

# Organogenesis in *C. elegans*: Positioning of Neurons and Muscles in the Egg-Laying System

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## Summary

One of the final stages in the development of egg-laying behavior in the nematode *C. elegans* is the organization of 8 motor neurons (2 HSN and 6 VC cells) and 8 muscles into a motor system to control the opening of the vulva. Using mutations that disrupt the development of specific components of the egg-laying system and laser microsurgery to ablate selected precursor cells, we have determined that the guidance of the egg-laying neurons and muscles, and in particular the VC neurons and vulval muscles, into the vulval region is dependent on interactions with surrounding epithelial and gonadal tissue and appears to be independent of neuron-neuron and neuron-muscle interactions. The development of the egg-laying system can be described as a series of cell interactions in which certain cells arise through induction and subsequently provide inductive cues themselves.

## Introduction

Organogenesis, the development of functional organs, requires the organization of diverse cell types into particular patterns. Such pattern formation involves series of coordinated cell interactions in which one group of cells may affect or direct the patterning of adjacent cells. Such interactions have been described in vertebrates, for example, in the formation of the eye (see Gilbert, 1988), but these interactions have not been examined at the cellular level.

The egg-laying system of the nematode *Caenorhabditis elegans* presents a simple and accessible system in which such interactions can be resolved at the level of individual cells. The system (Figure 1) consists of the gonad and vulva, which join to form a channel through which fertilized eggs pass out of the animal (Sulston and Horvitz, 1977; Kimble and Hirsh, 1979), 8 vulval muscles that control the opening of the vulva (White et al., 1976; Sulston and Horvitz, 1977), 8 uterine muscles that presumably function to propel eggs out of the uterus (the uterine muscles do not appear to receive any direct innervation, but have gap junctions with each other and with the vulval muscles [White et al., 1976]), and 8 neurons, 2 HSN cells and 6 VC motor neurons, that provide most of the direct innervation to the vulval muscles (see White et al., 1986; Desai et al., 1988).

Several cell interactions have been identified in the formation of the vulva. Interactions between cells within the somatic gonad determine which cell becomes the anchor cell (Kimble, 1981). The anchor cell, in turn, induces vulval formation (Kimble, 1981) and regulates the pattern of the vulval lineages from the 6 potential vulval hypodermal precursor cells (Sternberg and Horvitz, 1986). Cell interactions also take place among the vulval precursor cells to determine which vulval lineages are expressed among the 6 (Sulston and White, 1980; Sternberg, 1988).

In this paper, we investigate the formation of the motor unit. Using mutations that affect the development of components of the egg-laying system (Horvitz and Sulston, 1980; Sulston and Horvitz, 1981; Greenwald et al., 1983; Trent et al., 1983; Ferguson and Horvitz, 1985; Ferguson et al., 1987) and laser ablation of selected precursor cells, we have identified cell interactions that are necessary for the neurons, particularly the VC cells, to find their targets, the vulval muscles, and for the vulval muscles to assume their final positions. Neither neuron-neuron nor neuron-muscle interactions appear necessary in the guidance of the neurons and muscles into the vulval region; instead, signals from the vulva and gonad are critical to the formation of the motor system. The development of the egg-laying system thus involves a cascade of cell interactions in which certain elements arising through induction subsequently provide additional inductive cues.

## Results

### The VC Motor Neurons

Using indirect immunofluorescence with an antibody against the neuropeptide FMRFamide, we detect 6 cells in the ventral nerve cord that we identify as the VC motor neurons (Figure 1A; Figure 2A). The positions of these cells, 4 anterior and 2 posterior to the vulva, correspond to those of the 6 VC cells (White et al., 1976). In addition, in animals carrying the strong *lin-39* allele *n1760*, all 6 VC cells undergo cell death (S. Clark, H. Ellis, and H. R. Horvitz, personal communication), and no immunoreactive cells are present in the ventral cord of these animals. A variable number of VC cells die in the weaker *lin-39* allele *n709* (Horvitz et al., 1982), and similarly, we see variable numbers of immunoreactive cells in the ventral cord (data not shown).

Neuronal processes from the 6 VC cell bodies project along the ventral cord; at the vulva these processes branch from the ventral cord to innervate the vulval muscles (White et al., 1986). In this paper, we use the terms "processes" for the VC neurites in the ventral cord and "branches" for any VC neurites that extend from the ventral cord. Since the ventral cord also contains immunoreactive processes from other

