc and non-Unc animals from the following strain: *lin-22(mu2) unc-17(e245) osm-3(p820); him-5(e1490); mDp1*. The strategy was to look at amphid staining for patterns that would suggest that the free duplication was lost somatically in a particular branch of the lineage that included the V cells (the cells we wished to analyze for *lin-22* activity) For example, somatic loss of *mDp1* in AB.a would lead to no amphid staining in ADF, ADL, ASJ, ASK

or AWB on the left side, but would still allow staining in ASI and ASK on the left and in all the amphid cells of the right. If we saw this staining pattern we would predict that V1, V2, V4, and V6 on both the right and left sides would also have lost *mDp1* and should be mutant for *lin-22* while the V3 and V5 cells would be wild type. The figure shows the early *C. elegans* lineage with the relevant amphid neurons circled and the V cells boxed. This illustrates the various combinations of amphid staining and V cell phenotype that would be linked depending on where *mDp1* was lost in the lineage.

A total of 3857 animals were stained with fluorescent dyes and examined for alterations in the amphid staining pattern. No interpretable patterns were ever observed suggesting that either the *osm-3* gene does not act cell autonomously, or that the somatic loss rate of *mDp1* was negligible and the spurious patterns seen were simply due to background staining problems. Evidence against the first explanation comes from the recent cloning of *osm-3*, which turns out to be a *kinesin* gene that is expressed within the amphid neurons (Shakir *et al.*, 1993; Tabish *et al.*, 1995). Further support for this second explanation comes from the observation that *mDp1* has a germ-line loss frequency of only 35-40%. The germ-line loss frequency has been seen to reflect the somatic loss rate, and 35-40% compares unfavorably to the duplication loss frequencies seen in successful mosaic analyses, which range from >60-90% [see Herman (1984) and Hunter and Wood (1990)].

Figure 4-20

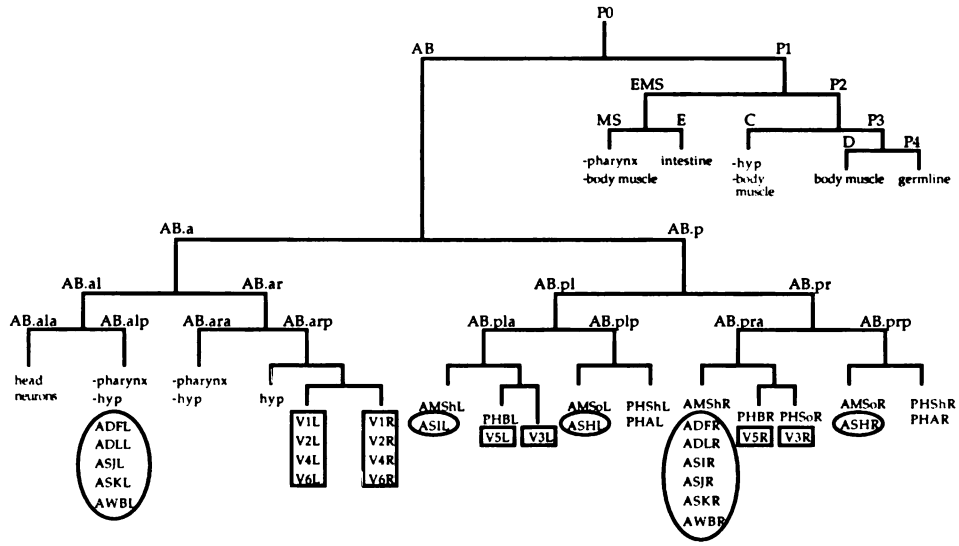

P0
AB
P1
EMS
P2
MS
E
C
P3
D
P4
-pharynx
-body muscle
intestine
-hyp
-body muscle
body muscle
germline
AB.a
AB.p
AB.al
AB.ar
AB.pl
AB.pr
AB.ala
AB.alp
AB.ara
AB.arp
AB.pla
AB.plp
AB.pra
AB.prp
head neurons
-pharynx
-hyp
ADFL
ADLL
ASJL
ASKL
AWBL
-pharynx
-hyp
hyp
V1L
V2L
V4L
V6L
V1R
V2R
V4R
V6R
AMShL
ASIL
PHBL
V5L
V3L
AMSoL
ASHL
PHShL
PHAL
AMShR
ADFR
ADLR
ASIR
ASJR
ASKR
AWBR
PHBR
V5R
PHSoR
V3R
AMSoR
ASHR
PHShR
PHAR

Figure 4-21. Anti-Mab-5 antibody staining during L2 in wild type and *lin-22* mutant animals.

a-b. a-Mab-5 staining in the anterior (**a**) and posterior (**b**) of a wild type animal. The location of the Vn.p daughters are indicated by the lineage drawings. In both the wild type male and the hermaphrodite all four daughters of V6.p express Mab-5, whereas only the posterior two daughters of V5.pp express Mab-5 (**b** and Salser and Kenyon, 1995). None of the anterior V1-V4 cells show any Mab-5 expression (**a**). The two strongly staining cells near the V5.pa daughters are the two left Q descendents.

c-d. Mab-5 expression in the anterior (**c**) and posterior (**d**) of a *lin-22* animal. As in wild type, all four V6 daughters and the two V5.pp daughters express Mab-5 protein (d). However, in *lin-22*(-) the daughters of V1-V4.pp also express Mab-5 protein (**c-d**). This indicates that *lin-22*(+) normally functions to prevent *mab-5* expression in the anterior V cells. Salser and Kenyon (1995) have shown that *mab-5* expression must be off in V5.pa in order to allow the postdeirid to form, and this pattern is conserved in V5 and the anterior cells in a *lin-22* mutant.

METHODS. *him-5(e1490)* and *lin-22(n372); him-5(e1490)* animals were staged and stained at 15-17 hours post hatching (20°C) with anti-Mab-5 antibody and DAPI according to S. Salser (pers. comm). V cell nuclei were located and identified using the nuclear stain DAPI.

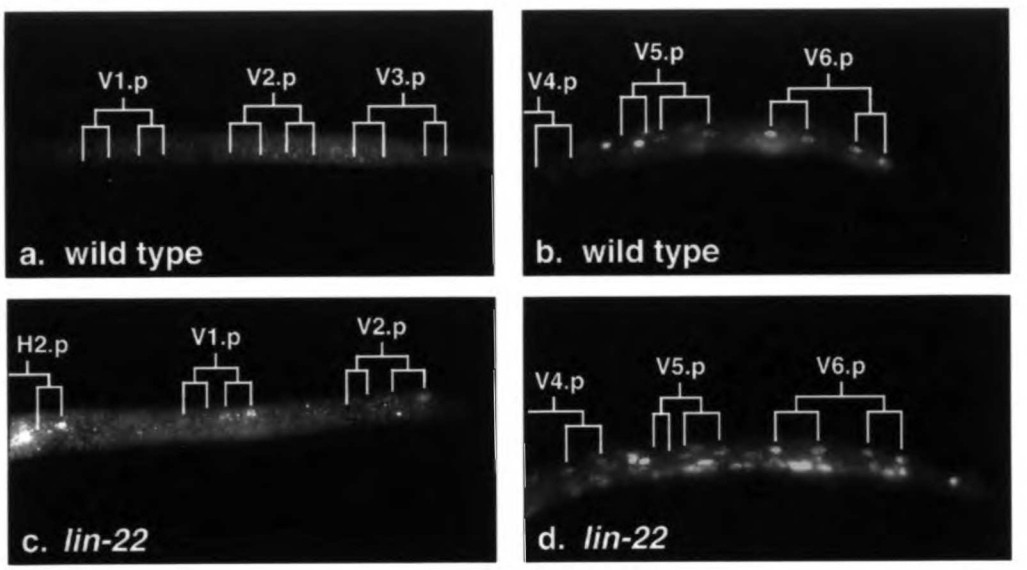

V1.p
V2.p
V3.p
a. wild type
V4.p
V5.p
V6.p
b. wild type
H2.p
V1.p
V2.p
c. lin-22
V4.p
V5.p
V6.p
d. lin-22

Table 4-1. Summary of V cell descendants staining with a-Mab-5 antibody at specific times up to the L3 proliferative division in *lin-22* mutants.

Time post-hatching is listed on the left, along with the actual V cell descendants scored, and the number of animals with staining in the daughters of a particular V cell are listed on the right. This pattern of expression in V1-V5 mimics the wild type pattern for V5 as seen by S. Salser (pers. comm.; see also Chapter 3 for further analysis). *him-5* controls showed no staining in any anterior V cell descendants at any timepoint (15-17hours, n=19; 25+hours, n=22; all others n>10).

METHODS. *him-5(e1490)* and *lin-22(n372); him-5(e1490)* animals were staged and stained with a polyclonal antibody to Mab-5 according to S. Salser (pers. comm.). Animals from timepoints with weak or nonexistent anterior staining were first selected on the basis on good morphology and clear V6 staining.

