Comparative studies of Vulva development in *C. briggsae*.

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The thesis is dedicated to all the minority and women scientists and engineers that have come before me. Thank you!

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ABSTRACT

As evolution progresses, developmental changes occur. Genes lose and gain molecular partners, regulatory sequences, and new functions. As a consequence, tissues evolve alternative methods to develop similar structures, more or less robust. How this occurs is a major question in biology. One method of addressing this question is by examining the developmental and genetic differences between similar species. Several studies of nematodes *Pristionchus pacificus* and *Oscheius* CEW1 have revealed various differences in vulval development from the well-studied *C. elegans (e.g.* gonad induction, competence group specification, and gene function.)

I approached the question of developmental change in a similar manner by using *Caenorhabditis briggsae,* a close relative of *C. elegans. C. briggsae* allows the use of transgenic approaches to determine developmental changes between species. We determined subtle changes in the competence group, in 1° cell specification, and vulval lineage.

We also analyzed the *let-60* gene in four nematode species. We found conservation in the codon identity and exon-intron boundaries, but lack of an extended 3' untranslated region in *Caenorhabditis briggsae.*

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INTRODUCTION

For many years, animal model systems have been studied to understand how genes control development. Because of their small size, limited cell number, and rapid growth, *Caenorhabditis elegans* has been proven to be a powerful genetic and developmental model system (Brenner, 1974). The development of every cell is well documented (Sulston and Horvitz, 1977; Sulston *et al.*, 1980; Sulston *et al.*, 1983) and most recently, its entire genome has been sequenced (*C. elegans Sequencing Consortium*, 1998). But the mass of information leaves more questions than answers. Another approach is to use evolutionary comparisons with different Nematode species to take advantage of naturally occuring variation in developmental mechanism. Two species being used as satellite model systems are *Pristionchus pacificus* and *Oscheius* CEW1 to study developmental differences (Sommer and Sternberg, 1995; Sommer and Sternberg, 1996b; Sommer and Sternberg, 1996a; Félix and Sternberg, 1997; Sommer, 1997; Félix and Sternberg, 1998). These studies have revealed several variations on a common theme (Sigrist and Sommer, 1999; Jungblut and Sommer, 2000; Dichtel *et al.*, 2001).

To focus on developmental issuses, I examined the development of the vulva, the adult egg-laying structure, in another nematode species, *C. briggsae*, and compared it with *C. elegans* to study the mechanism of cell signaling and fate specification. In different species, many changes have been identified that show evolutionary divergence from *C. elegans* in vulval induction, specification, and execution. These changes could be explained by alterations in the regulatory mechanisms that define and guide the VPCs

to develop into the vulva. To better understand these changes, I review vulva development in *C. elegans*, followed by a discussion of vulva development in several nematode species.

Vulva Development in C. elegans

Pn.p birth

A newly hatched nematode *C. elegans* larva has a ventrolateral row of six equivalent P cell nuclei on each side denoted P1/2(L/R), P3/4(L/R) etc. During the mid-L1 larval stage, these nuclei migrate to the ventral cord and interdigitate to form a single row, denoted P1-12 (Sulston, 1976; Sulston and Horvitz, 1977). Two genes, *unc-83* and *unc-84* (encodes a predicted novel transmembrane protein), have been identified that specifically affect these nuclear migrations (Horvitz and Sulston, 1980; Sulston and Horvitz, 1981; Malone *et al.*, 1999). P cells that fail in nuclear migration often die; therefore these genes are required for the birth of the Pn cells.

During the L1 stage, the P cells each divide along the anterior-posterior axis to generate Pn.a, a neuroblast and Pn.p, an ectoblast. The gene *lin-26* is required for the non-neuronal fate (Horvitz *et al.*, 1983; Labouesse *et al.*, 1994; Labouesse *et al.*, 1996). In hermaphrodites, the posterior daughter or P(1-2, 9-11).p fuse with the hyp7 epidermis in L1. P12.p divides once more and the anterior daughter, P12.pa, forms hyp12; while the posterior daughter dies. P(3-8).p remain unfused to form the vulva during L3-L4.

Vulval Precursor Cells specification

The Vulval Precursor Cells (VPC) are the cells competent to form the vulva in nematodes. The VPCs are a subset of the Pn.p cells, specifically P(3-8).p, which express LIN-39, a homeodomain protein homologous to Drosophila *Sex combs reduced/deformed/proboscipedia* (Salser and Kenyon, 1994; Hunter and Kenyon, 1995). LIN-39 is required to make the VPCs competent to become vulval tissue. Recently, a WNT signaling pathway has been implicated in the regulation of hox genes in *C. elegans*, including *lin-39*. Furthermore, the genes, *bar-1*, a ß-catenin/Armadillo-like protein, and *apr-1*, an APC-related gene, and *pry-1*, are implicated in *lin-39* regulation (Eisenmann *et al.*, 1998; Maloof *et al.*, 1999; Eisenmann and Kim, 2000; Hoier *et al.*, 2000). Without *lin-39*, the VPCs fuse to the hyp7 epidermis in the L1 stage.

Even though three of the six cells normally form the vulva, all six are capable of forming vulval tissue if induced.

VPC induction

In early L3, the anchor cell (AC), a specialized cell in the gonad, sends a graded signal to the closest VPCs, generally P(5-7).p. P6.p, due to its close proximity to the AC, receives the most signal (Fig. 1A). This signal is encoded by *lin-3*, a gene containing Epidermal Growth Factor (EGF) domains (Hill and Sternberg, 1992). LIN-3 acts as a morphogen: VPCs with low amounts of LIN-3 are induced to one fate, 2°, and VPCs with high amounts of LIN-3 are induced to another fate, 1° (Sternberg and Horvitz, 1986; Katz *et al.*, 1995). A Receptor Tyrosine Kinase (RTK) protein, encoded by *let-23*, serves as the receptor, (Aroian *et al.*, 1990). The LIN-3 inductive signal starts a cascade of

intracellular signals involving: *sem*-5 (Grb2 adapter protein), *sos-1* (Ras guanine nucleotide exchange factor), *let-60* (Ras), *lin-45* (Raf serine/threonine kinase), *mek-2* (Map kinase kinase), *sur-1/mpk-1* (Map kinase), and *lin-1* (ETS domain) (Fig. 1B; Beitel *et al.*, 1990; Han and Sternberg, 1990; Clark *et al.*, 1992a; Clark *et al.*, 1992b; Han *et al.*, 1993; Lackner *et al.*, 1994; Wu and Han, 1994; Beitel *et al.*, 1995; Kornfeld *et al.*, 1995; Wu *et al.*, 1995). Once again, *lin-39* acts downstream of *lin-1* to affect vulval fates (Clandinin *et al.*, 1997; Maloof and Kenyon, 1998).

In addition to this highly conserved Ras/Raf pathway, at least one other intercellular pathway is triggered. P6.p utilizes LIN-12, a *notch* homologue, (Yochem *et al.*, 1988; Tax *et al.*, 1994), to enforce 2° fates on neighboring cells, P5.p and P7.p (Simske and Kim, 1995).

VPC execution

Each induced vulval cell assumes a distinct vulval fate. After two rounds of division, the four cells undergo the third characteristic cell division that indicates a specific vulval fate (Sternberg and Horvitz, 1986; Katz *et al.*, 1995). The 1° cells all divide transversely , TTTT. Half the progeny of the presumptive 2° cell will divide longitudinally, another cell will divide transversely and the remaining cell will remain undivided (LLTU or UTLL).

Of the 2° cells, the LL cells adhere to the hyp7 epidermis while the TU cells detach and migrate up toward the gonad giving an asymmetric appearance. Several genes are involved with the polarity of the 2° cells (Ferguson and Horvitz, 1985; Ferguson *et al.*, 1987; Sternberg and Horvitz, 1988). *lin-18*, a RTK, (T. Inoué, W. Katz, H. Oz, S.

Gharib, and P. Sternberg, unpublished results) and *lin-17*, a *frizzled* homolog, (Sawa *et al.*, 1996) affect the polarity of the cell divisions of the 2° cells. *lin-11*, LIM homeobox gene (Freyd *et al.*, 1990), functions to make the TUs different from the LL cells (Ferguson *et al.*, 1987).

The 1° cell, P6.p, completely detaches from the hyp7 epidermis and invaginates dorsally toward the gonad. The eight cells give a symmetric appearance surrounding the vulva. Four great-granddaughters of P6.p, P6.ppa(l/r) and P6.pap(l/r), signal the developing uterus to make the final connections. *egl-38*, a *pax* gene, (Chamberlin *et al.*, 1997; Chamberlin *et al.*, 1999) is involved in this process through reciprocal signaling with the uterine cells (Chang *et al.*, 1999).

Finally during the L4 stage, morphogenesis occurs. The VPC progeny invaginate dorsally and fuse to form seven multinucleate toroidal rings: vulA, vulB1, vulB2, vulC, vulD, vulE, vulF (Sharma-Kishore *et al.*, 1999; Shemer *et al.*, 2000). Each 2° contributes half the cells for the outermost rings (vulA-vulD). The final two rings are composed entirely of P6.p progeny. vulF makes the vulval-uterine connection.

Studies in other nematode species indicate alternative developmental methods which achieve the same structure.

Vulval Induction in Nematode Species

Induction by the Gonad

Throughout evolution several methods of vulval induction have emerged. They range from nongonadal induction to continuous gonadal induction to develop the vulva in

nematode species. I will describe vulval induction in several species and focus on two species in depth, *P. pacificus* and *Oscheius* CEW1.

As mentioned above, in *C. elegans*, the AC sends the LIN-3 inductive signal to the VPCs and induces them to form the vulva. If the gonad is ablated in the L1, the vulva is not formed. In one Rhabditidae species, *Cruznema tripartum*, induction functions the same as in *C. elegans* (Sommer and Sternberg, 1994). However, previous studies show various nematode species may implement vulval induction without a gonad, in 2 or 3 steps, or continuously thoughout development (Table 1).

Brevibucca sp. SB261, a member of the Brevibuccidae family, *Mesorhabditis* sp PS1179, and *Teratorhabditis palmarum*, both members of the Rhabditidae family, have gonad-independent vulva induction (Sommer and Sternberg, 1994; Félix *et al.*, 2000). Gonad ablations in these species result in an intact vulva. In the case of *Mesorhabditis* and *Teratorhabditis*, they have a posterior vulva which may have necessitated the change in induction (Sommer and Sternberg, 1994). Induction may be intrinsic to the VPCs, perhaps pre-patterning by hox genes as part of their specification to become precursors. Or, vulval cell fates may still be regulated extrinsically, with the inductive source coming from another tissue(s). For example, in *C. elegans*, LIN-3 induces cells in other tissues (Chamberlin and Sternberg, 1994). Gonad independent induction could also have arisen independently in separate species.

Another variation in vulval induction is the requirement for two temporally distinct inductive steps. In *Oscheius*, another Rhabditidae family member, and two members of the Panagrolaimidae families, *Panagrolaimus* sp PS1732 and *Panagrellus redivivus*, there are two separate induction steps, though the mechanics are different.

Oscheius inductions are both mediated by the AC (Félix and Sternberg, 1997). The AC first specifies P(5-7).p to become vulval tissue in early L3. This first signaling step is sufficient to fully specify the 2° fates. The second induction comes in late L3 and specifies the inner T divisions of the 1° by directing the daughters of P6.p (P6.pa and P6.pp). In Panagrolaimidae, the induction steps are temporally different from *Oscheius*. A gonad derived signal specifies P(5-8).p to become vulval tissue throughout L2. In L3, a second signal specifies the inner T fates of P6.p and P7.p (Sternberg and Horvitz, 1982; Félix and Sternberg, 1997; Félix *et al.*, 2000). Neither induction signal originates from the AC or uterine cells. Because of the mechanistic differences, inductive paradigms may have evolved separately.

Halicephalobus sp. JB128 has yet another variation on induction (Félix, 1999; Félix *et al.*, 2000). The VPCs require a survival signal, originating in early L2, from the gonad; otherwise, the cells undergo Programmed Cell Death (PCD). In addition to repressing cell death, the survival signal specifies the outermost vulval fate. It is followed by another gonad-based signal to specify the inner vulval fates in early L3. Finally, the AC specifies the inner T fates of the 1° cell. These observations indicate that the VPCs in *Halicephalobus* have the noninduced fate of PCD instead of fusion with the hyp7 epidermis as in *C. elegans.*

One more modification to vulval induction can be found in *Pristionchus pacificus*, a member of the Diplogastridae family. The gonad begins signaling the VPCs just after hatching and continues throughout development until the birth of the AC, which specifies the 1° cell fate (Sigrist and Sommer, 1999).

Pristionchus pacificus *vulva development*

Dr Ralf Sommer and colleagues have initated molecular and genetic analysis of *P. pacificus* as a satellite model system to *C. elegans*. In *P. pacificus*, the vulva is formed from 3 cells, P(5-7).p, which are born during embyrogenesis. These cells form a characteristic pattern of 2° 1° 2° once induced, but the division pattern is different from *C. elegans* (Fig. 2). P8.p adopts a 4° fate and fuses to the hyp7 epidermis. The remaining Pn.p cells undergo PCD during embryogenesis (Sommer and Sternberg, 1996a). Ablation results combined with mutant analysis indicate that P8.p only joins the vulval group under the influence of a 1° cell. Furthermore, P8.p suppresses P(5,7).p from assuming 1° fates (Sommer, 1997; Jungblut and Sommer, 2000).

P(3,4).p are VPCs

In wild-type *P. pacificus*, P3.p and P4.p cells undergo PCD in embryogenesis, yet if they survive they are competent to form vulval tissue. In the *ped-5* mutant, P3.p and P4.p do not die, but they do not divide. Ablation experiments in this mutant reveal P3.p and P4.p are capable of responding to the inductive signal and contributing to the vulva. Also in the *ped-6* mutant, P(3,4).p not only survive, but also differentiate ectopically and gonad independently (Sommer and Sternberg, 1996a). Thus, *P. pacificus* reduces the size of the equivalence group by programmed cell death.

A novel cell type

P8.p represents a novel cell type in vulval formation. It provides a negative signal on the VPCs to suppress nongonadal inductive differentiation in J1 (Juvenile stage 1). In

addition, P8.p mediates a lateral inhibitory signal originating in the M (mesoblast) cell to suppress P(5,7).p to secondary fates. And finally, P8.p can form vulval tissue, but only in response to a lateral signal from the 1° cell (Jungblut and Sommer, 2000).

Ppa-lin-39

The Hox gene, *Ppa-lin-39*, is involved in the regulation of apoptosis in the ventral cord. In *Ppa-lin-39(lf)* mutants, P(5-8).p undergo apoptosis, indicating its function is to prevent the VPCs from PCD and quite possibly, to specify the VPCs (Fig. 2A; Eizinger and Sommer, 1997). In contrast to *C. elegans*, however, *Ppa-lin-39* is not used during vulval induction. In *Ppa-lin-39(lf); Ppa-ced-3(lf)* double mutants, P(5-8).p survive and form a wild-type vulva (Sommer *et al.*, 1998).

Ppa-mab-5

Another homeotic transcription factor, *Ppa-mab-5*, functions to restrict the developmental competence of P8.p. In *Ppa-mab-5* mutants, P8.p differentiates to form vulval tissue gonad independently (Jungblut and Sommer, 1998). Additionally, *Ppa-mab-5* functions to specify the M cell and transcribe the inhibitory signal expressed by M (Fig. 2A; Jungblut and Sommer, 2000). This is vastly different from *C. elegans mab-5.* Although *Ce-mab-5* is expressed in and inhibits P(7,8).p, loss of function mutants do not have ectopic vulva (Clandinin *et al.*, 1997).

Oscheius / Dolichorhabditis *CEW1 vulva development*

Oscheius has five VPCs P(4-8).p with P(5-7).p forming the vulva (Fig. 3; Sommer and Sternberg, 1995). Félix and colleagues has uncovered an entire class of

mutants in *Oscheius* uncoupling cell division from cell fate, a phenotype of which is rarely seen in *C. elegans* (Dichtel *et al.*, 2001). Phenotypically, these animals are viable, fertile, Pvl (protruding vulva), and Egl$^-$ (egg-laying defective). These mutants can be divided into two classes: (1) VPC hypoproliferation or (2) VPC hyperproliferation.

Hypoproliferation

This class consists of *dov-1* through *dov-6*. *dov-1* reduces the number of divisions of P(4,8).p indiscriminate of the vulval fates they assume. But gonad and AC ablations reveal a role in vulval specification. The mutant seems to be defective in autonomous specification of the VPCs. This defect is overridden in P(5-7).p, possibly by the inducing signal from the AC .

In *dov-2*, *-3*, and *-4* mutant animals, the presumptive 3° cells have a reduction in the number of cell divisions from two to one, or in some cases none. VPCs assuming 1° or 2° fates have wild-type division patterns including P(4,8).p when impelled to assume 1° or 2° cell fates. This indicates these genes normally drive the cellular divisions of presumptive 3° VPCs and not specifically cellular divisions of P(4,8).p. In previous studies, no other nematodes have revealed any 3° specific execution defects. This set of mutants may have not been identified in *C. elegans* due to 3° fate's single division.

In *dov-5* and *dov-6* animals, each VPC loses one or more rounds of division. In these mutant backgrounds, Pn.px cells behaved as their daughters, Pn.pxx. AC ablations seem to not affect the defective division patterns (Dichtel *et al.*, 2001).

Hyperproliferation

This class contains *dov-9* through *dov-15* plus one dominant mutant, *dov-(sy476d).* In mutants *dov-9*, *-10*, *-11*, and *-12*, P(5,7).p granddaughters have an additional division, mostly longitudinal. The outer cells of the presumptive 2° are more sensitive. P6.p inner granddaughters have an extra round of transverse divisions. In addition to these mutants, *dov-13* mutants have a fourth round of AC-dependent divisions of P6.p. In contrast, some P(5,7).p granddaughters have a non-vulval fate. Some *dov-14* and *dov-15* mutants have abnormal excess tranverse divisions in P6.p inner granddaughters. These defects are linked to a low penetrant P(5,7).p granddaughter defect of an occasional oblique/transverse division. It also appears that *dov-15* is AC dependent in regards to the 2° defects. In the dominant strain, *dov-(sy476d)*, P6.p divides four times with the last two division along the transverse axis and the inner P(5,7).p progeny divide again (Dichtel *et al.*, 2001). These cells behave as their mothers, a Pn.pxx -> Pn.px transformation.

Cell proliferation in *C. elegans*.

In *C. elegans*, very few genes have been identified molecularly that uncouple cell division from cell fate. *cye-1*, a cyclin E homolog (Fay and Han, 2000), has been identified that appears to mimic the defects seen in *dov-5* and *dov-6.* Additionally, in *cul-1* mutants, a *cullin* homolog (Kipreos *et al.*, 1996), has hyperproliferation of the VPCs. This *C. elegans* class differs somewhat from *Oscheius* in that they also have other pleiotrophic effects directly affecting fertility and viability (Kipreos *et al.*, 1996; Fay and Han, 2000). A quick scan of *C. elegans* (*i.e.* wormbase), reveals other genes with *pvl*

defects and sterility. One class of genes are the *evl* genes (Seydoux *et al.*, 1993). Some of them are defective in uterine development and uterine-vulval connection, but a few of them have vulval defects. Very few have been molecularly characterized. These mutants appear to uncouple the cell-cycle control and VPC fate suggesting that cell fate depends on a timing mechanism, rather than cell divisions.