Table 4-1

mab-5 staining in V cells

hours post-hatching	V cell daughters	V1	V2	V3	V4	V5	V6
4-6	Vn Vn.a, Vn.p	0/12	0/12	0/12	0/12	0/12	12/12
8-10	Vn.a, Vn.p	0/8	0/8	0/8	0/8	0/8	8/8
15-17	Vn.pa, Vn.pp Vn.paa, Vn.pap Vn.ppa, Vn.ppp	10/11	8/11	2/11	1/11	5/11	8/11
19-21	Vn.ppa, Vn.ppp	6/9	7/9	4/9	4/9	6/9	8/9
21-23	Vn.ppa, Vn.ppp	0/8	0/8	0/8	1/8	1/8	8/8

Table 4-2. The *lin-39: lacZ* L1 expression pattern does not dramatically change in a *lin-22* mutant.

Wild type and *lin-22* mutant animals containing a *lin-39: lacZ* fusion array were analyzed during L1 for differences in the RNA expression pattern. The frequency of V cell staining increased slightly in the *lin-22* mutant, but the domain of the expression pattern remained unchanged. The differences seen could be due to variation in fixing slides or to the effects of differences between the *lin-22* and wild type cuticle.

METHODS. *muIS5* (n=18) and *n372; muIS5* (n=22) L1 rollers (*muIS5* contains the *lin-39:lacZ* fusion array with the *rol-6* dominant coinjection marker; Wang *et al.*, 1993) and were fixed and stained for ß-galactosidase activity according to Fire (1992). Only animals with strong P cell staining were scored for V cell staining. Strains were *him-5(e1490)*. Staining frequencies for the V and P cells in *muIS5* are consistent with those seen in Wang *et al.* (1993).

Table 4-2

% *lin-39;lacZ* Staining in each cell type

	P3/4	P5/6	P7/8	V1	V2	V3	V4	V5	V6
muIS5	22	89	100	0	0	6	0	0	0
lin-22; muIS5	5	95	100	0	0	23	5	14	0

Table 4-3. The *lin-39* protein expression pattern does not dramatically change in a *lin-22* mutant.

The cells indicated were scored at the three different timepoints listed. Only V2-V5 stain with the anti-*lin-39* antibody in wild type animals (J. Maloof pers. comm.). Table 4-3 lists the number of animals with ectopic staining in V1 or H2. An "*" indicates the occurrence of weak staining in V1 which was never seen to be as intense as that found in V2-V5. This occurred in both wild type and *lin-22* animals. Mutations in *lin-22* do not appear to alter the *lin-39* domain of expression. The levels of staining appeared identical for both wild type and *lin-22*.

METHODS. *him-5(e1490)* and *lin-22(n372); him-5(e1490)* animals were staged according to Figure 4-20 and stained with purified anti-*lin-39* polyclonal antibodies according to J. Maloof (pers. comm.).

Table 4-3

	hours post-hatching		
	4-6	8-10	15-17
	Vn.a, Vn.p	Vn.a, Vn,p	Vn.pa, Vn.pp Vn.paa, Vn.pap Vn.paa, Vn.ppp
wild type	1/18*	0/>80	0/11
lin-22	1/14*	1/14*	1/15*

Figure 4-22. Utilizing bromodeoxyuridine to selectively label ray vs. postdeirid cell groups.

a. Male at 38-40 hours post-hatching stained with an antibody to BrdU and to MH27, a cell adhesion molecule (Francis and Waterston, 1991). The animal was fed BrdU-labeled bacteria starting at ≈25 hours after hatching. In the figure, only the V cells daughters that arise after this time are staining brightly. Co-staining with MH27 also allows the cell outlines to be observed. **b.** Same male stained with DAPI. Most of the V cell daughters are out of the plane of focus in order to better see the postdeirid cell nuclei, labeled and marked with a bracket. These neuronal cells, which arise during the L2, are not labeled with BrdU.

BrdU Staining Protocol

(adapted from MH27 protocol; Austin and Kenyon, 1994)

Labeling

1. Grow X2913 bacteria (*E. coli* strain mutant for thymidylate synthetase; LIGHT SENSITIVE so grow in the dark) at 37°C in 1L minimal media supplemented with 25 uM thymidine (or thymine) + 475 uM BrdU (this conc. ratio was found by Steve Salser to be a good balance between incorporation and growth)

2. Spin down cells and resuspend in 1/10 volume minimal media/thy/BrdU (can just use growth media; store at 4°C)

3. Grow worms to desired stage and then transfer to plates containing a dry lawn of X2913+BrdU. Cover plates with foil and grow for desired length of time (up to 17 hr. has worked)

Fixation

4. Harvest worms in M9 or H_2O + triton X-100 (approx. 1uL/200mL) and rinse 2x in cold 10.8% sucrose in H_2O + triton

5. Put worms on polylysine-coated slides (pre-labeled with PAP pen) in drop of 10.8% sucrose /H_2O+triton

6. Place coverslip over worms and aspirate off liquid until worms flatten slightly

7. Freeze slides on dry ice (> 5 minutes) and pop coverslips with razor blade. (Pop coverslips before storage at -70°C, as well)

8. -20°C methanol 7 min

9. -20°C acetone 7 min

Rehydration/DNA Hydrolysis

10. EtOH/H_2O series 100%/90%/60%/30% (2.5 min each at RT {room temp})

11. Rinse with PBS/Tw 2x (1 min 1st wash, 5 min 2nd; can leave at this step awhile longer)

12. Add 100 uL 2M HCl to worms and incubate in humidified chamber 40 min at RT

13. Rinse 2x 5min in PBS/Tw

Primary Antibody

14. Add 100 uL of 1% BSA/5% goat serum/PBS/TW to each slide and block 30 min RT

15. Dilute BrdU Ab 1/10 and MH27 Ab 1/500 together in 1% BSA/5% goat serum/PBS. Add 20 uL to each slide and place coverslip on worms. Incubate 1 hr at 37°C

16. Rinse 3x 20 min in PBS/Tw at RT

Secondary

17. Reblock 1% BSA/5% goat serum/ PBS 5 min RT

18. Dilute goat anti-mouse IgG rhodamine conjugate 1/100 in 1% BSA/5% goat serum/PBS. Add 20 uL to slides, coverslip, and incubate 1 hr at 37°C

19. Rinse 3x 20 min in PBS/Tw

Reagents

10X PBS

87.66g NaCl

11.94g Na2HPO4 or 22.49g Na2HPO4 +
2.5g NaH2PO4

adjust pH to 7.4

add tween20 to 0.2%

H2O to 1L

Minimal Media Prep

For 1 liter

250 mL 4x salts

250 mL 12% casamino acids (final conc. 3%)

5 mL 40% glucose

5 mL ea amino acid stock

490 mL H2O

4x salts:

28g K2HPO4-3H2O (21.75 w/o H2O)

8g KH2PO4

2g Na-citrate

0.4g MgSO4

4g (NH4)2/so4

to 1L w/ H2O

12% casamino acids:

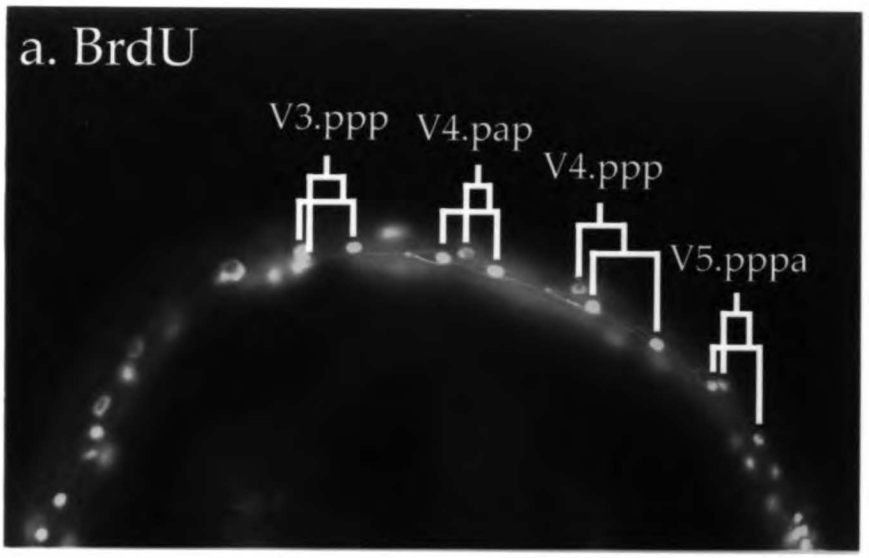
30g casamino acids in a total vol of 250 mL w/ H2O

-filter sterilize

amino acid stock:

5mg/mL stocks of proline, arginine, methionine, leucine, histidine, and threonine