Recent work reveals that VPC competency to respond to the inductive or lateral signal is dependent upon the cell cycle and fusion to the hyp7 epidermis (Ambros, 1999; Wang and Sternberg, 1999). This is in accordance with the results seen in *Oschieus*.

C. briggsae *as another genetic model system*

The evidence presented by these nematodes show there is much to learn from other species. However, the satellite model systems utilize thus far are deficient in genetic information. Another approach would be to transgenically express nematode genomic sequence into *C. elegans* (Krause *et al.*, 1994; Thacker *et al.*, 1995; de Bono and Hodgkin, 1996; Maduro and Pilgrim, 1996; Gilleard *et al.*, 1997a; Gilleard *et al.*, 1997b; Jan *et al.*, 1997; Harfe and Fire, 1998; Culetto *et al.*, 1999; Dufourcq *et al.*, 1999; Gilleard *et al.*, 1999; Liu *et al.*, 1999; Streit *et al.*, 1999; Thacker *et al.*, 1999; Vatcher *et al.*, 1999). This approach does not employ evolutionarily derived genetic networks like *Oscheius* and *Pristionchus* do. Here we use a third alternative combining the best of both approaches.

Caenorhabditis briggsae, a relative of *C. elegans* separated by about 23-40 million years, (Emmons *et al.*, 1979; Heschl and Baillie, 1990; Lee *et al.*, 1992; Kennedy

et al., 1993) can be a used as a model system which will provide evolutionary divergence as well as cross transgenic manipulation (Krause *et al.*, 1994; Thacker *et al.*, 1995; de Bono and Hodgkin, 1996; Maduro and Pilgrim, 1996; Gilleard *et al.*, 1997a; Gilleard *et al.*, 1997b; Jan *et al.*, 1997; Harfe and Fire, 1998; Culetto *et al.*, 1999; Dufourcq *et al.*, 1999; Gilleard *et al.*, 1999; Liu *et al.*, 1999; Streit *et al.*, 1999; Thacker *et al.*, 1999; Vatcher *et al.*, 1999). The study of development and gene expression in *C. briggsae* may provide clues to the evolution of development and help to uncover developmental processes. In addition the genomic sequence of *C. briggsae* is being determined (A. Coulson, personal communication) to compare it with *C. elegans* (*C. elegans Sequencing Consortium*, 1998).

Recent studies on whole genome alignment between *C. elegans* and roughly eight million bases of *C. briggsae,* using Wobble Aware Base Alignment (WABA), indicate only 40% of the genome is resistant to rearrangement (Kent and Zahler, 2000). Likewise, approximately 89% of nucleotides match in highly conserved regions and this includes introns, intergenic areas as well as coding regions (Kent and Zahler, 2000).

As a satellite model system to *C. elegans, C. briggsae* could prove to give better insight into gene function and development.

CONCLUSION

Many questions remain about the evolutionary changes between species concerning induction of the vulva and the vulva competence group. I will compare *C.*

briggsae vulva development to *C. elegans* to examine evolutionary changes in development. In chapter two, I will discuss the subtle evolutionary changes in vulva formation in *C. briggsae*: lineage of the vulva; competence of P3.p and P4.p to form vulva tissue; and AC induction of the 1° precursor cell. In chapter three, I will discuss the sequence comparison of *let-60* between four nematode species: intron boundary conservation and codon variation of the coding sequence.

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Table 1. Pn.p Cell Fates, Vulva Position, and Gonad Dependence

Species	1	2	3	4	5	6	7	8	9	10	11	Position
A. *Brevibucca* sp. SB261	S	S	S	S	V	V	V	V	S	S	S	P
Mesorhabditis sp PS1179	S	S	S	S4*	V	V	V	S4*	S	S	S	P
Teratorhabditis palmarum	S	S	S	S4*	V	V	V	S4*	S	S	S	P
B. *Cruznema tripartitum*	S	S	S2*	S2*	V	V	V	S2*	S	S	S	P
Caenorhabditis elegans	S	S	S/S2*	S2*	V	V	V	S2*	S	S	S	C
Caenorhabditis briggsae	S	S	S/S2*	S/S2*	V	V	V	S2*	S	S	S	C
Oscheius CEW1	S	S	S	S4*	V	V	V	S4*	S	S	S	C
Panagrolaimus sp PS1732	x	x	x	x/Sc	V	V	V	V	S	S	S	C
Panagrellus redivivus	S	S	S*	S*	V	V	V	V	S*	S*	S	C
Halicephalobus sp. JB128	x	x	x	x	V	V	V	V	X	X	x	C
Pristionchus pacificus	x	x	x	x	V	V	V	S	x	x	x	C

Table 1. The fates of P1.p to P11.p have been determined in the species listed. A) Strains have gonad-independent vulva induction. B) Strains have gonad-dependent vulva induction. x, cell death in the L1 stage. X, cell death in the L2 stage. S, syncytial epidermal fate. S2, one division in the L3 stage. S4, two divisions in the L3 stage. V, vulval fate (the different vulval fates are not distinguished here). Asterisks indicate additional cells competent to form vulval tissue. P, posterior gonad 70% or more of body length. C, center vulva 50 to 70% of body length.

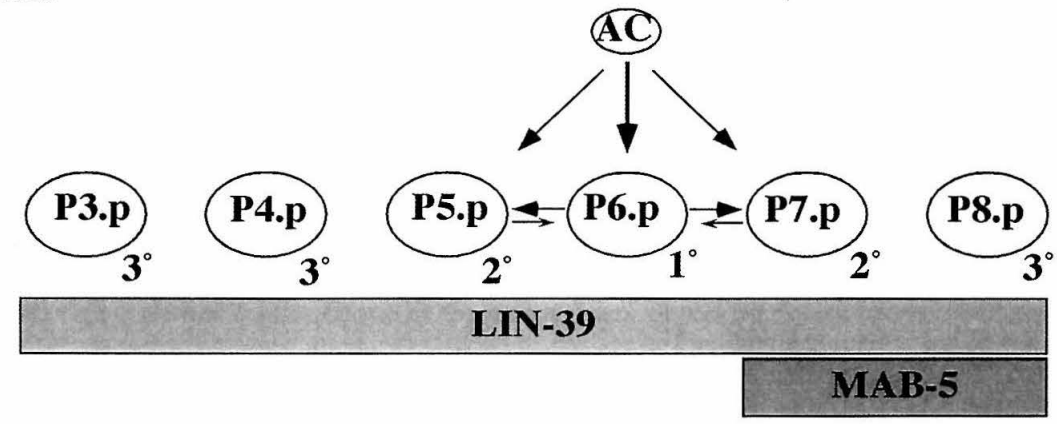
[f] P4.p survives in about one-third of the animals.

(data from Sommer and Sternberg, 1994; Félix and Sternberg, 1997; Félix *et al.*, 2000; Delattre and Félix, 2001).

Figure 1. **Vulva development in *Caenorhabditis elegans*.**

The vulva develops from Pn.p precursor cells in the ventral epidermis. A) The AC sends the inductive signal (*lin-3*) and P(5-7).p start an inter- and intracellular signal cascade. P(3,4,8).p form 3°; P(5,7).p form 2° and P6.p assumes 1°. B) The signal cascade in the Vulval precursor cells.

Figure 1. *C. elegans* Vulva Development



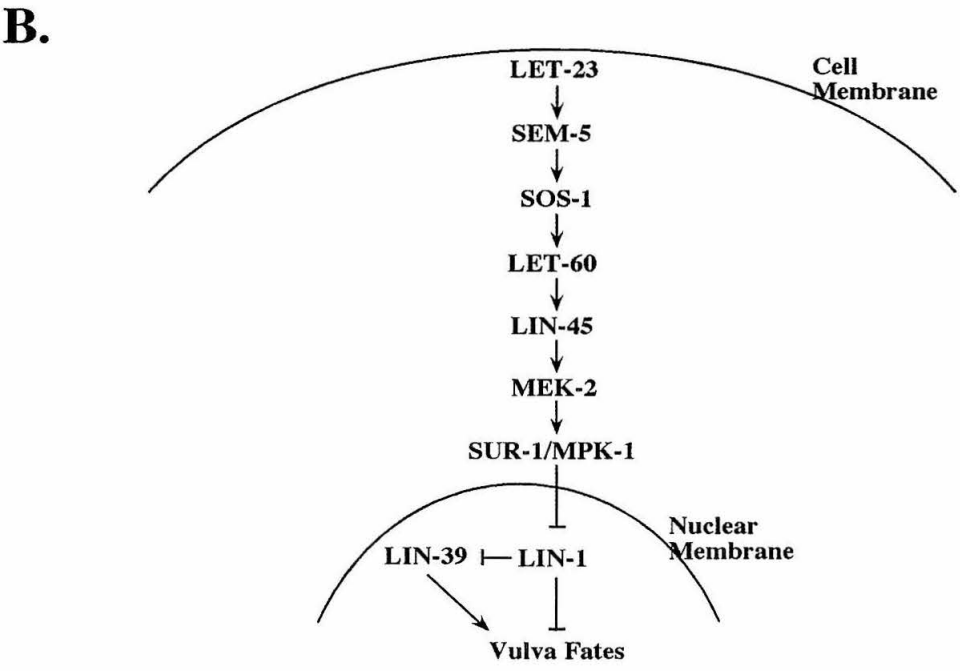


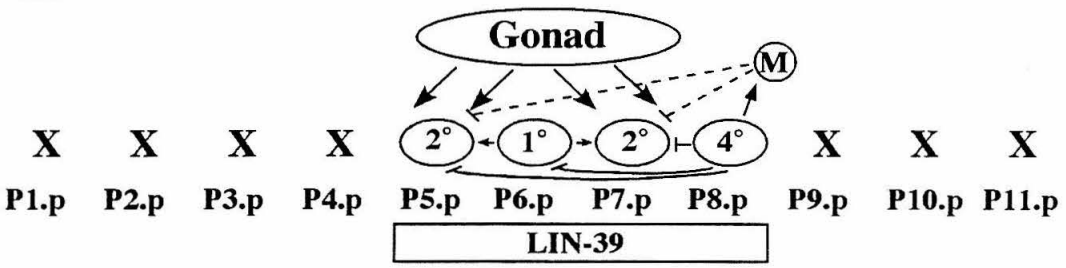
Figure 2. **Vulva development in *Pristionchus pacificus*.**

A) P(1-4,9-11).p undergo apoptosis during embryogenesis. The gonad is continously inducing the VPCs while P8.p is expressing *mab-5* which specifies M as well as a negative signal to prevent gonad independent differentiation. M inhibits P5.p and P7.p. P6.p is enforcing secondary fates on P5.p and P7.p.

B) Lineage of P(5-8).p; not drawn to scale; L – longitudinal, T – transverse, U-undivided, S – fuses with the hyp7 epidermis.

Figure 2. *Pristionchus pacificus* Vulva Development

A.



B.

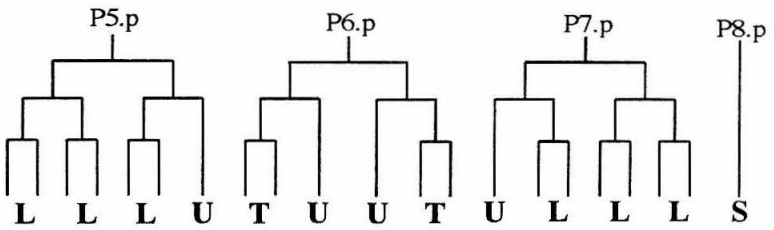


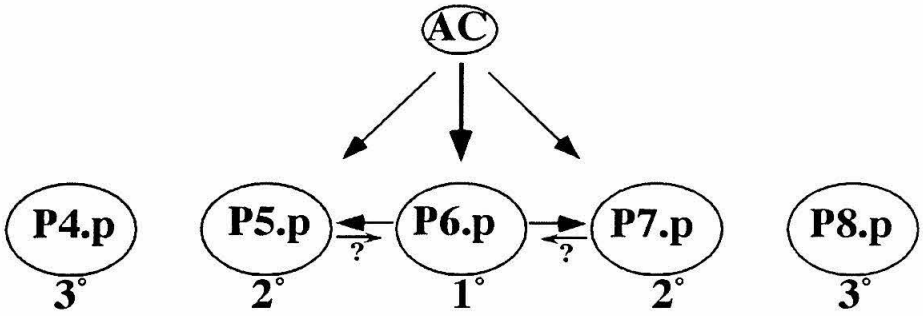
Figure 3. **Vulva development in *Oscheius / Dolichorhabditis* CEW1.**

A) P(4-8).p form the equivalence group. P(5-7) form the vulva. AC induces the outermost fates in the first step and specifies the inner T's of P6.p in the second step. P6.p is enforcing secondary fates on P5.p and P7.p.

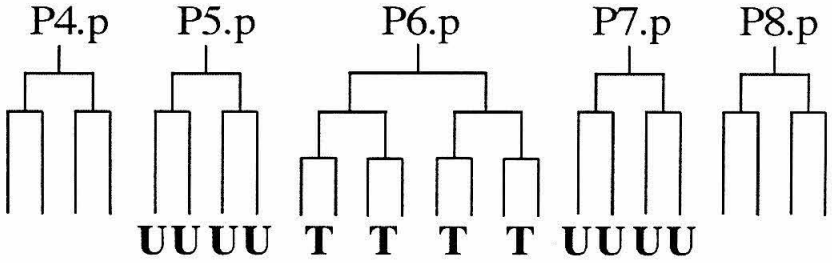
B) Lineage of P(4-8).p; not drawn to scale; U – Undivided, and T – transverse. Only 16 cells form the vulva in this species.

Figure 3. *Oscheius* Vulva Development

A.



B.



Chapter II

Vulva Development in *Caenorhabditis briggsae*

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(Prepared for Submission to ***Developmental Biology***)

ABSTRACT

C. elegans vulval development has served as a paradigm for understanding the genetic control of development; comparison with other nematode taxa has suggested ways in which development has evolved. As a step towards a microevolutionary analysis of development, we examined vulval development of *C. briggsae*, a species closely related to *C. elegans*. While *C. briggsae* and *C. elegans* share most features of vulval development, we found three differences. The vulval lineage has some slight changes in the order of cell divisions; in *C. briggsae*, transverse divisions occur in a wave toward the anchor cell. In comparison to *C. elegans*, P3.p is less competent to generate vulval cells in *C. briggsae* then *C. elegans*. Finally, the anchor cell proximal granddaughters (vulF parents) of the 1° precursor cell require the anchor cell for their division in *C. briggsae* but not in *C. elegans*, supporting the hypothesis that the single-step induction of vulval lineages is derived in *C. elegans*.

INTRODUCTION

Comparative studies of development can lead to an understanding of how developmental processes evolve, as well as offer a different perspective on an intensively-studied process. Vulval development in *C. elegans* has been studied as a paradigm for the genetic control of development (reviewed by Wang and Sternberg, 2001), while vulval development in other free-living nematodes has been studied to approach questions concerning the evolution of development (reviewed by Félix, 1999; Sommer, 2000).

Comparison of genomic sequences has proven a powerful way to identify conserved elements in a genomic sequence. *Caenorhabditis briggsae* serves this purpose for *C. elegans* (Krause *et al.*, 1994; Thacker *et al.*, 1995; de Bono and Hodgkin, 1996; Maduro and Pilgrim, 1996; Gilleard *et al.*, 1997a; Gilleard *et al.*, 1997b; Jan *et al.*, 1997; Harfe and Fire, 1998; Culetto *et al.*, 1999; Dufourcq *et al.*, 1999; Gilleard *et al.*, 1999; Streit *et al.*, 1999; Thacker *et al.*, 1999; Vatcher *et al.*, 1999), and it is for this and other reasons that the genomic sequence of *C. briggsae* is being determined (A. Coulson, personal communication) to compare it with *C. elegans* (*C. elegans Sequencing Consortium*, 1998). *C. elegans* and *C. briggsae* are close relatives within genus *Caenorhabditis*. Their molecular divergence is about one-half the evolutionary distance between mice and humans (i.e., 23-40 million years if the molecular clock was running at the same rate in the two groups) (Emmons *et al.*, 1979; Heschl and Baillie, 1990; Lee *et al.*, 1992; Kennedy *et al.*, 1993). Thus the *C. elegans-C. briggsae* comparison is exemplary for other close sequence comparisons, such as human-mouse. This

comparative sequence approach often relies on the assumption that the species being compared are, for all intents and purposes, identical in the process under study. We started an analysis of vulval development in *Caenorhabditis briggsae* to examine the extent to which this assumption is correct.

Previous comparative studies of nematode vulval development revealed a remarkable array of differences (Sternberg and Horvitz, 1982; Sommer *et al.*, 1994; Sommer and Sternberg, 1994; Sommer and Sternberg, 1995; Sommer and Sternberg, 1996; Eizinger and Sommer, 1997; Félix and Sternberg, 1997; Sommer, 1997; Félix and Sternberg, 1998; Jungblut and Sommer, 1998; Sommer *et al.*, 1998; Eizinger *et al.*, 1999; Félix, 1999; Félix *et al.*, 1999; Sigrist and Sommer, 1999; Félix *et al.*, 2000a; Félix *et al.*, 2000b; Jungblut and Sommer, 2000; Sommer, 2000; Dichtel *et al.*, 2001; Jungblut and Sommer, 2001), and suggested to us that there might be differences between *C. elegans* and *C. briggsae*, in spite of their similar morphology. In addition, examination of vulval induction in other free-living species suggested that the rapid vulval induction in *C. elegans* as opposed to the multi-stage or continuous induction might be a derived character.

During the early L3 stage, in *C. elegans*, the six ventral epidermal cells P(3-8).p form a group of cells competent to generate vulval tissue ("competence group") (Sulston and White, 1980). This group of cells was historically called the vulval "equivalence group", but since the recognition that the VPCs can be competent without being equivalent (Sommer *et al.*, 1994; Clandinin *et al.*, 1997), we prefer the term "competence group." The extent of this group is set by the domains of HOM-C gene expression; in particular, P(3-8).p express LIN-39, a protein related to Drosophila *Sex combs*

reduced/deformed/proboscipedia (Salser and Kenyon, 1994; Hunter and Kenyon, 1995). Without *lin-39*, the VPCs fuse to the hyp7 in the L1 stage. The expression of LIN-39 is regulated by downstream effectors of WNT signaling (Eisenmann *et al.*, 1998; Maloof *et al.*, 1999; Hoier *et al.*, 2000). P3.p is unique among the six cells because it is not capable of joining the competence group in about half of animals (Sulston and Horvitz, 1977; Eisenmann *et al.*, 1998). Although only three cells are required to form the vulva, these vulval precursor cells (VPCs) are capable of responding to an inductive signal generated by the anchor cell (AC) or a LIN-12-mediated signal from neighbors. This inductive signal is the epidermal growth factor-like protein LIN-3 (Hill and Sternberg, 1992; Katz *et al.*, 1995). The receptor is very likely LET-23, homolog of the EGF-receptor, a tyrosine kinase (Aroian *et al.*, 1990), which acts via RAS and a MAP kinase cascade which converge on the ETS domain protein LIN-1, among other transcriptional regulators (reviewed by Sternberg and Han, 1998). *lin-39* acts once again downstream of *lin-1* to effect vulval fates (Clandinin *et al.*, 1997; Maloof and Kenyon, 1998). A lateral signaling pathway, mediated by LIN-12 (Yochem *et al.*, 1988), ensures that P5.p and P7.p are 2° (Sternberg and Horvitz, 1989; Simske and Kim, 1995; Berset *et al.*, 2001). Also, two redundant pathways involving *C. elegans* Rb and other proteins (Ferguson and Horvitz, 1989; Lu and Horvitz, 1998; Fay and Han, 2000) repress induced fates in P(3, 4, 8).p (Ferguson *et al.*, 1987; Huang *et al.*, 1994).

In *C. elegans*, each induced vulval precursor cell undergoes three rounds of division to assume a distinct vulval fate 1° or 2° in a specific pattern, 2°-1°-2°. The 1° cells have a symmetric division pattern [TTTT] and the 2° cells have an asymmetric division pattern [LLTU] or [UTLL] named by their divisional axis (L – longitudinal, T –

transverse, U- undivided). The presumptive 1° cell, P6.p, detaches from the hyp7 epidermis and invaginates dorsally toward the gonad. Some of the 2° cells' progeny adhere to the epidermis while others detach and migrate up toward the gonad. During the L4 stage, the cells derived from the 1° and 2° lineages invaginate and eventually fuse to form seven multinucleate toroidal rings (from AC-distal to AC-proximal): vulA, vulB1, vulB2, vulC, vulD, vulE, vulF (Sharma-Kishore *et al.*, 1999; Shemer *et al.*, 2000). The first five rings are produced from the 2° cell progeny with each ring composed of cells from both the P5.p and P7.p lineages. The final two rings are composed entirely of P6.p progeny. The vulval-uterine connection is made by vulF (Newman *et al.*, 1996).

Here we describe the vulval cell lineages, analyze Pn.p cell competence and determine the role of the anchor cell in the specification of vulval cells in *C. briggsae* and compare them to *C. elegans*.

MATERIALS AND METHODS

General Methods and strains

Growth and handling of *C. briggsae* strain AF16 were according to Brenner (1974) and Wood (1988). All experiments were performed at about 20°C unless otherwise stated. Cell and tissue anatomy was observed with Nomarski differential interference contrast optics, as described by Sulston and Horvitz (1977). Standard cellular and genetic nomenclature is previously defined (Sulston and Horvitz, 1977; Horvitz *et al.*, 1979). *dev-2(sy5216)* (<u>dev</u>elopmental defective) was isolated in an

ethylmethylsulfonate mutagenesis of *C. briggsae* AF16 in a screen for visible mutants using conditions previously described (Brenner, 1974). Genetic nomenclature for *C. briggsae* has been proposed by D. Riddle, B. Herman, B. Horvitz, B. Waterston and B. Wood, with an addition by J. Hodgkin (personal communication).

Germline-mediated transformation by microinjection

Microinjection was performed as previously described for *C. elegans* (Mello *et al.*, 1991; Mello and Fire, 1995). Young adult hermaphrodites were placed on pads of 2% agarose under an inverted Nomarski DIC microscope and the DNA was injected into the gonad using an Eppendorf microinjector.

We chose pPD118.33, *Cel-myo-2::GFP* (kindly provided by Andy Fire), as a dominant transformation marker due to its pervasive expression pattern, low concentration requirements, and non-interference of other GFP expression constructs in the mid-section of the animal. *myo-2::GFP* expresses GFP at all stages in the pharynx of animals from the 3-fold stage to adulthood, including dauer. The *Cel-egl-17::GFP* promoter fusion construct, plasmid NH#293 (Burdine *et al.*, 1998), was co-injected at 100ng/μl with pPD118.33 at 10ng/μl and pPD10.46 at 60ng/μl (Fire *et al.*, 1990) as carrier DNA. We identified a spontaneously integrated transgenic array carrying the *egl-17::GFP* promoter fusion construct. This array, termed *syIs701*, conferred an expression pattern identical to that seen in the other non-integrated transgenic lines and was used throughout the remainder of this study. The complete genotype for *syIs701*-bearing strains is *syIs701* [*egl-17::GFP myo-2::GFP* pPD10.46] but will be referred to in the text either as *egl-17::GFP* or *syIs701* for brevity.

Cell Ablation & Lineage

Ablations of Pn.p cells and AC were carried out using a laser microbeam as previously described (Sulston and White, 1980; Epstein and Shakes, 1995). Cell lineage analysis of the *C. briggsae* vulva was performed as previously described (Sulston and Horvitz, 1977). The lineage in Figure 1 is a compilation of three complete lineages and three partial lineages.

Vulval Anatomy

Expression patterns of *egl-17::GFP* were observed using Nomarski optics and a Zeiss Axioplan microscope with a 200-watt HBO UV source, using a Chroma High Q GFP LP filter set (450 nm excitation/505 nm emission). Animals were anesthetized with 2-3 mM levamisole on 5% Noble agar pads. Photographs were taken with a Hamamatsu digital camera and the Improvision Openlab 2.0.6 software on a Zeiss Axioplan. Animals were scored during early L3 stage when the induced vulval cells were at the 2-cell or 4-cell stage.

RESULTS

C. briggsae *Vulval Lineage*

We observed the vulval lineage in *C. briggsae* by direct observation of cell divisions with Nomarski optics. In *C. briggsae*, P5.p, P6.p, and P7.p undergo three rounds of division to generate the vulva as in *C. elegans* (**Fig. 1**). Differences between

the species were only apparent late in the L3 stage in the third round of divisions of the induced VPCs.

The order of divisions between the cells that normally divide transversely (P5.ppa, P7.pap, and P6.pxx) is different from that described in *C. elegans*. In *C. elegans*, the presumptive 2° T cells (P5.ppa and P7.pap; precursors of vulD) divide after the presumptive 1° AC-distal T cells (P6.ppp and P6.paa; precursors of vulE) (Sulston and Horvitz, 1977). The presumptive 2° T cells (precursors of vulC) divide closely to the AC-proximal T cells (P6.pap and P6.ppa; precursors of vulF). In *C. briggsae*, the order of transverse divisions in the final round progresses in a wave from the distal cells to the proximal cells. P5.ppa and P7.pap divide with or just before P6.ppp and P6.paa.

In *C. briggsae*, the P6.pap and P6.ppa transverse divisions are late in the L3 or early in the L4, the last accomplished in the VPCs. These cells divide at ecdysis or within the hour following. In *C. elegans*, the presumptive 1° cell's AC-proximal T cells (P6.pap and P6.ppa) divide last, but the divisions are typically before ecdysis during the lethargus period.

The C. briggsae *vulval competence group*

In *C. elegans*, the vulval competence group was established through a combination of cell ablations, and the examination of multivulva mutants (Sulston and White, 1980; Kimble, 1981; Sulston and Horvitz, 1981; Sternberg and Horvitz, 1986; Thomas *et al.*, 1990). We employed both of these techniques to define the vulval competence group in *C. briggsae*.

To test whether ventral epidermal cells (Pn.p) in addition to P5.p-P7.p are part of the vulval competence group, we ablated P5.p-P7.p (**Table 1A**). P4.p and P8.p moved toward the AC and replaced the ablated cells. In 1 of 20 animals, P3.p generated one daughter that fused with the hypodermis and one cell (P3.pp) that generated vulval tissue. To ascertain whether P3.p and P9.p are part of the vulval competence group, we ablated P(4-8).p in late L1 or early L2 (**Table 1B**). Neither P3.p nor P9.p were induced in fifteen animals observed. P3.p is 4° in about 80% of animals, (Delattre and Félix, 2001, our unpublished results) Thus, in this experiment, they were not competent to adopt a vulval fate.

To further test the ability of P3.p and P9.p to generate vulval tissue, we examined the anatomy a multivulva mutant of *C. briggsae*, *dev-2(sy5216)*. *dev-2* has the general properties of a synthetic multivulva mutant such as the *lin-8; lin-9* double mutant in *C. elegans* (Ferguson and Horvitz, 1989): maternal rescue of the multivulva phenotype, gonad independence, ventral protrusions in the male, and P11 to P12 transformation (data not shown). In this *dev-2* mutant, P3.p is induced to form vulval tissue in ninety percent of the animals (n=20). By contrast, neither P9.p nor P2.p is induced in this background (**Fig. 2**). Therefore, assuming that this mutation corresponds to a hyperactivation of a vulval induction signal, we conclude that P3.p is able to respond to it and is part of the vulval competence group, as are P(4-8).p.

Vulval Induction

During the L3 stage in *C. elegans* and in *C. briggsae*, the somatic gonad is generated initially from two lineages, the dorsal uterine precursor cells (DU), and the ventral uterine precursor cells (VU), which surround the AC. The DU and VU lineages divide during the L3 stage and can be used as a convenient internal clock in *C. elegans* and in *C. briggsae*. Induction of the vulva by the AC in *C. elegans* occurs during the early part of the L3 stage (Kimble, 1981; Wang and Sternberg, 1999). We investigated the role of the gonad and AC in inducing the *C. briggsae* vulva.

Ablation of the gonad in *C. briggsae* results in animals with no vulval induction, indicating that an inductive signal is derived from the gonad (data not shown). The most likely candidate as the induction source is the AC. We next performed ablations in the L3 stage (**Table 2**). Animals with their AC ablated before the Dorsal Uterine (DU) precursor cells have fully divided have disrupted vulval fates with some amount of induction. If the ablation is done after this time, but before the VPCs have divided once, the presumptive 2° cells, P5.p and P7.p are fully induced and divide normally to give their characteristic pattern. The 1° cell, P6.p, is induced to a vulval fate but displays an abnormal pattern of cell divisions. The outer granddaughters, P6.paa and P6.ppp, divide transversely in 99% of cases (n=78) as in wild type.

However, the inner granddaughters, P6.pap and P6.ppa, fail to divide in 68% of the cases (n=78) (**Fig. 3**). If the AC is not ablated until after the first round of divisions in the VPCs, the division pattern for P5.p-P7.p appears wild type (**Table 2**). We conclude that the AC is required for an additional step in P6.p cell fate specification.

To further test if the 1° cell is misspecified, we utilized a 1° specific GFP marker, *egl-17::GFP* (**Fig. 4A-B**). In *C. elegans, egl-17::GFP* is expressed in the presumptive 1° VPC in the L3 stage. Expression begins in P6.p and continues through its granddaughters (P6.pxx). It fades away in the 1° lineage and, later, it turns on in the L4 stage in the TN cells (P5.ppxx and P7.paxx) of the 2° lineage (Burdine *et al.*, 1998). In *C. briggsae,* in our integrated line carrying the same *C. elegans* construct, GFP expression begins one stage later in P6.p daughters (P6.px), is maintained into their P6.p granddaughters (P6.pxx) and then fades away. *egl-17::GFP* expression does not reappear in the 2° lineage in L4 stage animals (data not shown).

We repeated the AC ablation experiments in this *egl-17::GFP* background. We chose two time points during the L3 stage: when P(5-7).p have not yet divided, and when P(5-7).p have divided only one round. Ablation of the AC at the first time point prevented expression of EGL-17::GFP in the daughters of P6.p, the presumptive 1°, and also the granddaughters (n = 16). Ablation of the AC at the second time point turned off EGL-17::GFP in the granddaughters (n = 15) (**Fig. 4C-D**). Persistance of *egl-17::GFP* expression appears to be a more sensitive assay of induction than is cell division, or requires the anchor cell for a longer time.

DISCUSSION

P3.p may be less competent in C. briggsae *compared to* C. elegans

P3.p assumes a 3° fate in 50% of wild-type *C. elegans*. By contrast, in *C. briggsae* animals, P3.p assumes a 3° fate in 15-20% of the animals (Delattre and Félix, 2001; our unpublished observations) and may not be a part of the competence group. The only vulval induction seen for P3.p was in a mutant background. One possible explanation is that *dev-2(sy5216)* may be involved with P3.p cell fusion with the hyp7 epidermis. Without cell fusion, P3.p may be capable of forming vulval tissue. This observation indicates that P3.p is quantitatively different from its counterpart in *C. elegans*.

In *C. elegans*, LIN-39 expression is required to specify the vulval cells and prevent fusion to the hypodermis (Clark *et al.*, 1993; Wang *et al.*, 1993). The simplest explanation for this change in *C. briggsae* could be a change in *lin-39* gene expression pattern. Two signaling pathways have been implicated in the regulation of LIN-39 expression in *C. elegans*. Effectors of a putative WNT signaling are required to maintain expression of LIN-39 (Eisenmann *et al.*, 1998). Similarly, the LET-23 (EGF-receptor) pathway is required to maintain expression of LIN-39 (Clandinin *et al.*, 1997; Maloof and Kenyon, 1998). The competency of *C. briggsae* P3.p might have changed due to reduced expression of LIN-39 in the anterior vulval cells, possibly by alterations in WNT or LET-23 signaling, or the response of LIN-39 to those signals.

1° Cell specification

Vulval precursor cell fates have been conveniently inferred by examining the cell lineage and cell types generated by each Pn.p cell. The vulval cell types have been defined by morphology, and more recently by gene expression (Burdine *et al.*, 1998; Wang and Sternberg, 1999; Wang and Sternberg, 2000). For example, of the VPCs, P6.p has a specific cell fate that is distinct from that of P5.p or P7.p. Although each VPC progresses through three rounds of cell division, P6.p produces two cell types, vulE (P6.paax and P6.pppx) and vulF (P6.papx and P6.ppax), which make the connection to the uterus, and P(5,7).p produce vulA, vulB1, vulB2, vulC, and vulD. P6.p great granddaughters completely detach from the cuticle and form a symmetric cone beneath the AC while half the progeny of P(5,7).p (P5.paxx and P7.ppxx) adhere and fuse to the hypodermis.

By employing these criteria, P6.p has a cell fate transformation after AC ablation in *C. briggsae*. The presumptive vulF cells fail to divide and make the proper connections to the uterus and both vulE and vulF lose expression of EGL-17::GFP. This observation indicates that vulE and vulF cell fate specification is not complete by the time the presumptive 2° cells are specified. Therefore, EGL-17::GFP expression is a more sensitive assay to anchor cell ablation than cellular division. When the same AC ablation experiments where conducted in *C. elegans*, the TUUT lineage was not observed (Kimble, 1981; M. Wang and P. Sternberg, unpublished results). However, AC ablation at the 2 to 4-cell stage of P6.p divisions does disrupt expression of another 1° specific marker, *zmp-1::gfp*, which is expressed in the vulE cells (P6.paax and P6.pppx) in late L4 to early adult (Wang and Sternberg, 2000). In addition to the AC ablations, mutations in

certain components in the *lin-3/let-23* pathway (*let-60* RAS, *let-23* RTK, and *lin-1* ETS) can also disrupt *zmp-1::GFP* expression in *C. elegans*, but whether this pathway is responsible for the induction is not yet determined. Furthermore, LIN-17, a WNT receptor, plays a role in distinguishing vulE and vulF, suggesting that the cell types coordinate with each other to determine proper cell fate (Wang and Sternberg, 2000). Overall, these data from *C. elegans* suggest that vulF requires contact with the AC until the 4-cell stage of P6.p for proper patterning of the 1° cell progeny.

We propose two models to explain the mechanism of vulE and vulF fate specification in *C. briggsae*: continuous or continual signaling of the LIN-3 inductive signal from the AC, or an AC dependent second signal (**Fig. 5**). Evidence from another nematode species more distantly related to *C. elegans* than is *C. briggsae* indicates apparently more complex induction. *Pristionchus pacificus* has an apparently continuous gonadal signal to induce the VPCs to differentiate and the AC serves to specify the vulval fates (Sigrist and Sommer, 1999). In *Oscheius* sp. CEW1, on the other hand, the existing evidence suggests the possibility of a second signaling step after P6.p division (Félix and Sternberg, 1997). There is an initial signal from the AC to induce the VPCs to divide and generate vulval cells, and a second induction (possibly by the same signal) to promote the 1° granddaughters to divide, much like in *C. briggsae*. These variations in induction mechanisms suggest the possibility of either model in *C. briggsae*.

It is conceivable that P6.pap and P6.ppa specification may be due to LAG-2 signaling from the AC. In *C. elegans*, the AC induces the uterine π fates in the ventral uterine cells via LAG-2 and LIN-12 approximately four hours after the induction of the VPCs (Newman *et al.*, 1995; Newman *et al.*, 2000). However, *lin-12(lf)* mutants in *C.*

elegans have no defects in 1° cells (Wang and Sternberg, 2000). It remains possible that the same mechanisms are present but serving different functions in *C. briggsae*.

CONCLUSION

C. briggsae will be useful for comparative development studies, although the differences with *C. elegans* are somewhat subtle. Comparison of sequences between relatively close species must take into account the existence of such possibly subtle but relevant changes in development.

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Table 1. Pn.p ablations in *Caenorhabditis briggsae*

A.	Animals	P3.p	P4.p	P5.p	P6.p	P7.p	P8.p	P9.p	Cells Induced
	4/19	4°	3°	X	X	X	1°	4°	1
	3/19	3°	2°	X	X	X	1°	4°	2
	2/19	4°	3°	X	X	X	2°	4°	1
	1/19	3°	1°	X	X	X	2°	4°	2
	1/19	3°	1°	X	X	X	1°	4°	2
	1/19	4°	6	X	X	X	2°	4°	2
	1/19	H	3°	X	X	X	1°	4°	1.5
	1/19	3°	H	X	X	X	2°	4°	1.5
	1/19	3°	3°	X	X	X	1°	4°	1
	1/19	4°	1°	X	X	X	3°	4°	1
	1/19	4°	2°	X	X	X	3°	4°	1
	1/19	3°	3°	X	X	X	3°	4°	0
	1/19	4°	3°	X	X	X	3°	4°	0

B.	Animals	P3.p	P4.p	P5.p	P6.p	P7.p	P8.p	P9.p	Cells Induced
	11/15	4°	X	X	X	X	X	4°	0
	4/15	3°	X	X	X	X	X	4°	0

Table 1. Pn.p ablations in *Caenorhabditis briggsae*. A. P(5-7).p ablation in the late L1/early L2 stage. X represents ablated cells. B. P(4-8).p ablation. Cell fate designations are determined on anatomy and morphology as in Katz *et al.,* (1995). H: one of the two Pn.p daughters generated vulval cells; the other joined hyp7 epidermis. 6, generated six progeny.