When thymine is required add 20 mL of 2.5 mg/mL thymine (or thymidine)


a. BrdU
V3.ppp
V4.pap
V4.ppp
V5.pppa

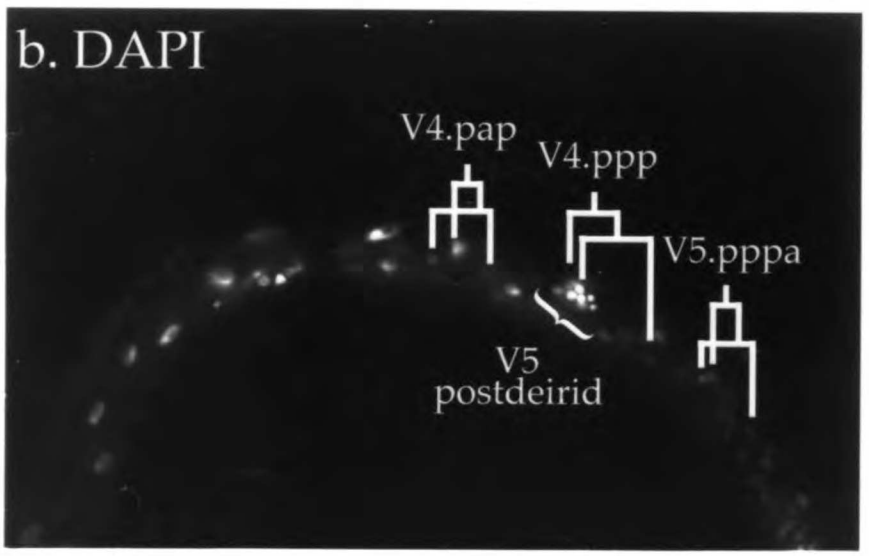

b. DAPI
V4.pap
V4.ppp
V5.pppa
V5
postdeirid

Figure 4-23. Isolation of a *Tc1* transposon as a linked RFLP to the *lin-22* gene.

The strategy was to cross a strain which only contains about 30 *Tc1* elements to a different *C. elegans* strain containing upwards of 300 *Tc1* elements, and then selectively maintaining only those extra *Tc1* elements contained within the region between *lin-1*, *lin-22*, and *unc-33* by careful backcrossing. The figure shows the position of each gene with the arrows indicating the location of the various recombination events that were used to map the *Tc1* (indicated as a stippled box). The number of animals of each class of recombinant is shown next to the arrow indicating the recombination event. The approximate genetic distance (in map units) between the three genes is shown above the map. The data indicate that this *Tc1* mapped to approximately 0.25mu to the left of the *lin-22* gene.

METHODS. *lin-1(e1275) unc-33(e204)* were crossed to *him-5(e1490)* males and the F1 males (*e1275 e204/ +*) were crossed to N62 and TR403 hermaphrodites. Hermaphrodite cross progeny were cloned out and their *e1275 e204/* N62 male progeny were identified and mated to *lin-1(e1275) lin-22(n372) unc-33(e204)* hermaphrodites in single male matings. The resultant *e1275 n372 e204*/N62 male cross progeny were backcrossed to *e1275 n372 e204* in single male matings, making sure that no recombination had occurred between *lin-1* and *unc-33*. This process was repeated for a total of eight backcrosses. Presence of the *Tc1* was determined by Southern analysis of recombinant DNA (Sambrook *et al.*, 1989; Church and Gilbert, 1984).

Figure 4-23

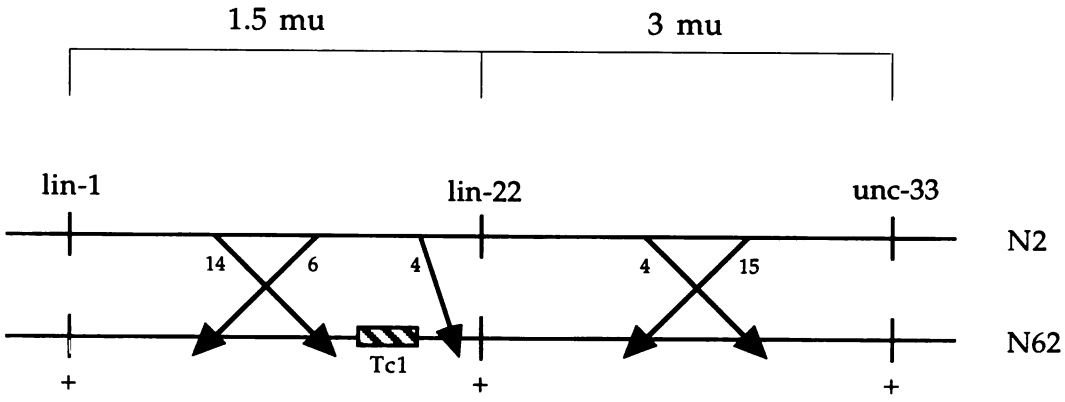


Table 4-4. The rescuing activities of the various YACs and l clones examined during the cloning of the *lin*-22 gene.

Since *lin*-22 rescue was never seen in the F1 progeny of injected animals, rescue was assayed on the basis of the number of stable roller-transmitting lines that rescued the *lin*-22 alae defect. DNA flanking the *Tc1* most closely linked to *lin*-22 was used to identify 8 separate Yeast Artificial Chromosomes (YAC), one of which rescued the *lin*-22 phenotype. This YAC was used to identify 28 genomic lambda clones from an unamplified library (in l2001; Stratagene), which were pooled together and injected. Two pools of l clones rescued the *lin*-22 alae phenotype. When the individual l clones were microinjected, only a single clone (l10) was capable of rescuing the *lin*-22 phenotype. An "*" indicates that these clones displayed an odd, partial rescue phenotype, in which rescue was most prominent after the animals had been allowed to starve for 1-2 months on their plates. By Southern analysis these clones (24 and 25, which are identical, and 38) did not have much, if any, homology with the l10 clone. A band from clones 24/25 and 38 was seen to hybridize very weakly to lambda 10 sequence, but this was not repeatable. These clones could perhaps represent other bHLH proteins with a weak ability to rescue *lin*-22.

METHODS. Microinjection of DNA was carried out according to the protocol of Mello *et al.* (1991) using 50-100 ng/ul of pRF4 as a coinjection marker.

Table 4-4

	DNA	Rescue
YAC DNA	Y94H6 (370 kb)	0/9
	Y55C10 (325 kb)	0/7
	Y54G2 (295 kb)	0/5
	Y68H6 (225 kb)	2/3
	Y50B4 (225 kb)	0/5
	Y43D2 (125 kb)	0/3
	Y77B2 (125 kb)	0/5
	Y87E10 (125 kb)	0/5
λ POOLS	5/19	0/6
	6/8/9/22/23/30	0/2
	21/24/25/38	2/2
	27/32/35/36/40	0/1
	4/10/16/31	2/4
λ CLONES	21	0/1
	24/25	0/2*
	38	0/2*
	4	0/4
	10	4/4
	16	0/2

Figure 4-24. A restriction map of the *lin*-22 rescuing lambda clone, its subclones, and various DNA fragments that were tested for rescue.

Dashed lines mark off the clone into 3 kb intervals. The "RV6" fragment was subcloned into the SmaI site of pIBI31 (IBI). The "8.5#6" fragment was cloned into the SalI and SmaI sites of pT7/T3a-18 vector (Pharmacia). The 8.5#6 subclones were all into pBluescript KS- (Stratagene). All fragments were gel isolated and purified before being injected. Aside from the entire 15kb lambda clone, only the 12kb Xho-Spe fragment was capable of rescuing the lin-22 alae defect. This suggested that the region between EagI and SpeI contained information important for rescuing activity. This region was later shown to contain helix2 of the *lin*-22 bHLH gene.
METHODS. Standard cloning procedures were utilized (Sambrook *et al.*, 1989). Plasmids were grown in SURE cells (Stratagene) to prevent the likelihood of deletions.