Table 2. Anchor cell ablations in *Caenorhabditis briggsae*

Cell(s) Ablated	Time of ablation	Descendants of P5.p	P6.p	P7.p	# of animals
-	-	LLTU	TTTT	UTLL	Many
AC	Early L3	S S	S S	S S	3/10
		LL S	S S	S ss	1/10
		S S	OT S	S S	1/10
		DO S	S TT	S S	1/10
		LOTU	S S	S Os	1/10
		LLss	S UU	S LL	1/10
		sLTU	TU S	S LO	1/10
		LLTU	TTUT	UTLL	1/10
AC	DU dividing	ss ss	DU ss	LU S	1/6
		LL S	OUUT	SS	1/6
		LOss	*O*TUO	ssLL	1/6
		LLOU	LUTO	UTLL	1/6
		LLTU	TTTT	UDUs	1/6
		LLTU	OOOO	UTLL	1/6
AC	DU divided VU 1-cell or dividing	LLTU	TUUT	UTLL	4/7
		LLTU	TTTT	UTLL	2/7
		LLTU	TTUT	UTLL	1/7
AC	P(3,4,8).p dividing or divided	LLTU	TUUT	UTLL	17/23
		LLTU	TUTT	UTLL	3/23
		LLOU	TUUT	UTLL	1/23
		LLTU	OOOT	UTLL	1/23
		LLTU	TDDT	UTLL	1/23
AC	P(5-7).p dividing	LLTU	TTUT	UTLL	2/9
		LLTU	TTOT	UTLL	2/9
		LLTU	TTTT	UTLL	2/9
		LLTU	TUUT	UTLL	1/9
		LLTU	TUTT	UTLL	1/9
		LLTU	TOTT	ULLL	1/7
AC	P(4-8).p 2-cell stage	LLTU	TTTT	UTLL	6/7
		LLOU	TTTT	UTLL	1/7

Table 2. Anchor cell ablations in *C. briggsae*. The anchor cell (AC) was ablated at different timepoints during the L3 stage and the vulval lineages then followed. S: fusion to hyp7. L: longitudinal division. T: transverse division. O: oblique division oblique. U: Undivided. *O*: the daughters of the oblique third division divided once more. D: divided,

axis not observed. Underline indicates that the cells adhered to the cuticle; bold indicates normal 1°-like fate.

Fig. 1. *C. briggsae* vulva lineage.

A. Lineage of *C. briggsae* vulval precursor cells in the wild-type strain AF16 during the L3 larval stage. Timing starts at zero from L2 ecdysis to L3 ecdysis. The hash marks are two hour intervals. B. Nomarski photomicrograph of a wild-type *C. briggsae* vulva in the L4 stage. Anterior is to the left and ventral is down. Scale bar is equal to 50 μm.

Caenorhabditis briggsae Vulva

A. Lineage

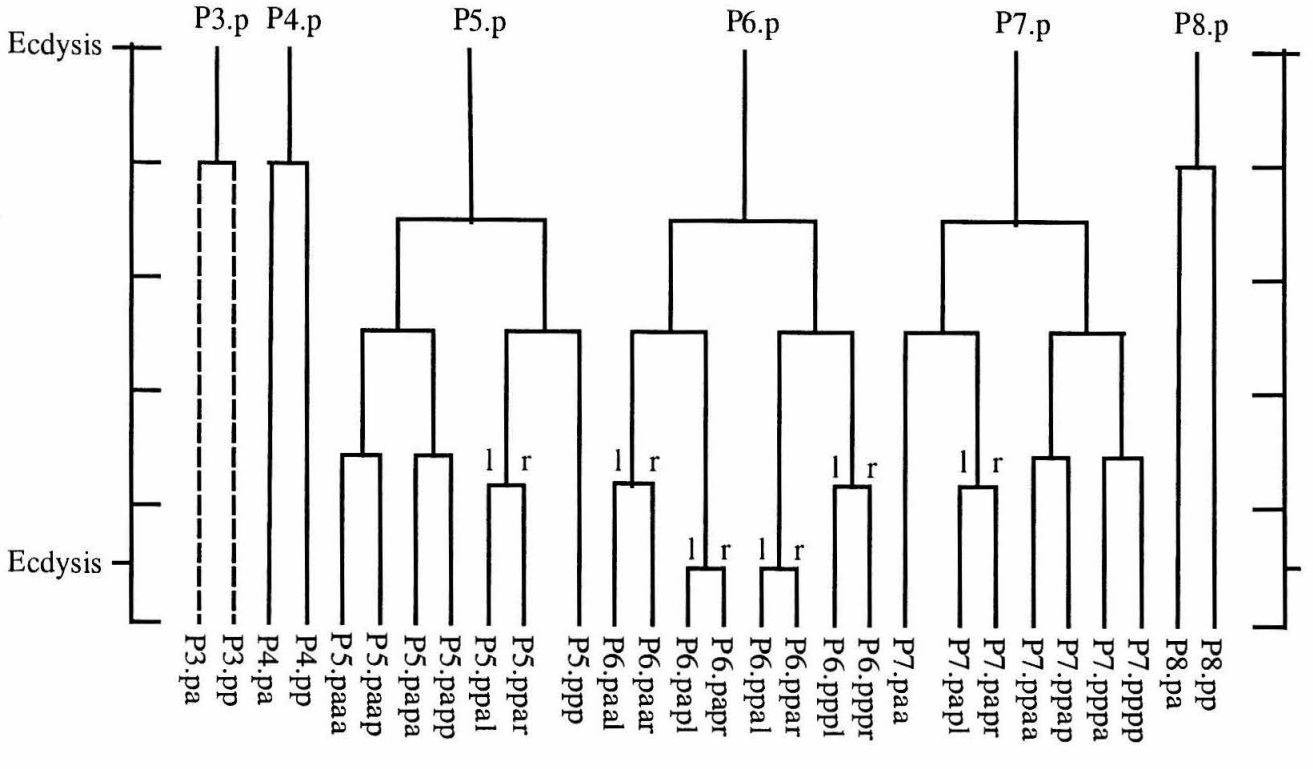


Figure 1A.

Figure 1B

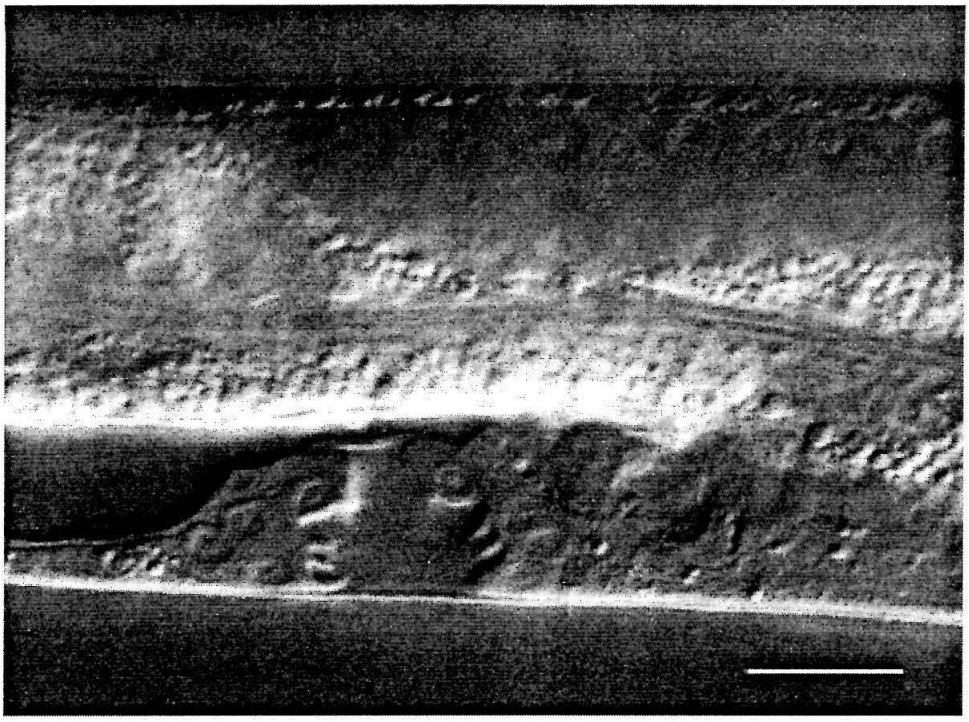


Fig. 2. Multivulva *dev-2* mutant animal.

Ventral region of a *dev-2* animal in the L4 stage. Anterior is to the left and ventral is down. The arrowhead is at the position of the main vulval invagination; the arrows indicate the supernumerary induction points. Scale bar is equal to 20 μm.

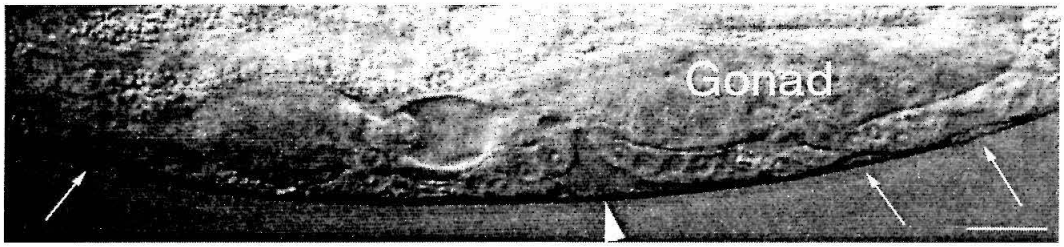

Gonad

Fig. 3. Undivided primary-fated cells after anchor cell ablation.

The anchor cell (AC) was ablated during the early L3 stage following P4.p division. Anterior is to the left and ventral is down. The arrowheads point to P6.pap and P6.ppa. The arrow points to the corpse of the AC. Scale bar is equal to 20 μm.

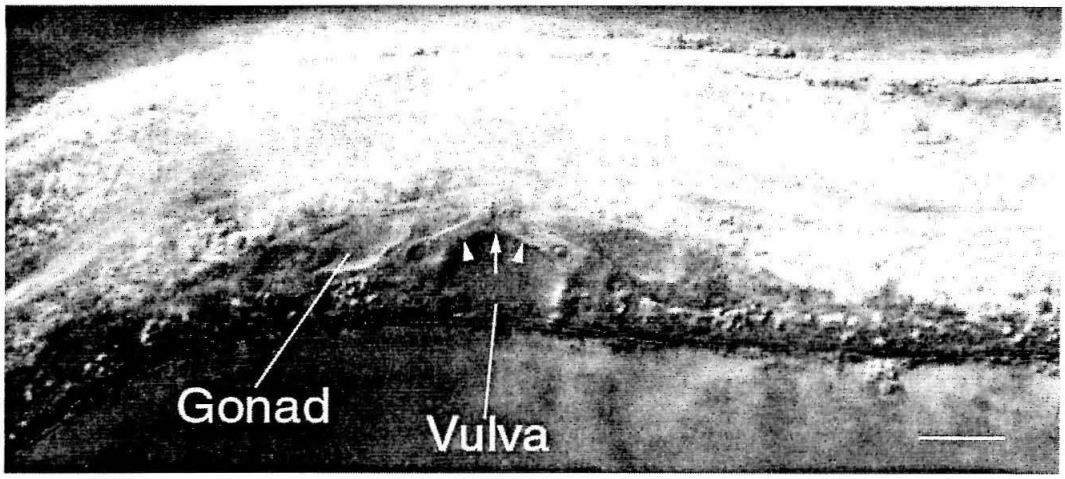

Gonad
Vulva

Fig. 4. EGL-17::GFP expression is disrupted by AC ablation in *C. briggsae*.

Anterior is to the left and ventral is down. (A,B) Nomarski micrographs. (C,D) GFP epifluorescence. A. Intact wild-type L3 stage larva at the 4-cell stage of the Pn.ps. Scale bar is equal to 20 μm and is the same in each panel. B. Same animal as in A, expressing *egl-17::GFP* in P6.p progeny. The arrowhead points to the Anchor Cell. C. Larva after ablation of the Anchor cell when P6.p was at the 2-cell stage. D. Same animal as in C, showing no *egl-17::GFP* expression.

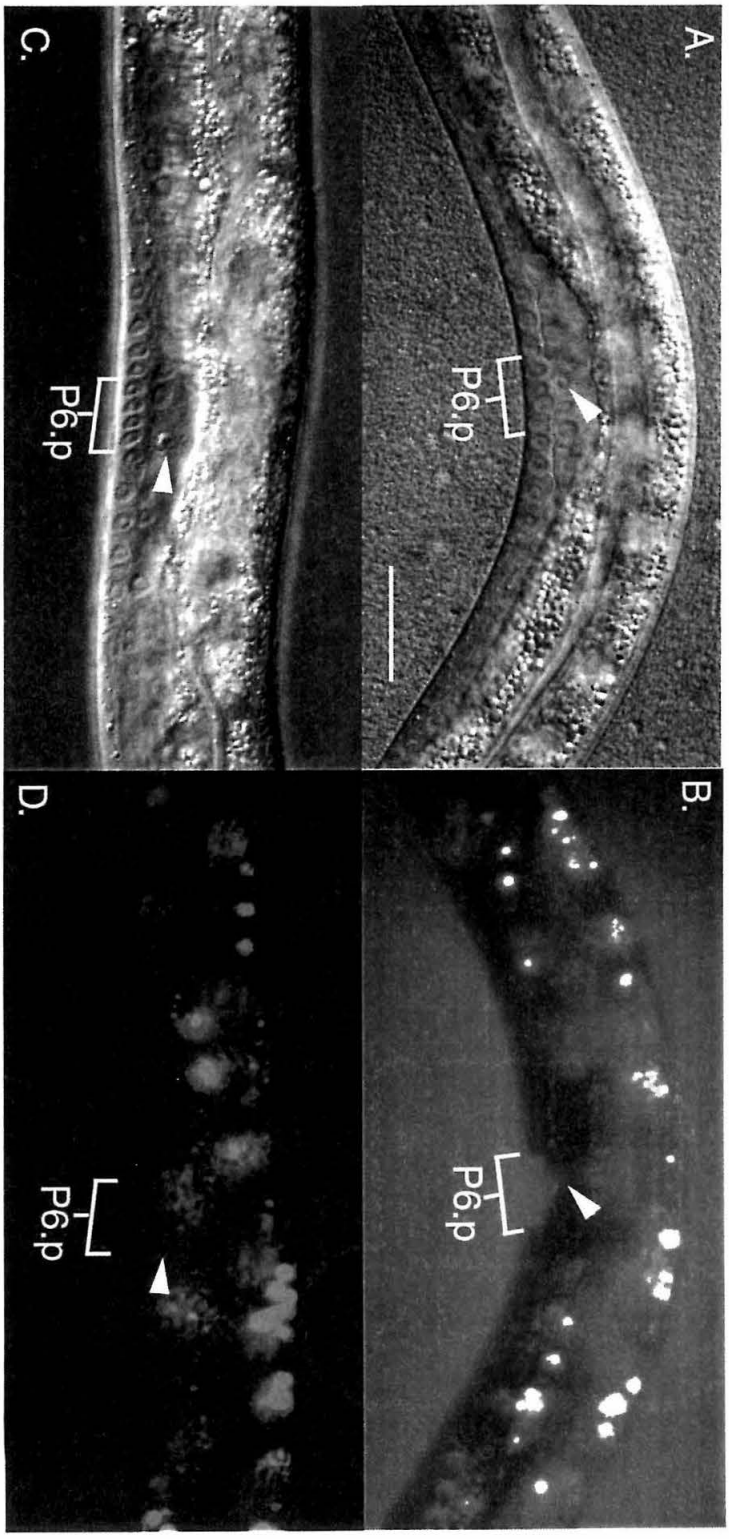
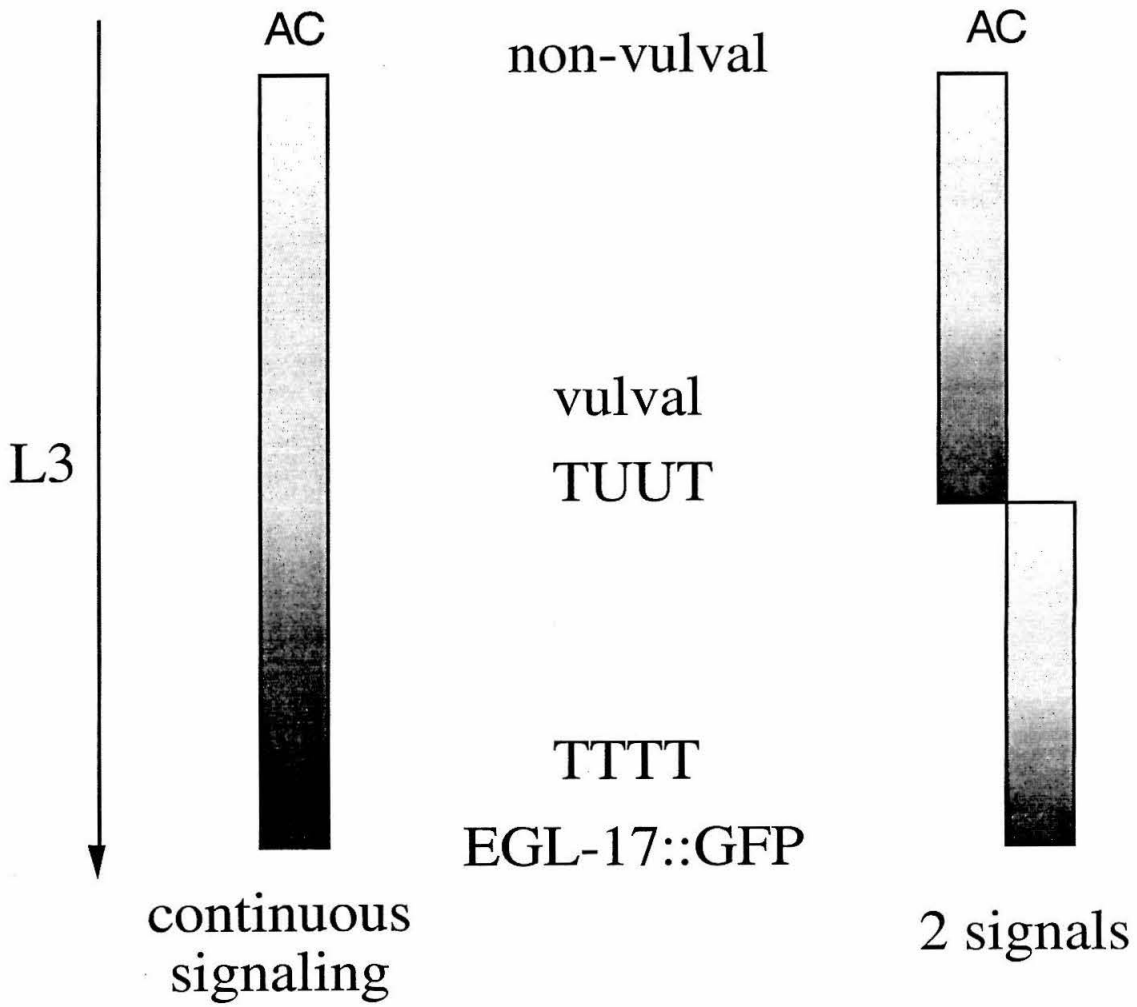

A.
P6.p
B.
P6.p
C.
P6.p
D.
P6.p

Fig. 5. Models for specification of the 1° lineage in *C. briggsae*.