Figure 4-24

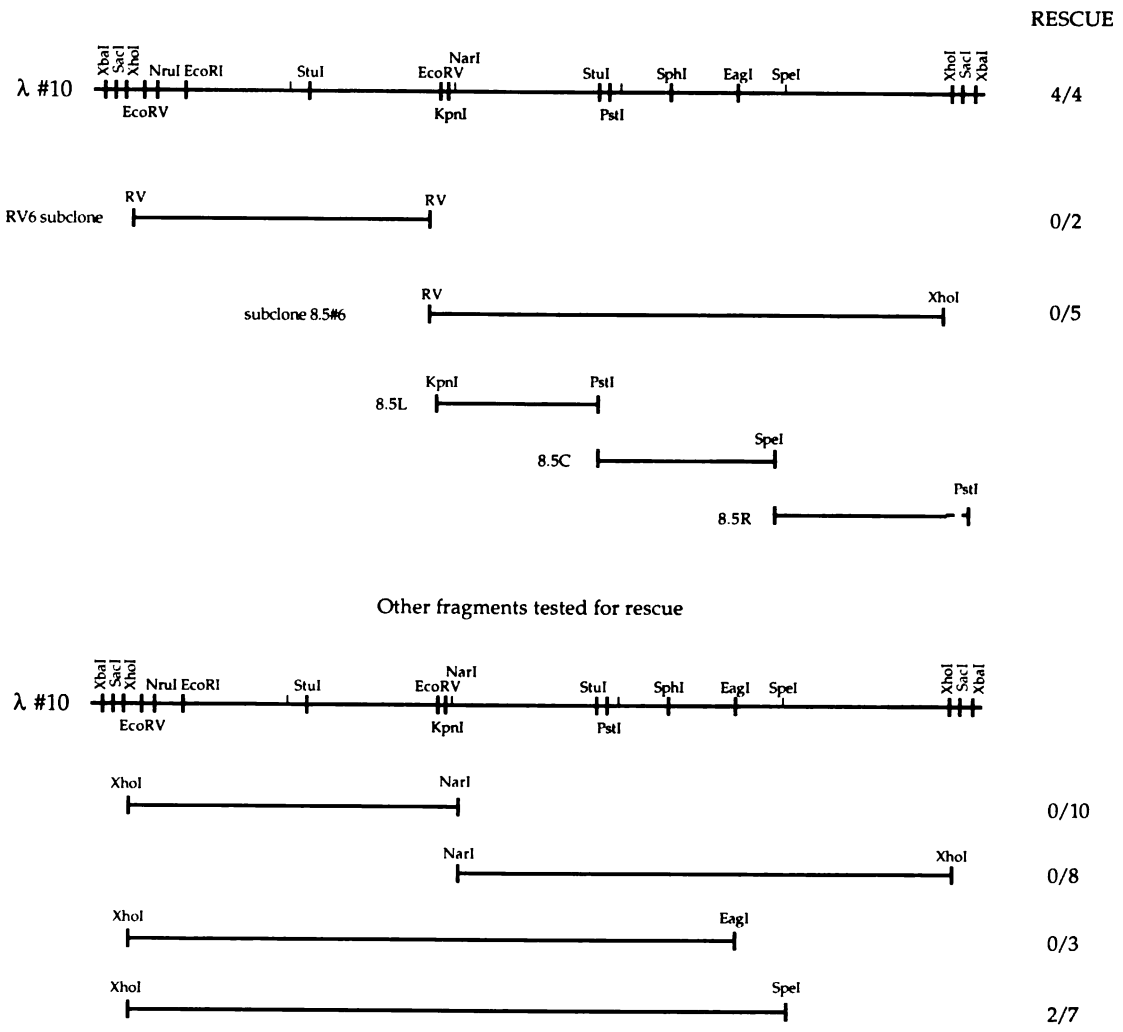

RESCUE
λ #10
XbaI SacI XhoI NruI EcoRI StuI NarI EcoRV StuI SphI EagI SpeI XhoI SacI XbaI
EcoRV
KpnI
PstI
RV6 subclone
RV
RV
4/4
0/2
subclone 8.5#6
RV
XhoI
0/5
8.5L
KpnI
PstI
8.5C
SpeI
8.5R
PstI
Other fragments tested for rescue
λ #10
XbaI SacI XhoI NruI EcoRI StuI NarI EcoRV StuI SphI EagI SpeI XhoI SacI XbaI
EcoRV
KpnI
PstI
XhoI
NarI
0/10
NarI
XhoI
0/8
XhoI
EagI
0/3
XhoI
SpeI
2/7

Figure 4-25. Genomic DNA from the lambda 10 subclones 8.5L, 8.5C, and 8.5R.

All sequences are 5' to 3' and progress from the left to the right of the lambda 10 clone. Primers used for sequencing are indicated above their DNA sequence. Oligonucleotides used in the PCR of *lin-22* coding sequence are indicated as well. The first sequences generated from each clone were obtained using primers that hybridize to vector sequence, such as the T3 promoter primer. The primers used are indicated at one end of the sequence. Only a single strand was sequenced, so errors will undoubtedly be found. The sequence corresponding to the coding region was confirmed by sequencing several RT-PCR products and PCR-amplified genomic DNA.

a. Sequence from the left side of the 8.5L clone (orientation the same as in Figure 4-23. The small underlined sequence is the KpnI site, so the sequence 5' to this was actually obtained from 8.5#6. The initial sequence was generated from the plasmid using the m13 reverse primer in the vector polylinker. All other primers listed were made and used to continue sequencing.

b. Sequence from the right side of 8.5L. The initial sequence was generated from the plasmid using the T7 promoter primer. All other primers listed were made and used to continue sequencing.

c-d. Sequence from the left side of 8.5C. Sequencing primers are labeled starting with a "C". The primers used to generate helix 2 of the cDNA were H1 and H1offset. H1seq was used to sequence the PCR product generated from H1 and H1off. The primers "genamp" and C2.2 were used to PCR this region from the genomic DNA of *n372*, and "genseq" was used to sequence

the resulting fragment. Coding sequence is underlined. The initial sequence was generated from the plasmid using a T3 promoter primer.

e. Sequence from the right side of 8.5C. Sequencing primers begin with "C". This sequence contains the H2 and H2offset primers that were used to get the 5' end of the cDNA. H2rev and the various SHR primers were used unsuccessfully in an attempt to find the C-terminus. Confirmed coding sequence is underlined. The dashed line indicates sequence that may or may not be coding, but was identified in a PCR screen of RT-RNA using pre-SHR/SHR and oligo dT.

f-i. the complete sequence of 8.5 R. Sequencing primers begin with "R". The protein sequence (WKIWVW) which represents the best candidate for a 3' "WRPW" motif yet identified is shown underneath its corresponding nucleotides. The primers UTR2.1 and UTR2.2 were used both to PCR screen oligo dT reverse transcribed RNA and to prime an RT reaction. Neither experiment produced a *lin-22* product.

Figure 4-25

a. 8.5L (left end)

Figure 4-25

b. 8.5L (right end)

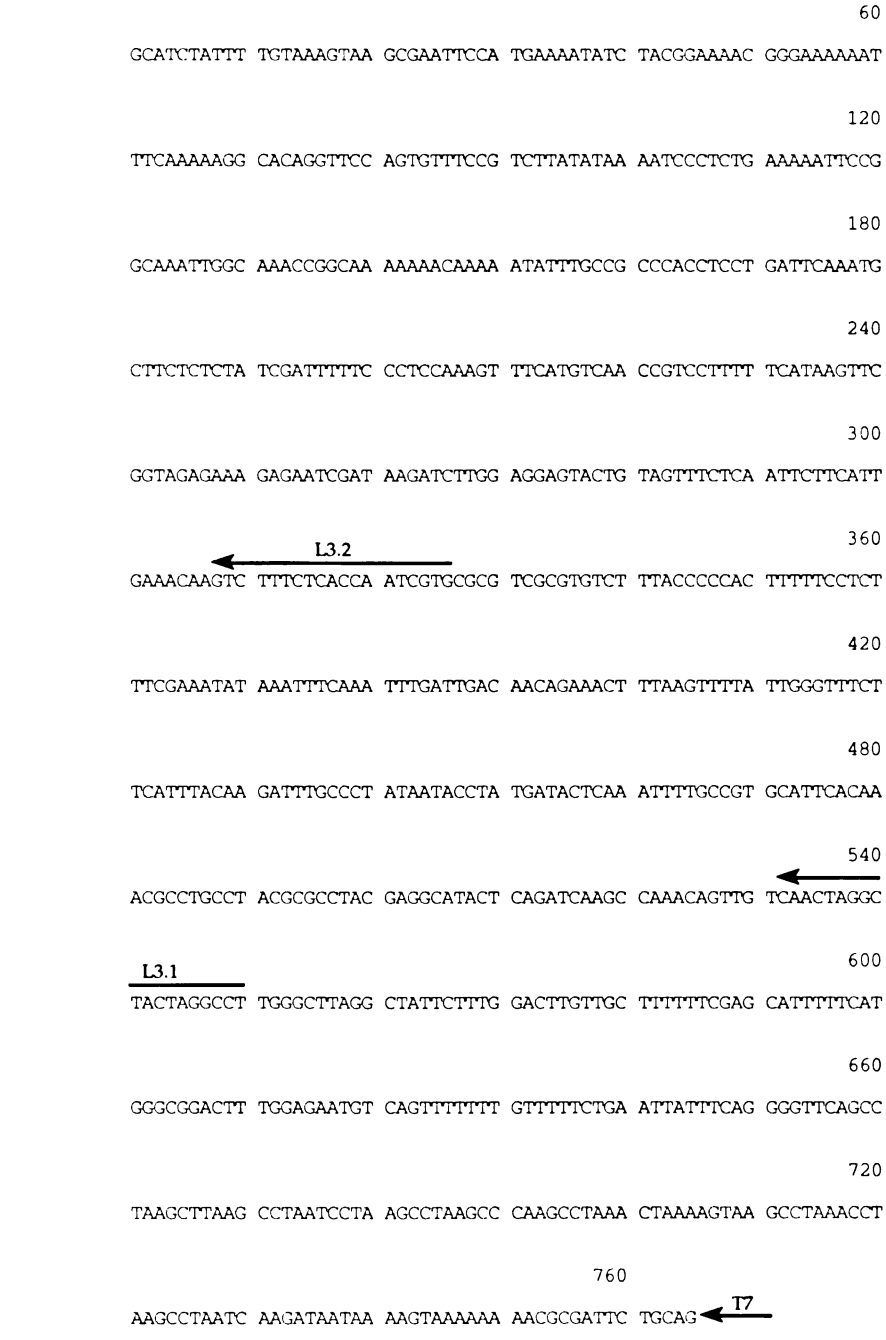


Figure 4-25

c. 8.5C (left end)

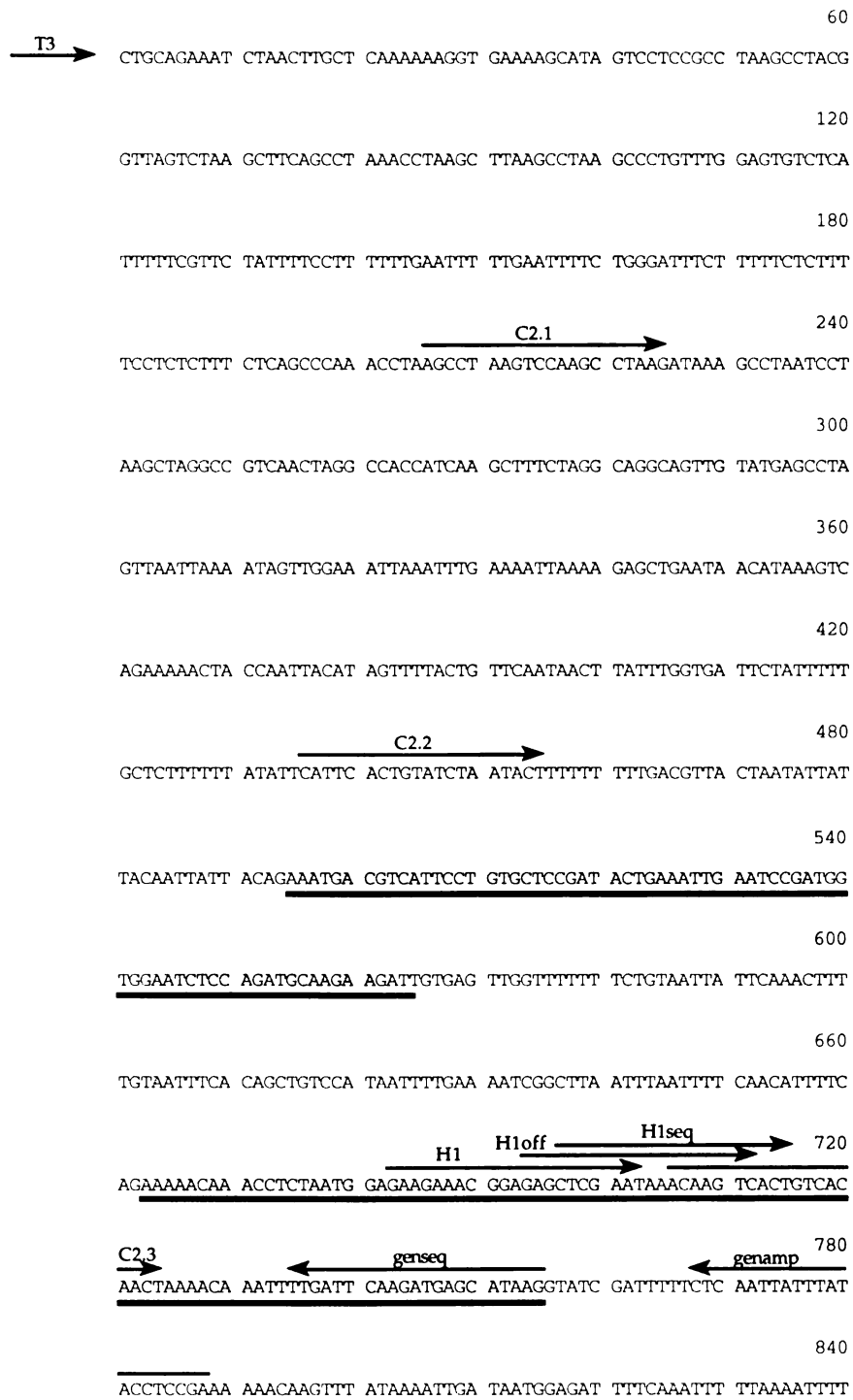


Figure 4-25

d. 8.5C (left end)

```
                                                                    900
AAGACGAAAT TTTGGTAAAT TGTCAAGTTT TTAAAAAAGT GAACTTTTTT AGCATTGCTT

                                                                    960
CAGATCAGGG GCCTCGCAAG TTCCGGAATT AAAAATTTCC GCCAAATTGT GGTTTTGCAC

                    980
TTCTTTTTGG AAATTTCAGA ATTTCAA
```

Figure 4-25

e. 8.5C (right end)