Each side represents a separate model of cell specification. On the left, a continuous signal from the AC (Anchor Cell) specifies the 1° cell. On the right, one signal induces the VPC to a vulval fate and a second signal specifies the 1° granddaughters; both signals are from the AC.

Steps in P6.p specification



Chapter III

Sequence Conservation Of *let- 60* In Nematode Species

ABSTRACT

Comparative genome analysis was used to examine *let-60(ras)* homologs between four species of nematode *C. briggsae*, *C. elegans*, *Oscheius* CEW1 and *P. pacificus*. There is evidence of strong evolutionary pressure to conserve the gene not only at the amino acid level but also at the nucleotide level. These four homologues have a 89% identity at the amino acid level and 56% identity at the nucleotide level. Upon examining the nucleotide sequence, we discover the third base of the codon has the most variability in the sequence. In addition, several exon-intron boundaries are conserved in the genomic sequences of the homologues.

INTRODUCTION

Systems can change to accommodate development. Gene mutations alter the function of gene products which result in modification of molecular complexes. These mutations, in essence, could be detrimental to the animal. Therefore, highly conserved genes indicate a strong dependence upon the gene product's specific function(s).

LET-60 has many functions in *C. elegans* development. In addition to vulva development, it functions during meiosis; spicule formation; and P12 specification to name a few (for more, see review Sternberg and Han, 1998). This requires the protein to be expressed in multiple tissues (*e.g.* VPCs, gonad, and pharynx) at multiple times (*e.g.* embyogenesis, L3, and adult) responding to multiple inducers (Sternberg and Alberola-Ila, 1998; Sternberg and Han, 1998).

To study evolutionary changes, we sequenced the *let-60* gene from *Caenorhabditis briggsae*. We determined the homologue is functionally equivalent in *C. elegans*. Comparisons of *C. briggsae let-60* (*cb-let-60)* were made to RAS homologues in *Caenorhabditis elegans* (Han and Sternberg, 1990), *Oscheius* CEW1 (Félix *et al.*, 2000), and *Pristionchus pacificus* (Sommer *et al.*, 1996). We found high conservation at both the amino acid level and the nucleotide level of the coding sequence. Also, the exon-intron boundaries in *C. elegans* and *C. briggsae* are conserved in *Oscheius* CEW1 and *P. pacificus*. And finally, we make determinations of which genomic structure is closer to the nematode ancestral gene.

MATERIALS & METHODS

Strains and Maintenance

Caenorhabditis briggsae AF16 and *Caenorhabditis elegans* N2 were gifts from the Caenorhabditis Genetics Center. The strains were maintained and culture according to Brenner (1974). All experiments were conducted at 20°C. Cell and tissue anatomy was observed with Nomarski differential interference contrast optics, as described by Sulston and Horvitz (1977). Standard cellular and genetic nomenclature is previously defined (Sulston and Horvitz, 1977; Horvitz *et al.*, 1979). The *C. briggsae let-60* plasmid, pBS.cblet60, is from David Baille.

Sequencing and Analysis

Sequencing was done by the California Institute of Technology sequencing facility using an ABI 373 DNA sequencer and ABI 3700 DNA sequencer (Perkin-Elmer, Norwalk, CT). The plus and minus strand were sequenced three times to remove ambiguous bases. The *cb-let-60* cDNA is predicted based on BLAST homology and predicted intron splice acceptor and donor sites. Macvector was the Macintosh software package used in addition to BLAST to conduct amino acid and nucleotide analysis.

RNAi

let-60 dsRNA was synthesized *in vitro* using Ambion's Megascript kit from plasmid pKB14, or pKB25 containing predicted exons 2 and 3 of the genomic clone. PCR was used to generate the fragments and ligated into the vector, pPD129.36

(Timmons and Fire, 1998) containing double T7 promoters. Transcripts were prepared using T7 RNA polymerase and annealed prior to injection (Fire *et al.*, 1998). Progeny of injected animals were assayed at 20°C unless otherwise noted.

let-60 *Germline-mediated transformation*

Microinjection was performed as previously described for *C. elegans* (Mello *et al.*, 1991; Mello and Fire, 1995). Young adult hermaphrodites were placed on pads of 2% agarose under an inverted Nomarski DIC microscope and the DNA was injected into the gonad using an Eppendorf microinjector.

We chose pPD118.33, *Cel-myo-2::GFP* (kindly provided by Andy Fire), as a dominant transformation marker due to its constant expression pattern. *myo-2::GFP* expresses GFP at all stages in the pharynx of animals from the 3-fold stage to adulthood, including dauer. *cb-let-60* was co-injected at 10ng/μl with pPD118.33 at 10ng/μl and pPD10.46 at 120ng/μl (Fire *et al.*, 1990) as carrier DNA. Stable lines were identified with a pervasive GFP expression past the second generation.

RESULTS

C. briggsae let-60 (RAS)

In *C. elegans*, *let-60(gf)* (Beitel *et al.*, 1990; Han *et al.*, 1990; Han and Sternberg, 1990), is capable of promoting vulval fates within the equivalence group. Similar effects can be achieved by overexpressing *let-60* from transgenic extrachromosomal arrays. To

determine if *cb-let-60* is sufficient to induce vulva formation in *C. briggsae*, we created extragenic arrays expressing cb-LET-60 in both *C. briggsae* and *C. elegans*. Several stable lines have hyperinduction of the VPCs and multivulva phenotypes (data not shown). Therefore, RAS is sufficient to produce vulva tissue in both wild-type *C. briggsae* and *C. elegans*.

Loss of *let-60* function can cause lethality in early larval stages (Beitel *et al.*, 1990; Han *et al.*, 1990; Han and Sternberg, 1990). Surviving animals have little or no vulva induction. RNA interference (RNAi) is used to create transient null phenotypes of desired genes by preventing translations of RNA to protein. We used this technique to remove LET-60 in *C. briggsae*. By injecting the RNAi into wild-type AF16 hermaphrodites we observed 96% lethality (n=120) with the escapers appearing wild-type and egg-laying competent.

Genomic Structure

C. briggsae let-60 has four exons and three introns; the same as *C. elegans* (Fig. 1 and Fig. 2). Moreover, the size of the exons is exactly the same as in *C. elegans* even though there is variation in the size of the introns. The respective lengths of intron-1 through intron-3 for *C. elegans* are 664 bps, 57 bps, and 726 bps (Han and Sternberg, 1990). In *C. briggsae*, the respective lengths of intron-1 through intron-3 are 383 bps, 47 bps, and 70 bps (Fig. 2). The exon-intron boundaries appear highly conserved even though the intron sequences have no significant homology.

Comparison with the partial genomic sequences from *Oscheius* CEW1 and *P. pacificus* indicate more conservation (Fig. 2). The first and second exon-intron

boundaries of *C. briggsae* and *C. elegans* are shared with the first and third exon-intron boundaries of *Oscheius* CEW1 and *P. pacificus*. The fifth exon-intron boundary of *Oscheius* CEW1 is shared with the final exon-intron boundary of *C. elegans* and *C. briggsae* as well. Additional sequence of *P. pacificus* may indicate that conservation of the 3 exon-intron boundaries is true for the four nematode genes.

Codon Variation

Both *Cel-let-60* and *cb-let-60* have 184 amino acids (Fig. 3; Han and Sternberg, 1990). Of the 184 amino acids, 182 (98.9 %) are identical; the remaining two amino acids are similar.

At the nucleotide level, they share 465 of 555 (83.8 %) conserved nucleotides. Codon analysis revealed conservations in the cDNA. We found that the two genes share 97 identical codons (52.7 %); 79 codons with a variable last base (42.9 %); 5 codons with a variable first base (2.7 %); 3 codons with only the second base conserved (1.6 %).

The two amino acid positions, 121 and 139, that are not conserved between *C. elegans* and *C. briggsae* have codons where the middle base is the only conserved base for the amino acid in position 121, and for the amino acid in position 139, the codons have different third bases.

We expanded our analysis to include two other nematode homologues of *let-60*, *Oscheius* CEW1 and *P. pacificus* (Fig. 3; Sommer *et al.*, 1996; Félix *et al.*, 2000). A protein alignment reveals an identity of 139 of 184 amino acids (75.5 %). We used the consensus sequence for our analysis with consensus defined as three of four positions identical. The consensus sequence for these proteins are conserved with 164 of 184

positions in the sequence (89.1 %). At the nucleotide level, 310 of 555 nucleotides are conserved (55.9 %). With three of four positions for consensus in the sequence, we have 423 of 555 nucleotides with conserved identity (76.1%).

The codon analysis reveals: 76 consensus codons with shared identity; 86 consensus codons with a variable last base (46.7 %); 4 consensus codons with a variable first base (2.2 %); 10 codons with only the second base conserved (5.4 %); 3 consensus codons with only the first base conserved (1.6 %), and 5 consensus codons with no bases conserved (2.7 %).

For the twenty positions not in consensus in the amino acid sequence alignment, six amino acid positions have codons with only the middle nucleotide conserved; five positions have codons with no conserved nucleotides; 8 positions have codons with either the first nucleotide or last nucleotide changed and the remaining position as a codon with three nucleotides conserved. The analysis indicates that the third base has the least amount of evolutionary pressure for conservation, as expected from the genetic code.

3'UTR

The *C. elegans let-60* cDNA has a 937 bp 3'UTR. Regulatory sequences in the 3' UTR are part of the post-transcriptional regulation machinery. Regulatory elements in the 3'UTR help control translation of RNA (Ahringer and Kimble, 1991; Wightman *et al.*, 1991; Goodwin *et al.*, 1993). Essentially, the 3'UTR increases the accuracy of spatial and temporal expression of proteins.

We compared the putative 3'UTR of *C. briggsae* with the 3'UTR of *C. elegans*. We found no significant similarity between the species. The fact that the 3'UTR of *C.*

elegans and *C. briggsae* share no similarities may mean a combination of factors: (1) *C. briggsae* employs different regulatory mechanisms; (2) perhaps *cb-let-60* does not serve as many functions as *Cel-let-60* and thus, requires less control mechanisms; (3) post-transcriptional control is a recent evolutionary development in *C. elegans*.

DISCUSSION

The *C. briggsae* and *C. elegans* homologs are highy conserved at the nucleotide level with 83.8% identity. Using Wobble Aware Base Alignment (WABA), Kent and Zahler (2000) show large highly conserved regions of *C. briggsae* genome shares 89% identity to *C. elegans*.

We believed the nematode ancestral *let-60* gene had three introns. The additional exons in *Oscheius* CEW1 and *P. pacificus* are the result of small introns inserted into the coding sequence and not the loss of introns in *C. elegans* and *C. briggsae*. This idea is supported by the lack of conservation of the second and fourth exon-intron boundaries of *Oscheius* CEW1 and *P. pacificus*. These intron insertions indicate more genetic manipulation, and thusly, further evolutionary divergence. Therefore we submit, that *C. elegans/C. briggsae let-60* gene is closer to the ancestral sequence of the gene.

The greatest variation between *C. elegans* and *C. briggsae let-60* is the size of the introns. Intron size is not enough to determine which gene is closer to the ancestral *let-60* gene.

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Figure 1. Sequence of *C. briggsae let-60*

A. The genomic sequence of *C. briggsae let-60*. The underlined and bolded sequence represents the predicted coding region. B. The putative cDNA sequence with amino acid translation.

Figure 1A. ***C. briggsae*** **Genomic Sequence**

GATTATGCCTGAAGTATTTATATCGTTCTCATTTTTCTTCACATTAACAG 50

ATTTATTCCCCGTCCCTTTTTGTTCAAAGTTTCTTATTACAGAAAGGGTA 100

ATGACGGAGTACAAGCTTGTGGTGGTTGGAGATGGAGGAGTGGGAAAGTC 150

TGCTCTCACTATCCAACTCATTCAAAACCACTTCGTCGAGGAATACGACC 200

CAACTATAGAGGACAGCTATCGAAAGCAGGTATGTGAGCACTTTGTGACA 250

ACTGAGAAGCACATCCGTTTCACACATGTGGAGATGGCAAATTGAACTAA 300

ACGGTGTCATTTGTAATAGCGAAGATATGAGTTAGAAAGAATTTGTTTCT 350

CGAAATCTTGCAGAGTGATTCACAGTTTCTAAACAATGTAGTCTAGACGT 400

TCTAATAAAGGGCCCGTTTTAAAATTACATAGTACATCTGATGACCAAAT 450

GTCCAGAACTGTGTACGTTACAAAGTCGGACAAGTCACAGAAATTTTTAG 500

AGAGTAGGTGAATTGTACAAAGTTCACGCAAGAATTGGGTAAGGAAAATG 550

TTGAGAGCTGTTCTTTGAAGTTATGCTGATTGGTTGAACTAACAACCCTA 600

TTTTTTATAG**GTAGTGATCGACGGAGAGACCTGCCTCCTCGATATATTGG** 650

ATACTGCTGGTCAAGAGGAGTACTCGGCGATGCGCGATCAGTATATGCGA 700

A**CTGGAGAGGGATTCCTTCTGGTCTTCGCCGTCAACGAGGCCAAATCGTT** 750

CGAAAACGTAGCCAACTACAGAGAGCAAATCAGGAGGGTGAAGGATTCAG 800

ATGATGTGAGTCTTAAAATATTCAAAAATAGACTAAATTGTAATATTCTC 850

AG**GTTCCAATGGTTCTGGTTGGAAACAAGTGCGATTTGGCTTCTCGGTCA** 900

GTGGACTTCCGAACAGTCAGCGAAACAGCCAAGGGATACGGAATGCCAAA 950

TGTGGATACTTCAGCCAAAACTCGCATGGGTGTCGATGAGGCATTCTACA 1000

CACTCGTTCGAGAGATACGCAAGGTACATATTAGATTTATATTATACGAA 1050

ATTTGCGAAACGAAATCTCAGTTTGGACAATTACGAATTACAG**CATCGCG** 1100

AGCGTCACGACAACAACAAACCACAAAAGAAAAAGAAGTGTCAAATTATG 1150

TGA 1200

Figure 1B. ***Caenorhabditis Briggsae let-60*** **cDNA**

```
ATG ACG GAG TAC AAG CTT GTG GTG GTT GGA  30
 M   T   E   Y   K   L   V   V   V   G

GAT GGA GGA GTG GGA AAG TCT GCT CTC ACT  60
 D   G   G   V   G   K   S   A   L   T

ATC CAA CTC ATT CAA AAC CAC TTC GTC GAG  90
 I   Q   L   I   Q   N   H   F   V   E

GAA TAC GAC CCA ACT ATA GAG GAC AGC TAT 120
 E   Y   D   P   T   I   E   D   S   Y

CGA AAG CAG GTA GTG ATC GAC GGA GAG ACC 150
 R   K   Q   V   V   I   D   G   E   T

TGC CTC CTC GAT ATA TTG GAT ACT GCT GGT 180
 C   L   L   D   I   L   D   T   A   G

CAA GAG GAG TAC TCG GCG ATG CGC GAT CAG 210
 Q   E   E   Y   S   A   M   R   D   Q
```

```
TAT ATG CGA ACT GGA GAG GGA TTC CTT CTG 240
 Y   M   R   T   G   E   G   F   L   L

GTC TTC GCC GTC AAC GAG GCC AAA TCG TTC 270
 V   F   A   V   N   E   A   K   S   F

GAA AAC GTA GCC AAC TAC AGA GAG CAA ATC 300
 E   N   V   A   N   Y   R   E   Q   I

AGG AGG GTG AAG GAT TCA GAT GAT GTT CCA 330
 R   R   V   K   D   S   D   D   V   P

ATG GTT CTG GTT GGA AAC AAG TGC GAT TTG 360
 M   V   L   V   G   N   K   C   D   L

GCT TCT CGG TCA GTG GAC TTC CGA ACA GTC 390
 A   S   R   S   V   D   F   R   T   V

AGC GAA ACA GCC AAG GGA TAC GGA ATG CCA 420
 S   E   T   A   K   G   Y   G   M   P

AAT GTG GAT ACT TCA GCC AAA ACT CGC ATG 450
 N   V   D   T   S   A   K   T   R   M
```

```
GGT GTC GAT GAG GCA TTC TAC ACA CTC GTT 480
 G   V   D   E   A   F   Y   T   L   V

CGA GAG ATA CGC AAG CAT CGC GAG CGT CAC 510
 R   E   I   R   K   H   R   E   R   H

GAC AAC AAC AAA CCA CAA AAG AAA AAG AAG 540
 D   N   N   K   P   Q   K   K   K   K

TGT CAA ATT ATG TGA                     570
 C   Q   I   M   *
```

Figure 2. Exon-Intron Genomic Structure

The four homologs of LET-60 protein. Each line represents the length of the protein. The arrowheads indicate the location of an intron within the gene. The number just above the arrowhead indicate the size of the intron. We only have partial genomic sequence for *Oscheius* and *P. pacificus*. The ? represents an intron on unknown length. The scale bar is equivalent to 20 amino acids.