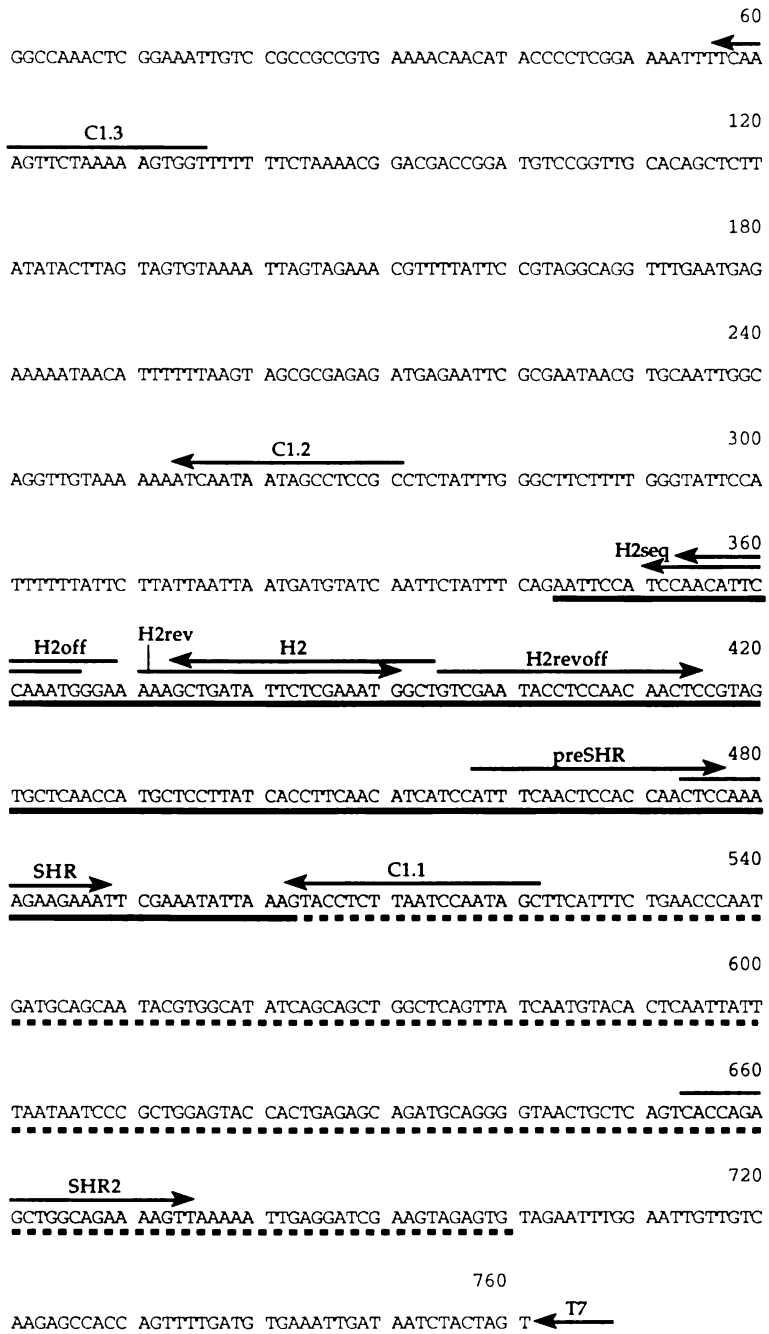


Figure 4-25

f. 8.5R

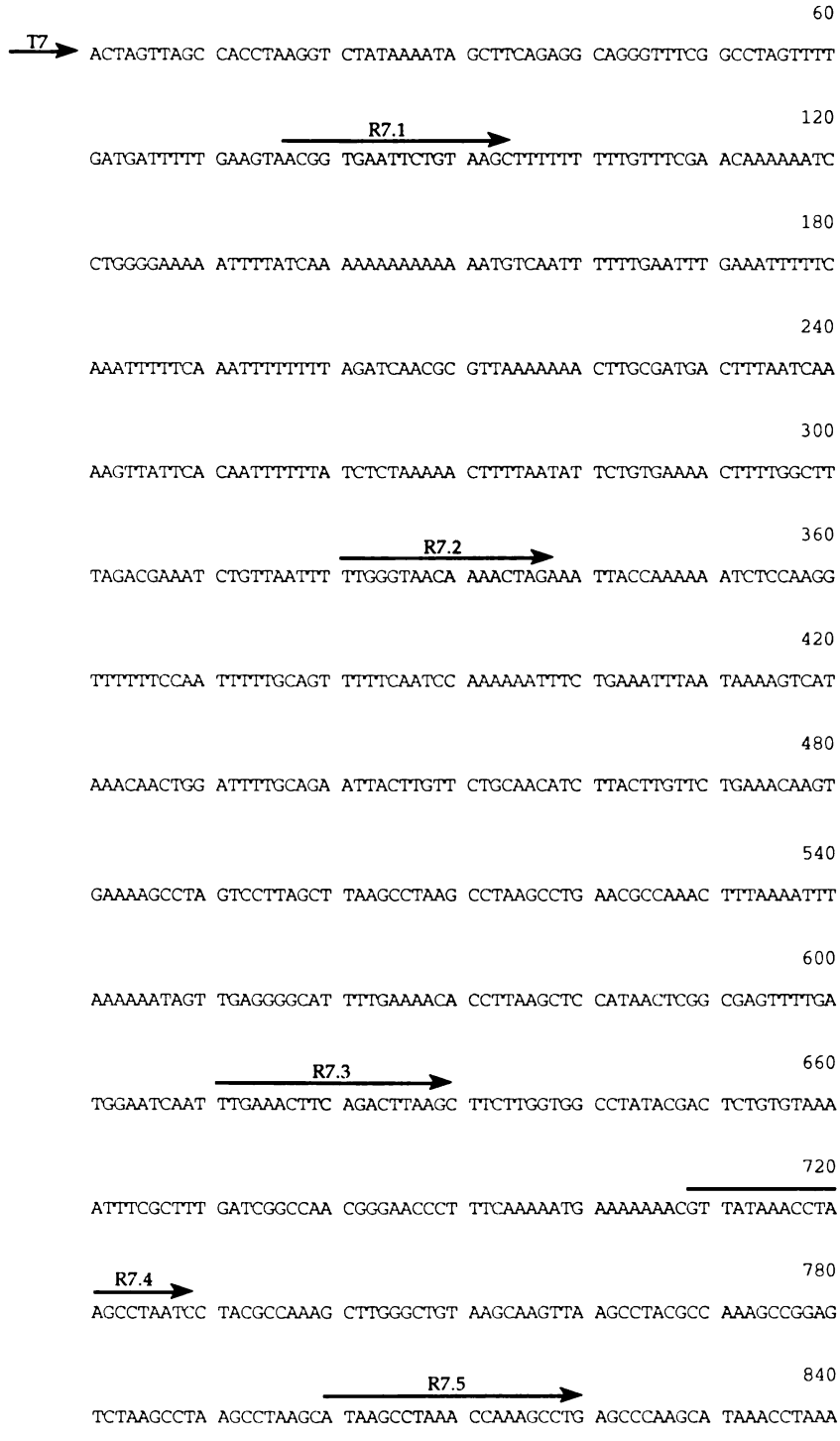


Figure 4-25

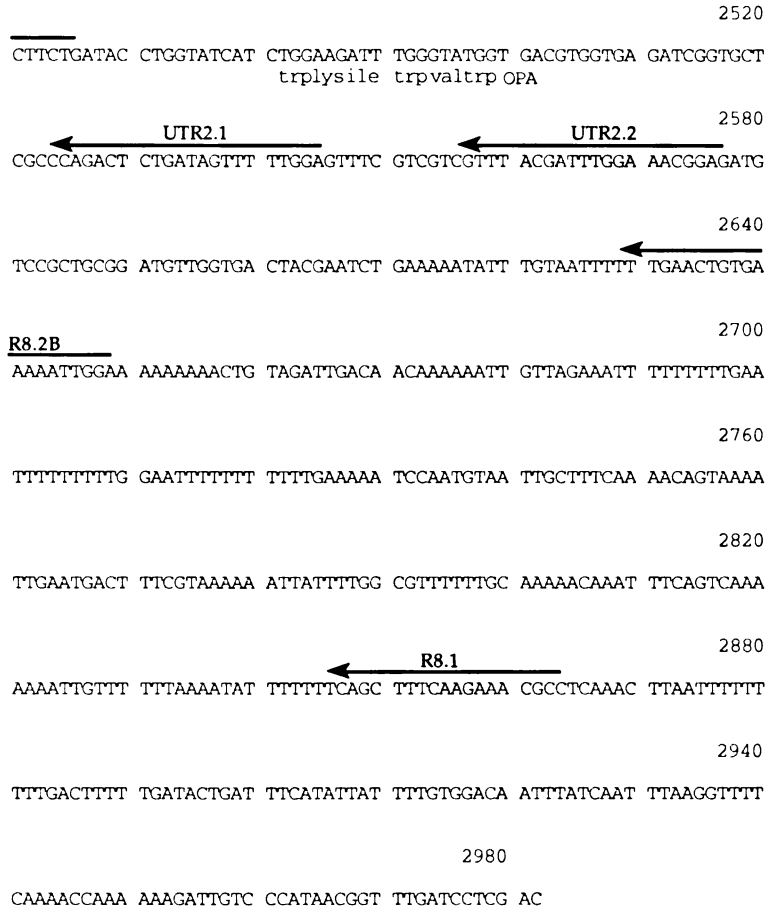
g. 8.5R

Figure 4-25

h. 8.5R

Figure 4-25

i. 8.5R



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CHAPTER 5

Discussion

DISCUSSION

As described in the introduction, several observations motivated us to pursue an analysis of the *lin-22* gene. First, the *lin-22* mutant phenotype indicated that this gene acts to block neurogenesis in the anterior cells of the lateral ectoderm (Horvitz, *et al.*, 1983; Fixsen, 1985). Second, genetic data suggested that a mutually inhibitory relationship exists between *lin-22* and the posterior-specific Hox gene *mab-5* (Kenyon, 1986). Finally, a study of signaling between the cells of the lateral ectoderm had suggested that these signaling events might regulate *lin-22* activity (Fixsen, 1985; Waring and Kenyon, 1990).

lin-22 is a homolog of the *Drosophila* gene *hairy*

We reasoned that the best way to find out how *lin-22* functions was to clone the gene. Cloning *lin-22* was arduous, but we were rewarded with the discovery that this gene encodes a basic helix-loop-helix protein with high homology to the *hairy* gene of *Drosophila* and *hairy* homologs from other organisms. Both *hairy* and the related *E(spl)* genes are negative regulators of the proneural genes of the AS-C (for review see (Ghysen, *et al.*, 1993)). In fact, *hairy* has been shown to bind directly to the upstream regulatory region of *achaete* (Ohsako, *et al.*, 1994; Van, *et al.*, 1994). By itself, this homology suggests that *lin-22* might act to directly regulate neurogenesis in the lateral ectoderm. More support for this model comes from the cloning of *lin-32*. This gene is required for the formation of both postdeirids and sensory rays

by the lateral ectoderm, and it turns out to be a *C. elegans* homolog of the AS-C genes (Zhao and Emmons, 1995).

So far, all *hairy* and *E(spl)* genes, as well as their identified homologs, contain a trp-arg-pro-trp tetrapeptide at or just before their C-terminus (Klambt, *et al.*, 1989; Bier, *et al.*, 1992; Knust, *et al.*, 1992; Ishibashi, *et al.*, 1993; Takebayashi, *et al.*, 1994). This is required for their interaction with the transcriptional corepressor *groucho* (Paroush, *et al.*, 1994). It is clear that this exact motif does not exist in >3kb of genomic sequence C-terminal to helix 2 in *lin-22*. The closest match was a trp-lys-ile-trp-val-trp-OPA sequence. To determine whether this sequence corresponded to the 3' end of *lin-22*, nested primers were made to the "putative" 3' untranslated region following this sequence. These primers were used in PCR screens of oligo dT-reverse transcribed RNA to see if they were really part of the *lin-22* coding sequence. RNA was also directly reverse transcribed using the "3'UTR" primers. Neither of these strategies was successful. This could imply that the actual 3'end of the gene is not on the l10 clone (entire genes are not always required for transformation rescue, see (Hill and Sternberg, 1992)), or that *lin-22* contains a unique 3' terminus. These questions will be resolved once the actual 3' end is identified.

lin-22 could act through the *C. elegans* AS-C homolog *lin-32*

lin-22 is a member of a family of genes that functions to block neurogenesis by inhibiting proneural gene function. This suggests that at least some of the effects of *lin-22* could simply be due to misregulation of *lin-32*. This is probably the case in postdeirid production. However,

examination of the *lin-22; lin-32* double mutant phenotype suggests that *lin-22* does more than negatively regulate *lin-32*, because neither the L3 proliferative divisions nor the formation of ray precursor cells seen in the *lin-22* mutant is eliminated when *lin-32* function is removed. One caveat to this argument is the observation that the three known *lin-32* alleles all become stronger when placed in trans to a chromosomal deficiency, implying that none of them are null, and the phenotypically strongest allele is a missense mutation (Zhao and Emmons, 1995). Nevertheless, a *lin-22; lin-32* double mutant containing the strongest alleles of both genes suggests that *lin-22* does not act solely through the regulation of *lin-32*. This leads us to the examination of the *mab-5* gene, the other primary candidate for regulation by *lin-22*.

lin-22 regulates the expression of the Hox gene *mab-5*

mab-5 is a Hox gene that specifies the fates of numerous cell types in the posterior body region of *C. elegans* (Kenyon, 1986). Among other effects, mutations in *mab-5* lead to the loss of the L3 proliferative divisions in V5 and V6. In addition, these cells no longer make sensory rays and instead adopt the epidermal seam cell fates of their anterior counterparts. This phenotype is roughly the opposite of that seen in *lin-22* mutants, so we were gratified to see that the expression pattern of the *mab-5* gene expands its domain into the anterior in a *lin-22* mutant, implying that the production of ectopic L3 divisions and sensory rays were due to a misregulation of *mab-5* activity in a *lin-22* mutant.

An interesting aspect of this ectopic *mab-5* staining is that in the *lin-22* mutant the anterior V cells express *mab-5* in the same complicated pattern as that seen in the V5 cell (S. Salser pers.comm.). S. Salser analyzed *mab-5* protein expression in wild type and found that it is expressed in a fairly straightforward fashion, generally on, in V6 and its daughters. However, the pattern of *mab-5* expression in V5 is quite complex: *mab-5* staining is first off in the L1, then comes on only in the posterior V5.pp branch of the lineage, eventually shuts off in V5.ppp just before the L3 proliferative division, and finally returns in the V5.pppp lineage that will generate the V5 ray. S. Salser has shown that this expression pattern is required for the daughters of V5 to adopt their correct fates. For example, *mab-5* must be off early to allow formation of the postdeirid, because early expression of *mab-5* in this cell blocks postdeirid formation. *mab-5* must also come on in V5.ppp to promote the L3 doubling division, and is later needed in V5.pppp to generate the sensory ray.

In a *lin-22* mutant the anterior cells H2-V4 all express *mab-5* in a V5-like pattern, but there is also a change within the V5 expression pattern itself. In *lin-22* mutants the V5.pppa branch of the lineage now expresses *mab-5*. This observation helps explain the generation of an extra V5 sensory ray from that anterior branch of the lineage. The novel *mab-5* expression in this V5.pppa branch is also adopted by the Vn.pppa branches of the anterior V cells as well. One could imagine that *lin-22* has a simple and direct regulatory relationship with *mab-5* in the V5.pppa branch of the V5 lineage, because this resembles a simple ON/OFF switch: *lin-22*(+) in on in V5.pppa, *mab-5* is repressed and alae is made; in a lin-22 mutant, where the activity of *lin-22*(+) in V5.pppa is lost, *mab-5* turns on and a ray is made. However, recall that the anterior V1-V4 cells in a *lin-22* mutant do not just turn on

mab-5 and keep it on. Instead, these cells adopt the complicated V5 expression pattern. At first this suggests that mutations in *lin-22* cause a complete cellular transformation of the anterior V cells into a V5 cell, complete with all the *mab-5* regulatory machinery. However, it may be that this machinery already exists in all the V cells, and all the *lin-22* mutation does is derepress *mab-5*.

Evidence for this latter hypothesis comes from the observation that the expression of the *lin-39* gene, which is normally expressed in V2-V5 (J. Maloof, pers. comm.), does not expand into the anterior in a *lin-22* mutant. This might be expected if *lin-22* mutations were causing a cellular transformation of H2-V4 into V5. *egl-5:lacZ* expression is also seen in V5.ppp, and is important for proper ray 1 migration into the tail fan (S. Salser, pers. comm.; (Chow and Emmons, 1994). We do not yet know if *egl-5* expression is found in the anterior V cells of a lin-22 mutant, but the direct analysis of *egl-5:lacZ* expression in *lin-22* will soon clear up this question.

In *Drosophila, hairy* does not appear to play a direct role in patterning the Hox genes

It is somewhat surprising that *lin-22* is required for the normal expression of *mab-5* because bHLH proteins with homology to *hairy/E(spl)* have not previously been seen to regulate Hox genes. This could indicate a novel activity for the *lin-22* bHLH protein. As a pair-rule gene, *hairy* is involved in setting up the metameric units of the fly embryo, and mutations in this gene remove seven "stripes" of patterning information (Ish, *et al.*, 1985). This can lead to indirect effects on Hox gene expression. For example,

hairy is important for patterning the pair-rule gene *fushi tarazu* (Howard and Ingham, 1986; Ish and Pinchin, 1987; Lawrence and Johnston, 1989), which is an important regulator of the Hox gene *Ultrabithorax* (Muller and Bienz, 1992). In another example studied, *hairy* was seen to affect the expression patterns of the genes *fushi tarazu* and *engrailed*, which then led to a change in the levels of expression of the Hox gene *Abdominal-A* without affecting its domain of expression (Macias, *et al.*, 1994). This observation has several implications for how we interpret the organization of the worm body plan. If *lin-22* affects the V cells as a "pair-rule" gene, affecting *mab-5* expression only within its domain of function, then we have to say that all the cells of the lateral ectoderm are effectively a single segment. If we take the opposite approach, and say that each V cell is in fact a metameric unit, then this suggests that the *lin-22* mutation has revealed a previously unknown function for this the *hairy* class of bHLH genes in patterning Hox gene expression.

Studies of the targets of bHLH proteins have uncovered canonical binding sites for various families of bHLH proteins, raising the question of whether there is evidence that *lin-22* binds directly to the *mab-5* and *lin-32* promoters. Most bHLH proteins can bind to the E-box sequence (CANNTG), with different families favoring specific versions of this sequence (such as the preference of the *myc* class of bHLH proteins for CACCTG; (Ohsako, *et al.*, 1994)). Two recent papers have described a canonical sequence for *hairy* binding: the favored sequence is CACGCG, with binding to CACGAG also seen(Ohsako, *et al.*, 1994; Van Doren, *et al.*, 1994). These papers also show that a CACGAG site ≈300 bp upstream of the start of *achaete* transcription is important for the negative regulation of *achaete* by *hairy*. No CACGCG sites have been seen upstream of the *mab-5* gene, but a CACGAG site is found

3917 bp upstream of the *mab-5* transcription start. The *mab-5* promoter is also riddled with CANNTG sites, indicating the possibility that a bHLH protein could bind upstream. Sequence information is not yet available for the *lin-32* promoter; however, recent data suggests that *lin-32* expression is altered in a *lin-22* mutant (Zhao and Emmons, pers.comm.). I have recently cloned the bHLH domain of the *hairy* protein into an expression vector and soon it will be possible to test whether *lin-22* actually binds upstream of these genes.

lin-22 is likely to act through a third target gene that regulates the L3 proliferative divisions

At first glance, the molecular data suggests that ectopic *mab-5* function could be contributing to both the generation of the ectopic L3 proliferative divisions and the sensory rays seen in the *lin-22* mutant. However, taking away *mab-5* function does not entirely eliminate ray production in a *lin-22* mutant, and has little or no effect on the generation of the *lin-22* ectopic L3 doubling divisions. Therefore, *lin-22* must regulate at least one other activity in addition to *lin-32* and *mab-5*. Since *lin-22* clearly affects the activity of one Hox gene, we wondered whether the residual rays (and perhaps the L3 proliferative divisions) were due to the misregulation of the remaining Hox genes, *lin-39* and *egl-5*. We found that the activity of these genes was responsible for some of the ectopic sensory rays seen in *mab-5*; *lin-22* males. However, since the expression pattern of *lin-39* is unaffected in a *lin-22* mutant, it appears that the loss of *lin-22* activity allows wild type levels of *lin-39* protein to promote neuronal development (perhaps by

raising *lin-32* levels). This is interesting because *lin-39* mutations do not affect either the L3 doubling divisions or ray formation. It is interesting to note that when *lin-39* is overexpressed using a heat-inducible promoter, it is capable of making rays (Hunter and Kenyon, in press). These observations might just illustrate the notion that there is a lack of specificity of Hox gene function that can only be seen in certain artificial situations, like overexpression or a *lin-22* mutant background. We as yet do not know if *egl-5* expression is altered in a *lin-22* mutant.

Neither the loss of *mab-5* nor *lin-39* appears to suppress the ectopic *lin-22* L3 proliferative divisions. However, the few interpretable V cell lineages from the *lin-39 mab-5; lin-22* triple mutant did show a decrease, but not the elimination, of L3 doubling divisions. One possibility is that the same promiscuity that allows *lin-39* and *egl-5* to promote ray formation in a *mab-5; lin-22* mutant is also allowing them to stimulate extra L3 divisions. The Hox genes could be responsible for this division, but their role might be masked by their ability to compensate for one another in a *lin-22* mutant. However, the L3 proliferative division could also be regulated by an entirely unknown gene, itself subject to regulation by *lin-22*.

Using a heat shock-*mab-5* construct, S. Salser has shown that the occurrence of this L3 proliferative division greatly promotes the subsequent formation of sensory rays. Therefore this proliferative division is tied to neurogenesis. To ask whether the neurogenic gene *lin-32* might be responsible for the ectopic proliferations, we analyzed the effects of *lin-32* mutations on the L3 doubling divisions. We found that both the wild type and ectopic *lin-22* doubling divisions were unaffected by a *lin-32* mutant. This extra L3 division took place in the *mab-5; lin-22; lin-32* triple mutant as

well. Thus, it seems likely that a gene other than *lin-32* regulates these divisions.