Figure 2. Genomic Structure Comparisons

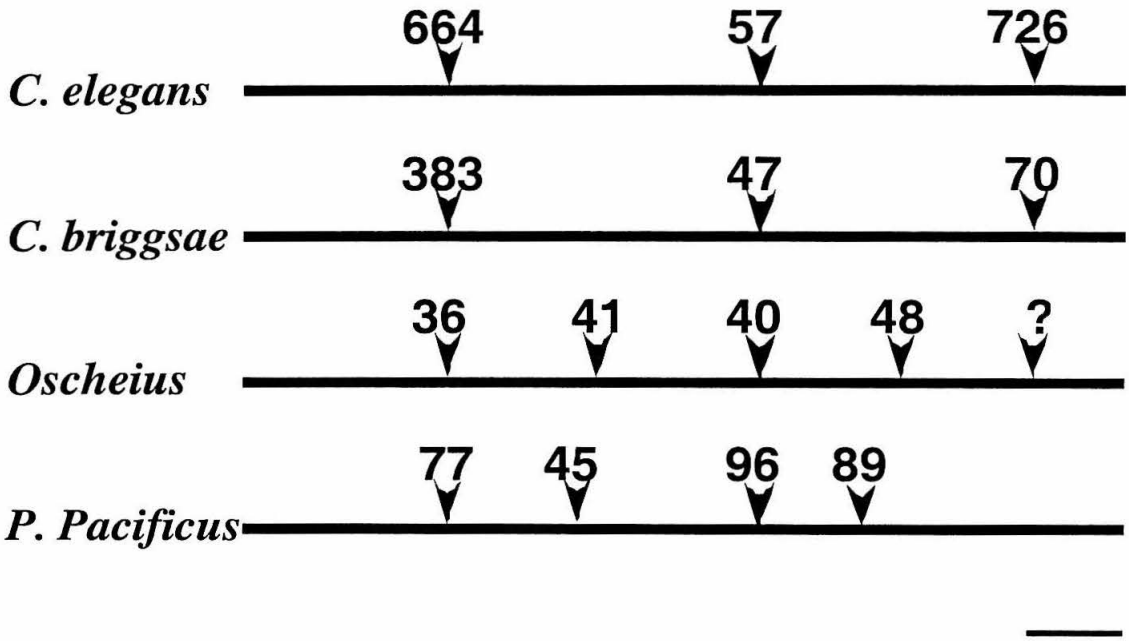
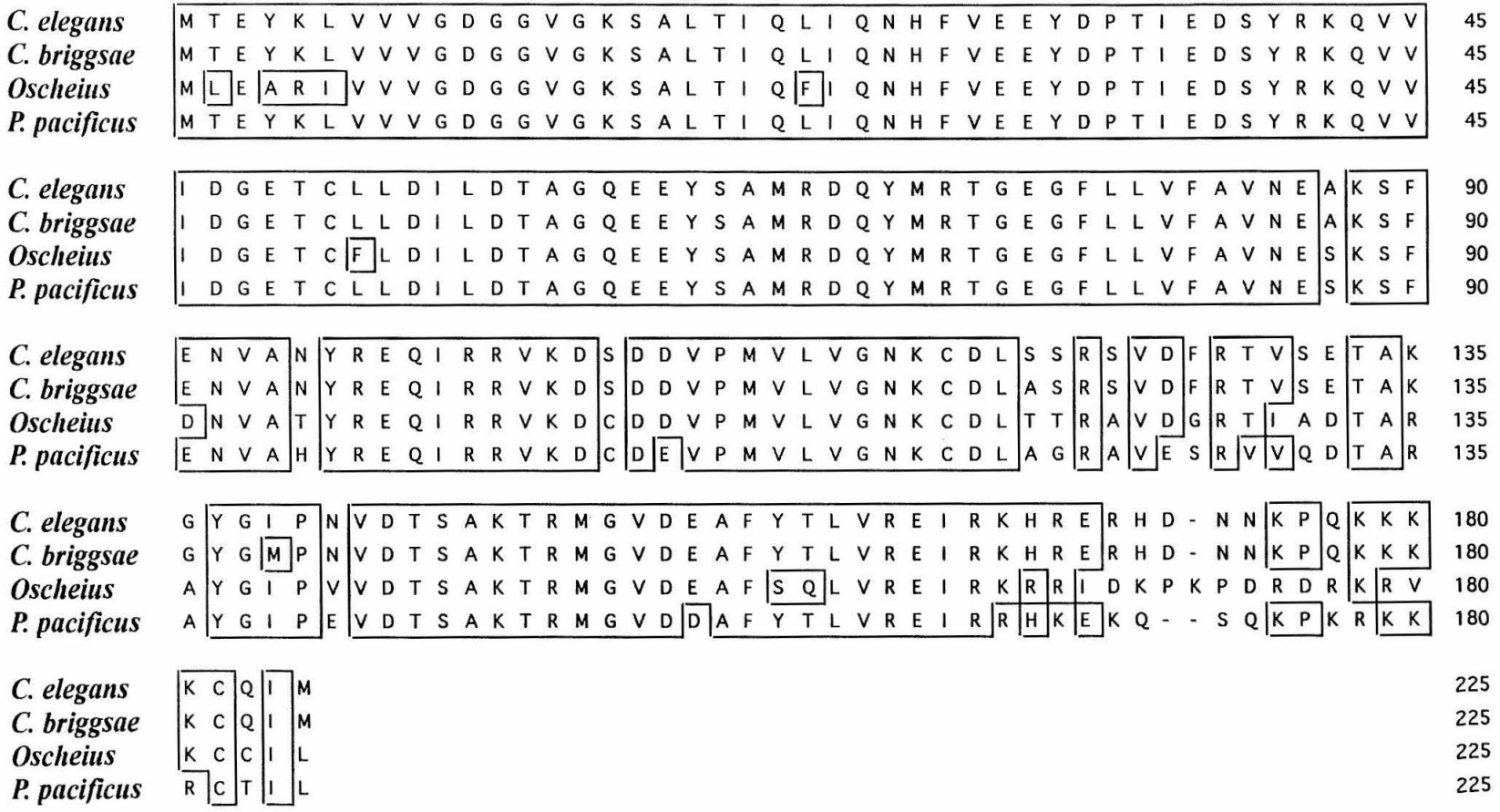


Figure 3. Amino Acid Alignment of *let-60* Homologues

A four sequence alignment of *C. elegans*, *C. briggsae*, *Oscheius* CEW1, and *P. pacificus*.

Amino acids conserved between three or more sequences are boxed.

Figure 3. Nematode *let-60* Alignments



Appendix

Characterization of Seven Genes Affecting *Caenorhabditis elegans* Hindgut

(I contributed genetic and molecular analysis of *egl-38(sy287)* previously known as *lin-50*.)

Characterization of Seven Genes Affecting *Caenorhabditis elegans* Hindgut Development

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ABSTRACT

We have identified and characterized 12 mutations in seven genes that affect the development of the *Caenorhabditis elegans* hindgut. We find that the mutations can disrupt the postembryonic development of the male-specific blast cells within the hindgut, the hindgut morphology in both males and hermaphrodites, and in some cases, the expression of a hindgut marker in hermaphrodite animals. Mutations in several of the genes also affect viability. On the basis of their mutant phenotypes, we propose that the genes fall into four distinct classes: (1) *egl-5* is required for regional identity of the tail; (2) *sem-4* is required for a variety of ectodermal and mesodermal cell types, including cells in the hindgut; (3) two genes, *lin-49* and *lin-59*, affect development of many cells, including hindgut; and (4) three genes, *mab-9*, *egl-38*, and *lin-48*, are required for patterning fates within the hindgut, making certain hindgut cells different from others. We also describe a new allele of the *Pax* gene *egl-38* that is temperature sensitive and affects the conserved β-hairpin of the EGL-38 paired domain. Our results suggest that a combination of different factors contribute to normal *C. elegans* hindgut development.

THE development of a digestive system is critical to animal viability and may include processes of organogenesis established early in animal evolution. Recent molecular investigations have identified similarities in the genes involved in development of the digestive system among vertebrate and invertebrate animals (reviewed in SIMON and GORDON 1995). Notably, there is a conservation of gene expression and function in hindgut development, despite classical distinctions in the embryonic source of hindgut tissues in different organisms (*e.g.*, endoderm *vs.* ectoderm; HOCH and PANKRATZ 1996; WU and LENGYEL 1998). Thus, genetic studies of hindgut development in invertebrates may identify genes with a common role in development as well as investigate factors important to organogenesis.

The *Caenorhabditis elegans* digestive system includes a foregut (pharynx), midgut (intestine), and hindgut (rectum; SULSTON *et al.* 1983). The cells of each structure derive from distinct embryonic precursors and have different developmental properties. All cells of the intestine are clonally derived embryonically from the endodermal founder cell, E. In contrast, pharyngeal cells are not born clonally and derive both from the mesodermal founder cell, MS, and an ectodermal precursor, ABa. The cells of the hindgut derive from another ectodermal precursor, ABp. Most of the hindgut cells are not closely related to each other by lineage, and cell ablation results suggest the proper specification of some hindgut cell types requires cell interactions during both early and late embryogenesis (BOWERMAN *et al.* 1992). To better understand the development and organogenesis of the *C. elegans* hindgut, we have identified and characterized mutations that disrupt this process.

The *C. elegans* hindgut is composed of 11 cells and eight distinct cell types (SULSTON *et al.* 1983; Figure 1). Together, these cells form an opening to the intestine, yet maintain the structural integrity of the animal. The eight hindgut cell types can be distinguished on the basis of cellular morphology, the expression of specific markers, and their behavior during postembryonic development. Although development of the *C. elegans* digestive system and the differentiation of hindgut cell types occurs during embryogenesis, the hindgut cells also contribute to larval development as one (in hermaphrodite animals) or five (in male animals) of the cells undergo further postembryonic cell divisions (SULSTON and HORVITZ 1977; SULSTON *et al.* 1980). Each of the dividing cells generally produces at least one progeny cell that continues as part of the hindgut, as well as other distinct cell types, such as neurons.

The four most posterior hindgut cells (F, U, B, Y) are termed male-specific blast cells. These four cells represent four distinct cell types in both males and hermaphrodites and can be distinguished on the basis of cellular morphology, behavior, and the expression of molecular markers. In addition, part of male sexual specialization includes the subsequent postembryonic

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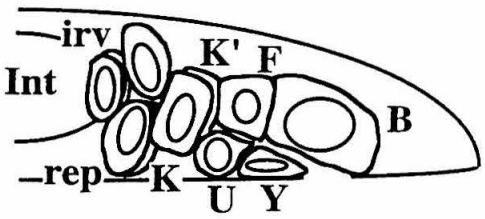

FIGURE 1.—Diagram of hindgut cells in early L1 larval stage (after SULSTON *et al.* 1983). The *C. elegans* hindgut cells form five tiers (or rings) of cells connecting the intestine to the anal pore. At hatching, the Y cell is part of the hindgut, but during larval development it is replaced by P12.pa. In hermaphrodites, Y moves anterior into the preanal ganglion to become PDA. In males, U, F, B, and Y cells divide during postembryonic development. K divides postembryonically in both males and hermaphrodites. Int, intestine; irv, intestinal rectal valve cells (also called vir); rep, rectal epithelial cells. Anterior left, dorsal up.

division of these cells. In males, each cell divides with a stereotypic pattern and produces a different set of differentiated progeny. For example, the B cell divides to produce 47 progeny, including all of the cells of the spicules, which are sensory structures important for male mating (LIU and STERNBERG 1995). The different cell division patterns and progeny produced in the male reflect the distinct cell types of the four precursor cells, and thus the male cell lineage can be used as an indicator of precursor cell type. In general, mutations that affect specification of cell type for these hindgut cells in both sexes can be identified and characterized because of the effects on the postembryonic male development (CHISHOLM and HODGKIN 1989; CHISHOLM 1991; CHAMBERLIN *et al.* 1997). Consequently, we have identified 12 mutations in seven genes that affect hindgut development in both males and hermaphrodites among a collection of mutants with abnormal male tail development. Using cell morphology, cell lineage, tissue differentiation, and gene expression criteria, we have subdivided the genes into several classes that suggest contributions from a combination of regional, local, and tissue-type factors in hindgut development.

MATERIALS AND METHODS

Strains: Nematode strains were cultured according to standard techniques (BRENNER 1974; SULSTON and HODGKIN 1988). Mutations used are described by HODGKIN (1997).

Linkage group (LG) I: *egl-30(n686), mek-2(q425), fog-1(e2121), unc-11(e47), unc-74(e883), dpy-5(e61), sem-4(n1378), unc-13(e51). qDf3.*
LG II: *mab-9(e1245), rol-6(e187), unc-4(e120).*
LG III: *unc-93(e1500), unc-103(n500), dpy-17(e164), egl-5(n945), unc-32(e189). yDf10, sDf121, sDf130.*
LG IV: *dpy-13(e184), unc-5(e53), unc-24(e138), mes-6(bn66), lin-49(s1198), fem-3(e1996), elt-1(zu180), egl-20(n585), egl-38(s1775), egl-38(n578), daf-14(m77), unc-43(e408), mec-3(e1338), let-312(s1234), lin-3(n378), dpy-20(e1282), dpy-20(e1362), unc-22(s7), unc-31(e169). eDf19, eDf18, mDf7.*
LG V: *dpy-11(e224), him-5(e1490).*
LG X: *lon-2(e678).*
Extrachromosomal array: *pkEx246* (PETTITT *et al.* 1996).

Isolation of mutations: The mutations described in this article were isolated in a screen for mutants with abnormal male tail morphology. We used the strain CB1490 *him-5(e1490)* as a convenient source of phenotypically wild-type males, since *him-5* mutant hermaphrodites (XX) segregate about 40% male (XO) self-progeny (HODGKIN *et al.* 1979). *him-5(e1490)* hermaphrodites (P0) were mutagenized with 50 mM EMS for 4 hr (SULSTON and HODGKIN 1988), placed individually on agar plates, and allowed to produce self-progeny. After 4 days, four to five F_1 L4 hermaphrodite progeny were removed from each parental plate and placed individually on fresh plates. After another 4–5 days, each F_2 brood was screened for presence of 1/4 or more morphologically abnormal individuals among the male self-progeny. Sibling hermaphrodites were selected from plates that yielded abnormal males to recover a homozygous mutant strain. In screens of over 25,000 mutagenized gametes, we identified 86 mutations that result in a *m*ale *ab*normal (Mab) phenotype. These mutants were then observed using Nomarski optics and placed into different phenotypic classes based on the morphological defects of larval and adult males.

Genetic mapping and complementation tests: All mutations were backcrossed at least twice to N2 (wild-type) stocks, usually during the process of mapping. Assignment to specific chromosomes was performed as described by BRENNER (1974), generally using the following markers: *dpy-5* I, *rol-6* II, *unc-32* III, *unc-5* IV, *dpy-11* V, and *lon-2* X. Once a mutation was assigned to a specific chromosome, it was either tested for complementation with known hindgut genes (in the case of *mab-9* and *egl-5* alleles) or mapped further using multipoint and deficiency mapping (Table 1). Linkage and complementation tests with two mutations, *sa417* and *sa423*, showed that they were alleles of *mab-9*. These two alleles were subsequently lost. They are included in this study for completeness and to correctly represent the frequency of allele recovery.

Cell lineage analysis and laser ablation: Divisions of nuclei in the male F, U, and B lineages were observed directly in living animals using Nomarski differential interference contrast optics as described by SULSTON and HORVITZ (1977). Nomenclature follows the standard of SULSTON and HORVITZ (1977), with modifications of CHAMBERLIN and STERNBERG (1993). For F and U cell lineages in Figure 4, the initial cell division was inferred on the basis of observations from other mutant animals and the position of the presumptive F.l/r and U.l/r cells in the animals. All lineages were followed from early L3 through the L3 molt. For B cell lineages in Table 3, all lineages were followed from the first divisions of the B.a(l/r)xx cells (early to mid-L3 larval stage; x represents both progeny of a division) through the L3 molt.

Laser killing of cells was performed by the method of SULSTON and WHITE (1980) using the laser microbeam system and procedure of AVERY and HORVITZ (1987). Animals were anesthetized on pads of 5% agar in water containing 5 mM sodium azide. F, U, and B or B.a were killed in late L1 or early L2 larval stage males. Animals were recovered, checked several hours later for successful surgery, and then raised to adulthood and scored for differentiation of spicule socket cells. Socket cell differentiation was scored on the basis of the production of refractile spicule cuticle (JIANG and STERNBERG 1999). In wild type, this material is expressed by both spicule cells (derived from B.a) and proctodeal cells (derived from

TABLE 1

Genetic mapping and complementation tests of mutations

Gene	Heterozygote[a]	Recombinants	Results
egl-5	*egl-5(sy279)/egl-5(n945); unc-24/+; him-5*		*sy279* fails to complement *n945* (Egl, Mab)[b]
sem-4	*sem-4(sa416)/unc-74 dpy-5; him-5*	Unc non-Dpy	*unc-74* (9) *sem-4* (0) *dpy-5*
		Dpy non-Unc	*unc-74* (6) *sem-4* (0) *dpy-5*
	sem-4(sa416)/dpy-5 unc-13; him-5/+	Unc non-Dpy	*dpy-5* (9) *sem-4* (0) *unc-13*
		Dpy non-Unc	*dpy-5* (10) *sem-4* (1) *unc-13*
	sem-4(sa416)/dpy-5 sem-4(n1378); him-5/+		*sa416* fails to complement *n1378* (Egl, Mab)
lin-49	*lin-49(sy238)/unc-24 mec-3 dpy-20; him-5*	Unc non-Dpy	*unc-24* (15) *lin-49* (6) *mec-3* (3) *dpy-20*
		Dpy non-Unc	*unc-24* (4) *lin-49* (3) *mec-3* (4) *dpy-20*
	lin-49(sy238)/unc-24 fem-3	Unc non-Fem	*unc-24* (19) *lin-49* (10) *fem-3*
	lin-49(sy238) unc-22/unc-24 mes-6 dpy-20	Unc-24 non-Dpy	*unc-24* (6) *mes-6* (0) *lin-49* (18) *dpy-20*
		Dpy non-Unc-24	*unc-24* (13) *mes-6* (2) *lin-49* (51) *dpy-20*
	unc-24 lin-49(sy238)/eDf18; him-5/+		*eDf18* deletes *lin-49* (Let)
	unc-24 lin-49(sy238)/eDf19; him-5/+		*eDf19* deletes *lin-49* (Let)
lin-59	*lin-59(sa489)/unc-11 dpy-5; him-5*	Unc non-Dpy	*unc-11* (0) *lin-59* (10) *dpy-5*
		Dpy non-Unc	*unc-11* (0) *lin-59* (5) *dpy-5*
	lin-59(sa489)/egl-30 unc-11; him-5/+	Egl non-Unc	*egl-30* (8) *lin-59* (2) *unc-11*
	lin-59(sa489)/mek-2 unc-11; him-5/+	Unc non-Mek	*mek-2* (7) *lin-59* (0) *unc-11*
	lin-59(sa489)/fog-1 unc-11; him-5/+	Unc non-Fog	*fog-1* (8) *lin-59* (1) *unc-11*
	lin-59(sa489) unc-11/qDf3; him-5/+		*qDf3 deletes lin-59* (Let)
mab-9	*mab-9(sa473) unc-4/mab-9(e1245); him-5*		*sa473* fails to complement *e1245* (Mab)
egl-38	*egl-38(sy294)/unc-24 dpy-20; him-5/+*	Unc non-Dpy	*unc-24* (6) *egl-38* (6) *dpy-20*
		Dpy non-Unc	*unc-24* (7) *egl-38* (4) *dpy-20*
	egl-38(sy294)/elt-1 dpy-20; him-5/+	Dpy non-Elt	*elt-1* (7) *egl-38* (35) *dpy-20*
	egl-38(sy294)/unc-24 daf-14; him-5/+	Unc non-Daf	*unc-24* (50) *egl-38* (6) *daf-14*
	egl-38(sy287)/let-312 lin-3; him-5	Lin non-Let	*let-312* (0) *egl-38* (5) *lin-3*
	egl-38(sy294) unc-22 unc-31/elt-1 unc-43	non-Elt non-Unc-22(d)	*elt-1* (14) *egl-38* (4) *unc-43* (88) *unc-22*
	egl-38(sy294) unc-22 unc-31/unc-24 egl-20 dpy-20	Unc-24 non-Dpy	*unc-24* (23) *egl-20* (3) *egl-38* (21) *dpy-20*
		Dpy non-Unc-24	*unc-24* (15) *egl-20* (5) *egl-38* (19) *dpy-20*
	dpy-13 egl-38(sy294)/eDf18 unc-22; him-5/+		*eDf18* does not delete *egl-38*
	egl-38(sy294) unc-22 unc-31/eDf19		*eDf19* deletes *egl-38* (Let)
	egl-38(sy294) unc-22 unc-31/dpy-13 mDf7		*mDf7* deletes *egl-38* (Let)
lin-48	*lin-48(sa469) unc-32/unc-93 dpy-17; him-5/+*	Unc-93 non-Dpy	*unc-93* (6) *lin-48* (2) *dpy-17*
		Dpy non-Unc-93	*unc-93* (1) *lin-48* (3) *dpy-17*
	lin-48(sa469)unc-32/unc-103 dpy-17; him-5/+	Dpy non-Unc-103	*unc-103* (0) *lin-48* (6) *dpy-17*
		non-Unc-32 non-Unc-103(d)	*unc-103* (0) *lin-48* (7) *dpy-17* (18) *unc-32*
	lin-48(sa469) unc-32/yDf10 unc-32; him-5/+		*yDf10* deletes *lin-48* (Mab)
	lin-48(sa469) unc-32/sDf130 unc-32; him-5/+		*sDf130* deletes *lin-48* (Mab)
	lin-48(sa469) unc-32/sDf121 unc-32; him-5/+		*sDf121* does not delete *lin-48*

[a] Alleles are listed in MATERIALS AND METHODS.

[b] The phenotypes observed in *trans*-heterozygotes are listed in parentheses.

B.p). Although both types of cells must be removed to completely eliminate refractile cuticle expression, killing the B cell is not tolerated well by the animals, and many do not survive to adulthood. To enhance viability of the operated animals, B.a, rather than B, was killed in many animals, and B.p was allowed to develop normally. These animals produce proctodeal cells, but these are distinct from both the normal and the ectopic spicule cells. In all cases, production of spicule socket cells in B.a-killed and B-killed animals was the same.

Analysis of *cdh-3::gfp* expression: The strain NL1008 *dpy-20(e1362); pkEx246* (PETTITT *et al.* 1996) was used as a parent strain for all crosses and as the wild-type control. *pkEx246* was crossed into strains and maintained on the basis of its ability to rescue *dpy-20* mutations. To score expression, L1 and L2 larvae from non-Dpy parents were anesthetized on pads of 5% agar in water containing 5 mM sodium azide and scored for larval stage and green fluorescent protein (GFP) expression at ×1000 magnification. Transgenic animals were verified by confirming expression of *cdh-3::gfp* in nonhindgut cells prior to scoring. Cells were scored positive for expression if any GFP fluorescence was detected above background.

Tests for strain viability: The viability of different strains was tested by counting the full brood of three to four homozygous mutant hermaphrodites. All strains contained the mutation *him-5(e1490)*, which causes a background of ~5% X-aneuploid progeny that fail to hatch (HODGKIN *et al.* 1979). CB1490 *him-5(e1490)* was used as the wild-type control for comparison. To score viability at 20°, L4 hermaphrodites were placed singly

TABLE 2

Summary of hindgut genes recovered in Mab screen

Gene	LG	Alleles isolated	No. other alleles	References
egl-5	III	*sy279*	>10	CHISHOLM (1991); WANG *et al.* (1993)
sem-4	I	*sa416*	6	BASSON and HORVITZ (1996)
lin-49	IV	*sy238, sa470*	1	CLARK (1990)
lin-59	I	*sa489*	0	
mab-9	II	*sa417, sa423, sa473*	3	CHISHOLM and HODGKIN (1989)
egl-38	IV	*sy287, sy294*	2	TRENT *et al.* (1983); CHAMBERLIN *et al.* (1997)
lin-48	III	*sy234, sa469*	1	JIANG and STERNBERG (1999)

on plates and then transferred to fresh plates every 24 hr until they stopped producing self-progeny. Twenty-four hours after transferring the parent, unhatched eggs and dead L1 stage larvae were counted and removed from the plate. Forty-eight or more hours after removing the dead eggs, the remaining progeny were counted and assessed for stage of development. These times were modified for tests at 15° and 25° to compensate for altered growth rates. In all cases, however, animals were scored when the wild-type control animals were all hatched and again when they had reached adulthood. Because many dying larvae disintegrate rapidly, dead larvae, especially L1s, may be underrepresented when counted by this method.

Temperature-sensitive tests with *lin-49*, *lin-59*, and *egl-38* mutants: *lin-49(sy238)*, *lin-49(sa470)*, and *lin-59(sa489)* are all temperature sensitive, and homozygous mutant strains cannot be maintained at 25°. To test *cdh-3::gfp* expression and animal viability at 25° and 15°, parents were shifted from 20°, and then their progeny were analyzed. *egl-38(sy287)* is temperature sensitive, but viable. Homozygous strains were maintained at least two generations at the experimental temperature prior to scoring phenotypes.

Scoring hindgut, tail, and egg-laying phenotypes in *egl-38* mutants: Hindgut (Scar, Con) and male tail (Mab) morphology were scored in adult animals at ×1000 magnification using Nomarski optics. Adult hermaphrodites were scored as egg-laying defective (Egl) if they retained more than the normal single row of eggs in the uterus and the retained embryos had developed past the gastrulation stage.

DNA sequencing: We used PCR to amplify DNA including the exons coding for the paired domain (exons 2–5; CHAMBERLIN *et al.* 1997) of *egl-38* from *egl-38(sy287)* mutants according to the single worm PCR method of BARSTEAD *et al.* (1991). PCR products were directly sequenced by the California Institute of Technology sequencing facility using an ABI 373 DNA sequencer (Perkin-Elmer, Norwalk, CT). Both strands of DNA were sequenced from three independent reactions to confirm the mutations.

RESULTS

Male tail-defective mutants identify genes affecting hindgut development: In a genetic screen for mutations that disrupt male tail development, we identified several distinct classes of mutants (H. M. CHAMBERLIN, unpublished results). One class consisting of 12 mutations shared several features that suggested a common effect on hindgut development (Table 2). These mutants display profoundly abnormal male tail development and morphology (Figure 2) and less penetrant phenotypes of abnormal hindgut morphology apparent at hatching and in hermaphrodites as well as males at all stages (Figure 3). These morphological features can result in constipation of larvae and adults; animals can be sufficiently deformed or damaged to result in lethality. Since the defects are present at hatching and are in both males and hermaphrodites, we infer that these genes function in the embryonic development of the hindgut in both males and hermaphrodites. The defects in male tail development and morphology reflect the effects on hindgut development, but they may also reflect additional male-specific functions of these genes.

Genetic mapping and complementation tests placed these 12 mutations into seven complementation groups. Three genes (*egl-5*, *sem-4*, and *mab-9*) had previously been identified by other researchers as affecting hindgut development (CHISHOLM and HODGKIN 1989; CHISHOLM 1991; BASSON and HORVITZ 1996). One gene (*egl-38*) had been identified for other functions but not hindgut development (TRENT *et al.* 1983). Three genes (*lin-48*, *lin-49*, and *lin-59*) were novel and were not previously identified in screens for developmental mutants in *C. elegans*.

Altered male cell lineage in hindgut mutants: To investigate the cause of the male tail defects we observed the development of the four hindgut cells that divide in males (F, U, B, and Y). Initial observations indicated that all the mutants had abnormal divisions of some or all of these cells. We carried out cell lineage analysis of F, U, and B in *lin-48* and *lin-49* mutants (Figure 4; Table 3). Cell lineage analysis for *egl-5*, *mab-9*, and *egl-38* has been reported previously (CHISHOLM and HODGKIN 1989; CHISHOLM 1991; CHAMBERLIN *et al.* 1997).

In *lin-48* mutants, the presumptive F and U cells produce more progeny than in wild type, and both cells produce a cell division pattern similar to each other (Figure 4B). Since the cell division pattern in *lin-48* mutants is abnormal and not similar to a cell lineage normally found in wild type, it alone does not suggest a specific role for *lin-48* in the development of F and U cells. Cell ablation experiments (see below) were used to further investigate the role of *lin-48* in F and U development.

We also followed the development of the B cell in *lin-48* mutants. Normally the B cell develops in three steps: (1) An initial set of divisions produces 10 progeny. (2) Eight of these progeny (the progeny from B.a) then

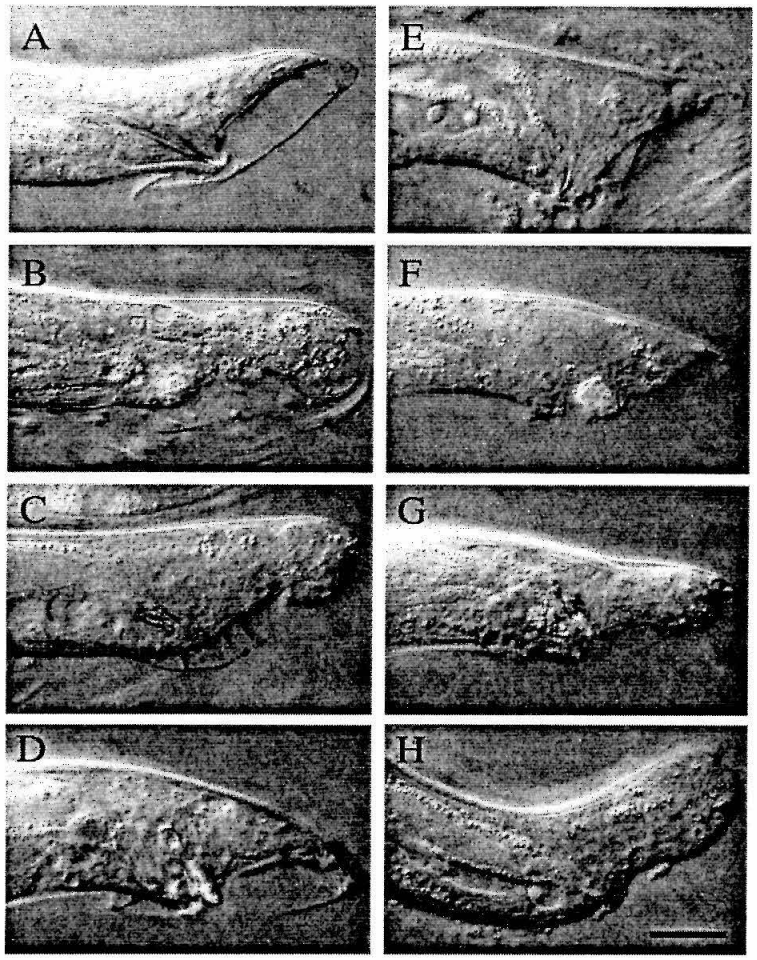

FIGURE 2.—Nomarski photomicrographs comparing the wild-type *C. elegans* adult male tail and those of mutants recovered in the genetic screen. (A) Wild type; (B) *mab-9(sa473)*; (C) *egl-38(sy294)*; (D) *lin-48(sa469)*; (E) *egl-5(sy279)*; (F) *sem-4(sa416)*; (G) *lin-49(sy238)*; (H) *lin-59(sa489)*. Anterior left, dorsal up. Bar, 20 μm.

participate in short-range migrations. (3) All 10 progeny then undergo a second set of cell divisions (SULSTON *et al.* 1980). The third phase of B cell development reflects response of the eight migrating cells to a collection of cell interactions. In particular, the eight cells form pairs of cells in which one cell is more anterior and the other more posterior. These cell pairs are subject to competing signals: signals that promote the anterior fate and signals that inhibit it (or promote posterior fate; Figure 5; CHAMBERLIN and STERNBERG 1993). The F and U cells (or their progeny) are a source of the anterior-promoting signal. Normally, of the two cells in the pair, the physically anterior cell is closer to the progeny of F and U, and it adopts the anterior fate. However, experimental conditions that result in ectopic anterior-promoting signal or eliminate the inhibiting signal can result in physically posterior cells behaving like their more anterior neighbors (CHAMBERLIN and STERNBERG 1993, 1994). We find that in *lin-48* mutants the first set of B cell divisions and the cell migrations are generally normal. However, the subsequent development of some B progeny is abnormal. For example, the posterior cell of the B.a(l/r)aa (**aa**) cell pair normally produces a cell lineage (β) that results in 6 progeny, while the anterior **aa** cell produces a cell lineage (α) that results in 4 progeny. In *lin-48* mutants the posterior cell often produces 5 or 4 progeny rather than the normal 6, suggesting it is developing in a manner more similar to its

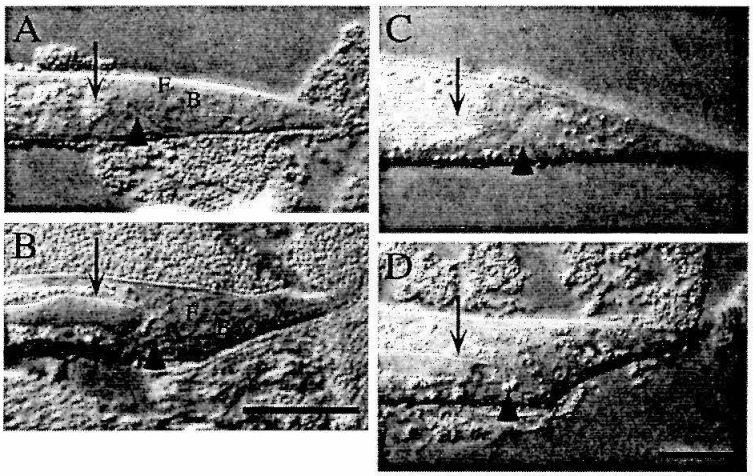

FIGURE 3.—Nomarski photomicrographs comparing larval tail morphology in wild type and representative mutants. (A) Wild-type L1 larval stage male animal. Although there are some cellular differences, L1 larval males and hermaphrodites are morphologically similar. (B) *lin-49(sy238)* L1 male. (C) Wild-type L4 larval stage hermaphrodite. (D) *egl-38(sy294)* L4 hermaphrodite. Arrows indicate lumen of the intestine, which is distended in mutants, indicating constipation. Arrowheads indicate the hindgut, which is morphologically abnormal and sometimes blocked in mutants. Anterior left, dorsal up. Bars, 20 μm.

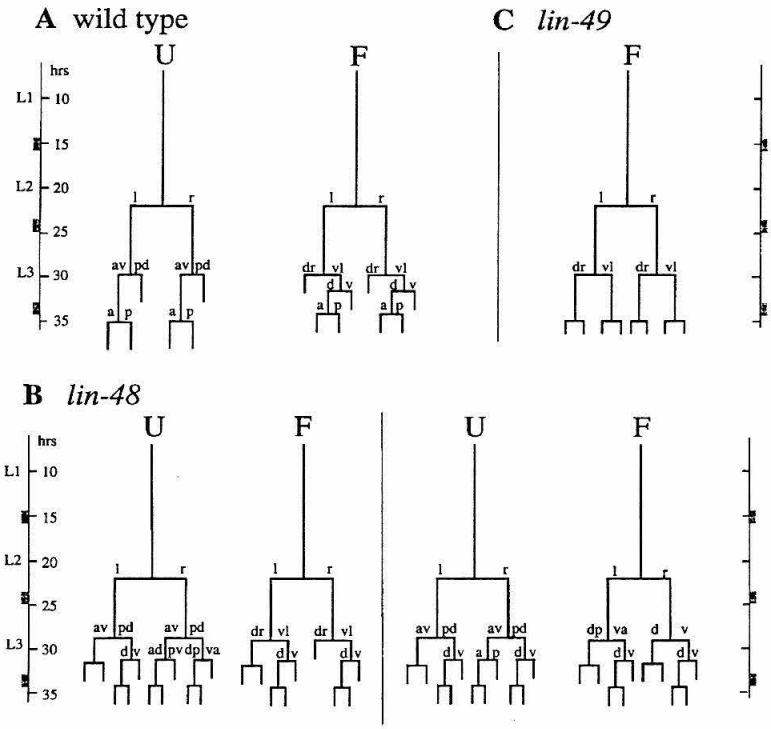

FIGURE 4.—Abnormal F and U cell lineage in hindgut mutants. (A) Wild-type male F and U cell lineages (after SULSTON *et al.* 1980). The U.(l/r)aa cells do not divide in all animals. (B) The F and U cell lineages from two *lin-48(sy234)* mutant males. (C) The F cell lineage from one *lin-49(sy238)* male. The U cell in this animal did not divide. Side bars indicate approximate developmental time from hatching (in hours), and larval stage. Shading indicates the lethargus period prior to molting.

TABLE 3

Abnormal male B cell lineage in hindgut mutants

Genotype	aa ant.	aa post.	pp ant.	pp post.
Wild type	α (4)	β (6)	γ (6)	δ (2)
lin-48[a]	α	abn-6[b]	abn-7	δτ
	ατ[c]	α	γτ	δτ
	abn-5(l)[d]	abn-5(r)	γτ	δτ
	ατ(r)	abn-5(l)	γτ	δτ
	α	abn-5	abn-7	δτ
lin-49[e]	α(l)	β(r)	γ*[f]	δ

Each line represents the observed cell lineage for B.a(l/r)aa (**aa**) and B.a(l/r)pp (**pp**) from one male animal. Other B cell progeny developed normally in all animals. For wild type, the number of progeny from each cell type is indicated in parentheses. Abnormal cell lineages are indicated by underscoring.

[a] Genotype: *lin-48(sy234); him-5(e1490).*

[b] abn-*n* indicates that the cell lineage was abnormal, but produced *n* progeny.

[c] τ indicates the cell produced the normal pattern of progeny, but cell division axes were abnormal.

[d] (l) and (r) indicate that the cells failed to migrate to their normal anterior/posterior positions. One cell (l) remained on the left, and one cell (r) remained on the right.

[e] Genotype: *lin-49(sy238); him-5(e1490).*

[f] γ* is a commonly observed abnormal lineage (CHAMBERLIN and STERNBERG 1993). It results in four progeny instead of the normal six.

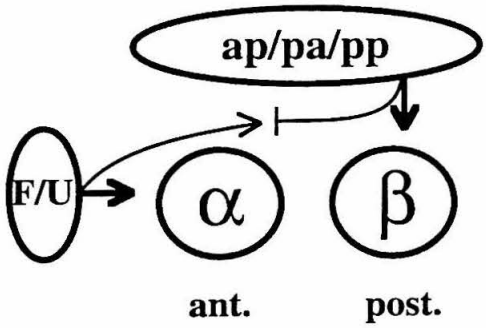

FIGURE 5.—A model for some cell interactions that affect fate choice of B.a(l/r)aa (**aa**) cells (figure after CHAMBERLIN and STERNBERG 1993). Normally, the two **aa** cells adopt distinct fates that correspond to their relative anterior/posterior position. The anterior cell adopts the fate and produces the cell lineage termed α. The posterior cell adopts the fate and produces the cell lineage termed β. Killing F and U cells results in both cells adopting the posterior fate (β). Killing the cousin and sibling cells (**ap**, **pa**, and **pp**) or ectopic expression of an anterior-promoting signal (*lin-3*; CHAMBERLIN and STERNBERG 1994) results in both cells adopting the anterior fate (α).

anterior neighbor. The abnormal cell lineage observed in the anterior **pp** cell is also consistent with ectopic signal. Even though F and U cells develop abnormally in *lin-48* mutants, the B cell progeny do not behave as if the F and U cells have been removed. Instead, the B cell progeny in *lin-48* mutants behave like cells exposed to either increased anterior-promoting signal or removal of signal that inhibits anterior fate.

lin-49 mutant males also have cell lineage defects in F, U, and B cells. We find that the presumptive U cell (or sometimes the presumptive F cell) often fails to divide, and the other cell divides without asymmetry and produces fewer progeny than normal (Figure 4C). We observed similar effects in *lin-59* mutants (data not shown). We followed the B cell lineage in one *lin-49* mutant (Table 3). The B cell lineage defect in this animal is consistent with a reduction of the signal that promotes anterior fate (see above). Lineage defects of this sort are observed in wild-type animals in which F and U cells have been killed (CHAMBERLIN and STERNBERG 1993).