The role of *hairy* in cell proliferation in *Drosophila*

hairy has recently been shown to be important for inhibiting cell cycle progression and differentiation in the region just anterior to the morphogenetic furrow in the eye imaginal disc (Brown, *et al.*, 1991; Brown, *et al.*, 1995). *hairy*, along with *extramacrochaete*, keeps these cells from dividing and differentiating into neurons prematurely. This parallels the role of *lin-22*(+) in blocking cell proliferation and preventing neurogenesis. Ultimately, signaling molecules such as *hedgehog(hh)* and *decapentaplegic* (*dpp*)promote neurogenesis in the morphogenetic furrow (Heberlein, *et al.*, 1993; Ma, *et al.*, 1993), allowing progression of the furrow and the activation of such proneural genes as *atonal* (Jarman, *et al.*, 1994).

The role of cell signals and *lin-22* function

Interestingly, one of the models to explain how *hairy/emc* act to keep cells undifferentiated is that *hairy* and *emc* regulate (and inhibit) the expression of genes that act to receive or interpret the *dpp* signal. This is very reminiscent of our view of *lin-22* function in blocking postdeirid signaling in the V cells: *lin-22* is expressed in a pre-pattern in the anterior V1-V4 cells and prevents them from responding to cell signaling. A strange observation from the fly eye data is that ectopic *hh* appears to induce both *atonal* (an eye proneural gene) and *hairy* expression, necessary perhaps for

the correct timing of the many developmental events involved in furrow progression. Perhaps a *hh* or *dpp* homolog will be shown to be important for V cell signaling events (and might have an as yet unknown effect on *lin*-22 activity).

Does *lin*-22 play a role in *C. elegans* sex determination?

Aside from their role in neurogenesis, the bHLH genes of *Drosophila* also play an important role in sex determination. The *daughterless* (*da*) gene is a bHLH protein that forms heterodimers with members of the AS-C genes, and it is these heterodimers which have proneural function. *da* is an autosomal denominator in counting the X chromosome/autosome ratio that determines sexual identity in flies (Caudy, *et al.*, 1988a; Caudy, *et al.*, 1988b). The *scute* gene of the AS-C is a numerator in the counting of the X/A ratio (Erickson and Cline, 1991; Torres and Sanchez, 1991; Parkhurst, *et al.*, 1993). Overexpression of the *hairy* gene also disrupts sex determination, but this is thought to be an indirect effect of ectopic *hairy* activity on the AS-C genes (Parkhurst, *et al.*, 1990). This brings up the question of whether *lin*-22 could be part of an X/A counting mechanism is worms. Interestingly, the *lin*-22 mutation causes the somatic Pn.p cells (the vulval precursor cells) of the male to adopt hermaphrodite fates (Fixsen, 1985). There is also a high level of spontaneous male degeneration after only two to three days in the *lin*-22 strain. The X/A ratio ultimately regulates the activity of the gene *tra-1*, which is required for proper hermaphrodite development (Hodgkin, 1987). If the *lin*-22 Pn.p defect was due to a misregulation of *tra-1* (through altering the X/A ratio), then the loss of *tra-1* activity should suppress this phenotype,

and male Pn.p cells should be made. Fixsen (1985) made the *tra-1; lin-22* double using a *tra-1* mutation (*e1488*) that turns normal hermaphrodites into somatic males with hermaphrodite gonads. In this double mutant it was found that the Pn.p cells still adopt the hermaphrodite fate seen in *lin-22* mutants, so it is unlikely that *lin-22*(-) causes this transformation by misregulating the *tra-1* gene (since there is presumably no *tra-1* activity in the soma of these animals to misregulate). This question will be fully answered when the *tra-1*(null); *lin-22* double mutant is analyzed.

What are the likely regulators of *lin-22* activity?

Finally, we have the question of what genes could be regulating *lin-22* activity. The early dosage experiments between *lin-22* and *mab-5* suggested that these two genes might be mutually inhibitory (see Figure 4-9); however, given the number of other genes that influence ray formation in *lin-22* mutants, the implication of these dosage studies is not entirely clear. The analysis of *lin-22* expression in *mab-5* animals will be an important experiment to answer this question.

Sequence produced by the *C. elegans* genome project has uncovered the presence of a number of genes with homology to the gap and pair-rule genes of *Drosophila*. Among these homologies are some known regulators of *hairy* in flies, including the gap genes *hunchback, Kruppel,* and *knirps,* and the pair-rule gene *even-skipped* (Howard and Ingham, 1986; Ish-Horowitz and Pinchin, 1987; Hooper, *et al.*, 1989) all of which regulate the formation of particular *hairy* stripes in the embryo (Pankratz, *et al.*, 1990; Warrior and Levine, 1990; Riddihough and Ish, 1991). Perhaps these genes play a role in the potential position-specific expression of *lin-22* gene in the

anterior V cells. I have generated antibodies against the bHLH domain of *lin-22* and an analysis of *lin-22* expression will soon tell us if *lin-22* is indeed localized to the anterior cells of the lateral ectoderm. Expression studies will also be useful in such analyses as the effects of V cell ablations on *lin-22* activity.

In conclusion, the cloning of the *lin-22* gene has appeared to generate as many possibilities for *lin-22* function as it cleared up. However, we now have a starting point, the *hairy* homology, from which we can interpret lin-22 activity, and which will direct us in the course of future experiments.

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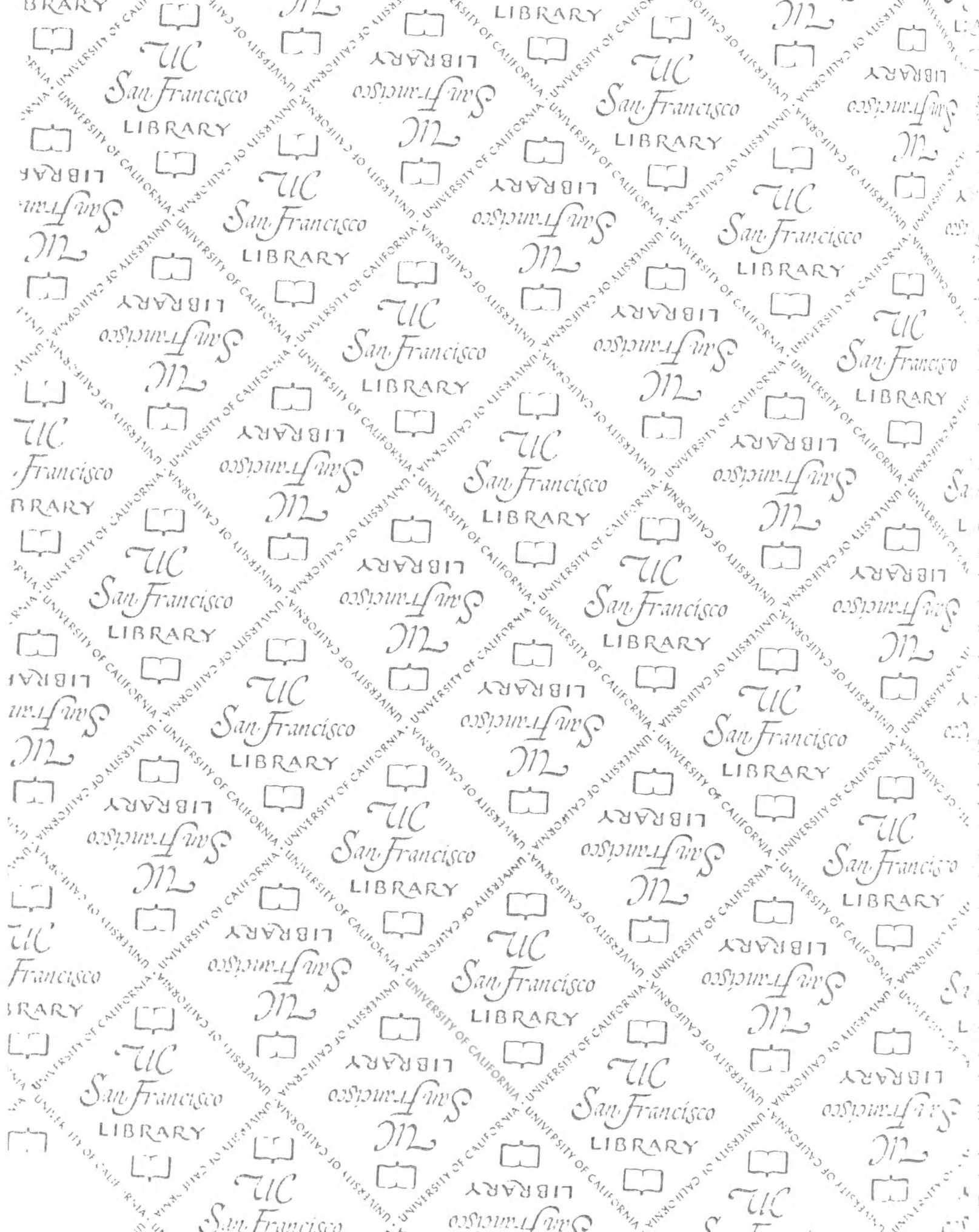
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