***lin-48* mutants produce ectopic spicule cells from U:** In addition to the cell lineage defects (see above), we observed that some adult *lin-48* mutant males produced ectopic refractile spicule cuticle (Figure 6). The spicule cuticle is made by spicule socket cells (JIANG and STERNBERG 1999) and thus serves as a marker for the differentiation of these spicule cells. In wild type, all of the cells of the spicules derive from a single precursor, the B cell (SULSTON *et al.* 1980). To test whether the ectopic socket cells derive from some cell other than B, we killed the B cell (or the B.a daughter; see MATERIALS AND METHODS) in *lin-48* mutants and found that 14 of 21 mutants still produced ectopic spicule socket cells (Table 4). Since U and F cells produce abnormal lineages in *lin-48* mutants, we killed these cells each in combination with the B cell and found that killing the U cell essentially eliminated the production of ectopic spicule socket cells. We infer that U is producing the ectopic spicule socket cells. Since spicule socket cells are a cell type normally produced only by the B cell, we conclude that *lin-48* plays a role in making the presumptive U cell different from B.

Altered *cdh-3::gfp* expression in some hindgut mutants: To further investigate the role of these genes in hindgut development, we tested whether expression of *cdh-3::gfp* could serve as a marker for specific hindgut cells. *cdh-3* is a *C. elegans* cadherin gene described by PETTITT *et al.* (1996), who found that *cdh-3::gfp* is expressed in the F and U cells of the hindgut in both males and hermaphrodites. We confirmed the expression of this construct in F and U, but detected expression at lower frequency and level in other hindgut cells in hermaphrodites grown at 20° (Table 5). We also found that detectable expression is greatly increased in animals grown at 25°. For example, we detected expression of *cdh-3::gfp* in K or K′ (or both) in 19% of wild-type hermaphrodites grown at 20°, but in 84% of those grown

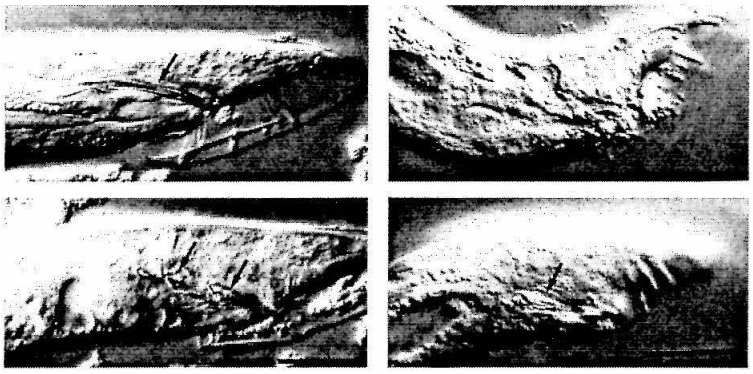

FIGURE 6.—Ectopic production of refractile spicule cuticle in *lin-48* mutants. Arrows indicate cells expressing spicule cuticle. Intact adult males: (Top left) wild type; (bottom left) *lin-48(sa469)*. Note the second, ectopic clump of cells in the *lin-48* mutant. Adult males in which the B cell was killed in early larval development: (Top right) wild type; (bottom right) *lin-48(sa469)*. The *lin-48* male still produces spicule cuticle. Bars, 20 μm.

at 25°. Detectable expression also increased in F and U, as well as B and repD, at the higher temperature. These observations suggest that *cdh-3::gfp* expression in the hindgut is not restricted to F and U, but that it is expressed at different levels or with different stability in different hindgut cells.

We tested expression of *cdh-3::gfp* in different hindgut mutants (Table 5). We found that mutations in *sem-4* and *egl-5* did not affect expression, despite the fact that morphological and cell lineage results indicate that these genes profoundly affect the fates of hindgut cells (CHISHOLM 1991; BASSON and HORVITZ 1996). Our observations suggest that these genes act independently (or possibly downstream) of the processes that affect hindgut *cdh-3::gfp* expression.

We found that only about half of *egl-38(sy294)* mutants that express *cdh-3::gfp* in the hindgut express the marker in both presumptive U and F (Table 5). Of animals expressing in only one cell, F was the expressing cell 87% of the time. This is consistent with our conclusion that in *egl-38* mutants the U and F cells adopt fates more similar to their posterior neighbors, Y and B, respectively (CHAMBERLIN *et al.* 1997). In wild type, *cdh-3::gfp* expression is never observed in Y; so a U to Y cell fate transformation should result in failure to express (or reduced expression) in the presumptive U cell, as observed. In contrast, the B cell expresses *cdh-3::gfp* at low levels in wild type; so a transformation of F to B cell fate might affect expression in the presumptive F cell to a lesser extent. It is also possible that the *sy294* mutation affects the development of U to a greater extent than F (*sy294* reduces *egl-38* function, but is not a null allele; CHAMBERLIN *et al.* 1997). *egl-38* mutants were the only tested mutants that exhibit a marked asymmetry in expression between F and U.

Mutations in *lin-49* and *lin-59* modestly affect *cdh-3::gfp* expression in cells expressing threshold levels of detectable GFP. Expression in U and F cells is affected at 20°, and expression in K and K′ cells is affected at 25°. This suggests that *lin-49* and *lin-59* may play either a role in establishing the fates of several hindgut cells, or a more general role in gene expression.

Hindgut mutants can have reduced viability: We observed that the hindgut morphological defects reduced the health of affected animals and in some cases appeared to be a cause of lethality due to blockage or damage to the hindgut and surrounding tissue. Previously, we have shown that *egl-38* is an essential gene, with strong mutations resulting in lethality at hatching (CHAMBERLIN *et al.* 1997). We tested whether the genes identified in our screen similarly affect the animals' viability. Both mutations in *lin-48* confer only a slight decrease in viability compared to wild-type controls (Table 6). Animals bearing either allele *in trans* to deficiencies that delete *lin-48* are also viable (Table 1). Thus, existing *lin-48* alleles affect viability to a modest extent. Similar modest effects on viability are seen in *mab-9* mutants (Table 6; CHISHOLM and HODGKIN 1989).

In contrast, we found that the alleles of *lin-49* and *lin-59* recovered in our screen cause sharply reduced viability and are temperature sensitive. In particular, *lin-49* mutants are inviable at 25° (Table 6 and data not shown). *lin-49* and *lin-59* alleles are also lethal *in trans* to deficiencies that delete the gene (Table 1). We identi-

TABLE 4

Production of ectopic spicule cuticle in *lin-48* mutants

Genotype	Cell(s) killed	Percentage with spicule cuticle	*N*
Wild type	B[a]	0	10
lin-48[b]	B	67	21
lin-48	F, B	57	14
lin-48	U, B	5	20

[a] B includes animals in which B or B.a was killed (see MATERIALS AND METHODS).

[b] Includes both *lin-48(sy234)* and *lin-48(sa469)* animals.

TABLE 5

Altered expression of *cdh-3::gfp* in some hindgut mutants

		Percentage of animals								
		U, F expression[a]			K, K′ expression[a]			Other cells		
Temp.	Genotype	2	1	0	2	1	0	B	repD	*N*
20°	Wild type[b]	72	15	13	11	8	81	8	13	109
	egl-5	85	9	6	23	24	52	3	18	103
	sem-4	79	12	9	12	13	75	3	6	107
	lin-49(sy238)	50	25	25	4	9	87	4	5	109
	lin-49(sa470)	62	23	15	10	10	79	3	7	106
	lin-59	79	13	8	17	19	64	2	13	107
	egl-38	47	43	10	6	0	94	16	25	108
	lin-48	70	19	11	5	14	81	6	13	109
25°	Wild type	89	7	4	65	19	15	42	32	113
	egl-5	87	7	6	61	21	17	14	21	103
	lin-49(sy238)	86	3	10	26	24	50	22	34	58
	lin-49(sa470)	86	9	5	10	29	58	18	36	112
	lin-59	86	7	8	39	25	37	14	22	106

[a] 2, 1, and 0 indicate expression in two of the cells, only one, or none, respectively.

[b] All genotypes include *pkEx246* and a *dpy-20* allele (*e1282* or *e1362*) for maintenance of the transgene. Alleles tested: *egl-5(sy279)*, *sem-4(sa416)*, *egl-38(sy294)*, *lin-48(sa469)*, *lin-59(sa489)*. *egl-5*, *lin-59*, and *lin-48* strains included *him-5(e1490)* in the background. In these strains, both male and hermaphrodite L1 and L2 larvae were scored for expression.

fied a nonconditional early larval lethal allele of *lin-49*, *s1198*, among lethal mutations linked to *unc-22* and left of *sDf2* recovered by CLARK (1990). We conclude that *lin-49* and, possibly, *lin-59* are essential genes, and the alleles of these genes isolated in our screen are non-null.

TABLE 6

Lethality associated with hindgut mutations

		Developmental stage[a] (percentage of animals)			
Temp.	Genotype	e/L1	L2/L3	L4/Ad	*N*
15°	Wild type[b]	6	1	93	1407
	lin-49	29	21	50	272
	lin-59	11	9	80	263
20°	Wild type	3	1	96	1334
	lin-49	35	35	30	601
	lin-59	13	23	65	559
	mab-9	6	12	82	955
	lin-48(sy234)	14	6	81	1094
	lin-48(sa469)	14	5	81	775
25°	Wild type	14	3	84	642
	lin-49	59	41	0	175
	lin-59	38	29	33	173

[a] Developmental stage at which animals arrest or the stage they had reached when counted. e/L1 indicates animals arrest as embryos or L1 larvae. L2/L3 indicates animals arrest as L2 or L3 larvae (also includes slow-growing animals). L4/Ad indicates animals had reached L4 or adulthood.

[b] All genotypes include *him-5(e1490)*. Alleles tested: *lin-49(sy238)*, *lin-59(sa489)*, *mab-9(sa473)*.

***egl-38(sy287)* is a temperature-sensitive allele:** The *Pax* gene *egl-38* is required for both the development of the hindgut and the development of the hermaphrodite egg-laying system. Previously we have shown that two viable mutations in *egl-38*, *n578* and *sy294*, preferentially disrupt different functions of the gene and represent distinct amino acid substitutions within the DNA-binding paired domain of EGL-38 (CHAMBERLIN *et al.* 1997). To investigate whether *egl-38(sy287)* showed similar defects we tested hermaphrodite egg laying, male and hermaphrodite hindgut morphology, and adult male tail morphology in mutants at different temperatures (Table 7). We find that *egl-38(sy287)* is temperature sensitive for these functions. However, in general, *egl-38(sy287)* preferentially affects male tail development compared to egg laying. For example, at 20° 100% of males have abnormal spicule morphology, whereas only 4% of hermaphrodites are egg-laying defective. We sequenced genomic DNA from *egl-38(sy287)* mutants and found that it has a missense mutation affecting the β-hairpin portion of the EGL-38 paired domain (Figure 7). *In vitro* studies have identified this domain as important for protein interactions between Pax and Ets transcription factors (WHEAT *et al.* 1999).

DISCUSSION

Several classes of genes affect hindgut development: In a genetic screen for mutations that affect male tail development, we identified mutations in seven genes that affect the hindgut in both males and hermaphro-

TABLE 7

***egl-38(sy287)* is a temperature-sensitive allele**

		Hermaphrodite			Male				
Temp.	Genotype	Egl	Scar	*N*	Scar	Con	Mab (sp)	Mab (hook)	*N*
	Wild type	0	0	Many	0	0	0	0	Many
15°	*sy287*[a]	0	0	17	0	0	50	0	10
20°	*sy287*	4	32	25	0	50	100	0	10
25°	*sy287*	69	31	26	100	100	100	100	11
20°	*sy287/s1775*[b]	56	ND	16	97	97	97	97	40
	sy287/n578	0	ND	10	ND	ND	20	ND	10

Numbers represent percentage of animals with indicated phenotypes. Phenotypes: Egl, hermaphrodite egg-laying defective; Scar, damage, blockage, or scarring of the hindgut; Con, constipated; Mab (sp), male spicules morphologically abnormal; Mab (hook), male hook morphologically abnormal; ND, not determined.

[a] Full genotype: *egl-38(sy287); him-5(e1490).*

[b] Full genotype: *unc-24(e138) egl-38(sy287)/unc-24(e138) egl-38(s1775) unc-22(s7) unc-31(e169); him-5(e1490)/+.*

[c] Full genotype: *egl-38(sy287) dpy-20(e1282)/egl-38(n578); him-5(e1490).*

dites. We have combined our analysis of these genes and that of other investigators to suggest that these seven genes represent four distinct classes. (1) *egl-5* encodes a homeodomain protein and is part of a *C. elegans* HOX gene cluster (Wang *et al.* 1993). Phenotypic analysis suggests *egl-5* is required for regional identity in the tail (Chisholm 1991). (2) *sem-4* encodes a zinc finger protein and is required for the normal development of several mesodermal and ectodermal cells throughout the animal (Basson and Horvitz 1996). *sem-4* may contribute to the tissue-type identity of hindgut cells. (3) Mutations in the two genes *lin-49* and *lin-59* affect the development of hindgut cells, but they are highly pleiotropic. We have observed that mutant animals are variably Unc, Sma, and Egl, and some cell lineages in both males and hermaphrodites can be disrupted (data not shown). Thus, although we initially identified these genes as affecting hindgut development, they may function in the development of many cells of *C. elegans.* (4) The three genes *mab-9*, *egl-38*, and *lin-48* function to make certain hindgut cells different from each other and thus contribute to the patterning of cell fates within the hindgut. In particular, the activity of these three genes reflects the three spatial axes involved in distinguishing the four posterior-most hindgut cells. *mab-9* is required to make the two dorsal cells (F and B) different from their ventral neighbors (U and Y; Chisholm and Hodgkin 1989). *egl-38* is required to make the two anterior cells (F and U) different from their posterior neighbors (B and Y; Chamberlin *et al.* 1997). *lin-48* plays a role in distinguishing the presumptive U cell from B (this work). Embryonically, U and B are lineal homologs, with each cell being born from a similar pattern of cell divisions, but one born from a precursor on the right of the animal, and one from a precursor on the left (Sulston *et al.* 1983; U is ABplppppapa, B is ABprpppapa). Thus *lin-48* functions to make left/right symmetrical cells different from each other.

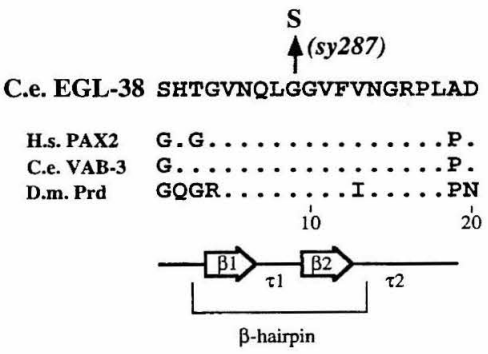

Figure 7.—Missense mutation in *egl-38(sy287).* The paired domain is composed of an amino-terminal β-hairpin followed by two helix-turn-helix motifs (Xu *et al.* 1995). The figure includes the first 20 amino acids of the paired domain from EGL-38 and other Pax proteins (from Chamberlin *et al.* 1997) and indicates the β-hairpin domain. *egl-38(sy287)* is a temperature-sensitive missense mutation of Ggc to Agc. This results in a serine substitution at a normally invariant glycine within the β-hairpin.

Developmental consequences of *lin-48* mutations on male tail development: We have observed cell lineage defects in the F, U, and B cells in *lin-48* mutant males. The F and U cells produce more progeny than in wild type and develop in a manner similar to each other. However, the consequences for each are not identical, as the presumptive U cell can produce differentiated spicule socket cells whereas the presumptive F cell cannot. Since spicule socket cells normally derive only from the B cell, one function of *lin-48* is to prevent the presumptive U cell from developing like B. Although the presumptive U cell can produce differentiated B progeny, it did not develop with a cell lineage similar to that

of B in the two animals we followed. In addition, the presumptive U cell in *lin-48* mutants does not produce all types of B cell progeny (JIANG and STERNBERG 1999). Thus, we find that certain differentiated cell types can be produced in the absence of a complete transformation of the developmental potential of the precursor cell.

In *lin-48* mutants development of the B cell in males is affected in a manner consistent with overproduction of an anterior-promoting signal. In wild type, the F and U cells (or their daughters) are required for this signal, and B progeny act antagonistically to it (CHAMBERLIN and STERNBERG 1993). Although we have not directly tested whether the B cell lineage effect results from the F and U defects in *lin-48* mutants, our observations are not consistent with a reduction of F and U signaling in *lin-48* mutants, since removing these cells causes B cell lineage defects opposite to those we observed. Thus the B cell lineage defect is consistent with F and U retaining some of their wild-type developmental features in spite of abnormal cell lineage and abnormal differentiated progeny. Alternatively, the *lin-48* mutant effect on B cell development may reflect a function independent of its effect on the development of F and U.

***cdh-3::gfp* expression and hindgut cell fates:** We have extended the observations of PETTITT *et al.* (1996) and found that *cdh-3::gfp* is expressed in many hindgut cells, with highest levels in the F and U cells, and that the expression of the transgene or stability of the product is sensitive to temperature. *cdh-3::gfp* expression is reduced in the presumptive U cell in *egl-38* mutants, consistent with cell lineage analysis that suggests *egl-38* is required to make U different from its posterior neighbor Y (CHAMBERLIN *et al.* 1997). *cdh-3::gfp* expression is also moderately affected in *lin-49* and *lin-59* mutants. In contrast, expression is not affected in *egl-5* or *sem-4* mutants, even though mutations in these genes affect the morphology and the development of hindgut cells. This suggests that these two genes function independently (or downstream) of the processes that regulate *cdh-3::gfp* expression in the hindgut. These genes may affect certain aspects of hindgut development, but not others. Alternatively, *cdh-3::gfp* expression may coincide with hindgut cell fate, but not actually reflect specific cell fate choices.

Hindgut development and viability: Mutations in genes affecting hindgut development can significantly affect viability (CHISHOLM and HODGKIN 1989; BASSON and HORVITZ 1996; CHAMBERLIN *et al.* 1997; this work). In this work we have recovered non-null alleles of essential genes, and other essential genes that affect hindgut development have been identified by other researchers (*lag-1* and *lag-2*, LAMBIE and KIMBLE 1991; BOWERMAN *et al.* 1992; *pha-4*, MANGO *et al.* 1994; HORNER *et al.* 1998; KALB *et al.* 1998). Although multiple developmental defects may contribute to the inviability of the mutant animals, our observations suggest that common defects associated with abnormal development of the hindgut, including blocking of the intestine and compromising the structural integrity of the tail, are sufficient to cause lethality. In our genetic screen we have identified viable mutations in seven genes that play a role in hindgut development, representing potential regional, local, and tissue-type factors. These genes provide the genetic framework for beginning the study of different factors that contribute to hindgut development. However, further screens to directly identify essential genes that contribute to hindgut development will be required to understand the full repertoire of factors involved in coordinating the development of the 11 distinct hindgut cells into a functional organ.

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