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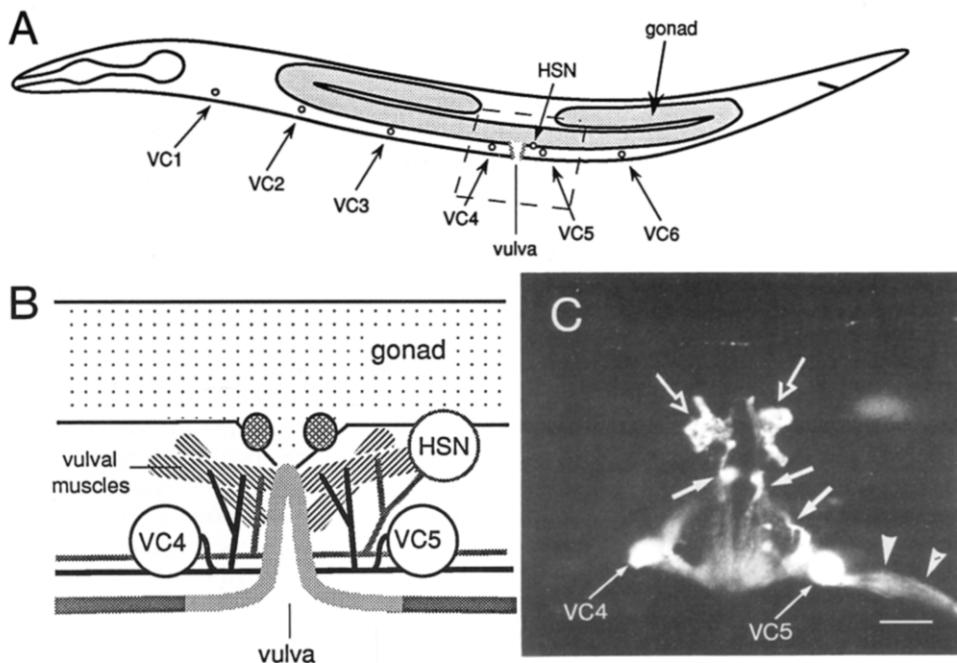


Figure 1. The Motor Neurons of the Egg-Laying System in *C. elegans*

(A) Schematic diagram of *C. elegans* hermaphrodite showing the positions of the gonad, vulva, and cell bodies of the 6 VC cells and 1 of the 2 HSN cells (the other is in a similar position on the other side of the animal). Anterior is to the left, and a lateral view of the animal is shown. The vulval region enclosed by dotted lines is enlarged in (B). (B) Schematic diagram of the vulval region. The cross-hatched cells indicate the putative gonadal cells that stain with the anti-FMRFamide antiserum. The uterine muscles, which we do not detect with the anti-myosin antibody, are not shown. (C) The morphology of the VC cells in the vulval region visualized immunocytochemically with an anti-FMRFamide antiserum tagged with a rhodamine-conjugated secondary antibody. The VC processes, VC branches, and cell bodies of the putative gonadal cells are indicated by arrowheads, unlabeled thick arrows, and open arrows, respectively. The cell bodies of only 2 VC neurons, VC4 and VC5, are seen in this field. All VC cell bodies are approximately the same size (White et al., 1986), but their fluorescence image here and in subsequent figures can be distorted because of differences in their plane of focus. Bar, 10  $\mu$ m.

neurons, such as the DVB and perhaps the PVT neurons (C. Li, unpublished data), the processes of the VC cells are often difficult to identify unambiguously, except near the cell body, where the processes can be seen emerging. The VC branches, however, appear as a thick plexus that can be easily seen immunocytochemically (Figure 1C). When the precursors of the VC neurons (Pn.a) are ablated, this plexus is no longer detectable ( $n = 7$ ; data not shown). Wispy branches, which are morphologically distinguishable from branches of the plexus and presumably arise from the putative gonadal cells (see below), are also present in the vulval region. The HSN cells also appear to stain with the antibody (G. Garriga, personal communication), but their vulval processes are rarely detectable with this antibody in our preparations.

VC neurons arise during the first larval stage (Sulston and Horvitz, 1977) and begin to extend processes probably late in the third larval stage (J. White, personal communication). The VC staining observed in animals from different larval stages correlates roughly with this temporal developmental pattern. During the fourth larval stage, the branches of the VC cells become visible. The VC cell bodies first stain in young adults. Immunoreactive processes from other neu-

rons are visible in the ventral cord as early as the first larval stage; no branches are observed, however, until the fourth larval stage, and these presumably belong to the VC cells.

#### The Branching of the VC Neurons Requires External Cues

The VC neurons normally branch into the vulval region between VC4 and VC5 (Figure 1; Figure 2A). To determine whether this branching is cell-intrinsic or dependent on external cues, we examined VC branching in *dig-1* mutants. The gonad, vulva, and vulval muscles are displaced anteriorly to a variable extent in these animals, but the positions of the VC and HSN cell bodies do not appear affected (J. Thomas and H. R. Horvitz, personal communication; C. Li, unpublished data). In *dig-1* animals VC branching is displaced anteriorly to the ectopic vulval region (the position of the vulva was determined by examining the animal under Nomarski optics); no branching is detected between VC4 and VC5 (Figure 2B). The presence of branches at the ectopic vulval position suggests that VC branching from the ventral cord is dependent on cues from cells in the vulval region.

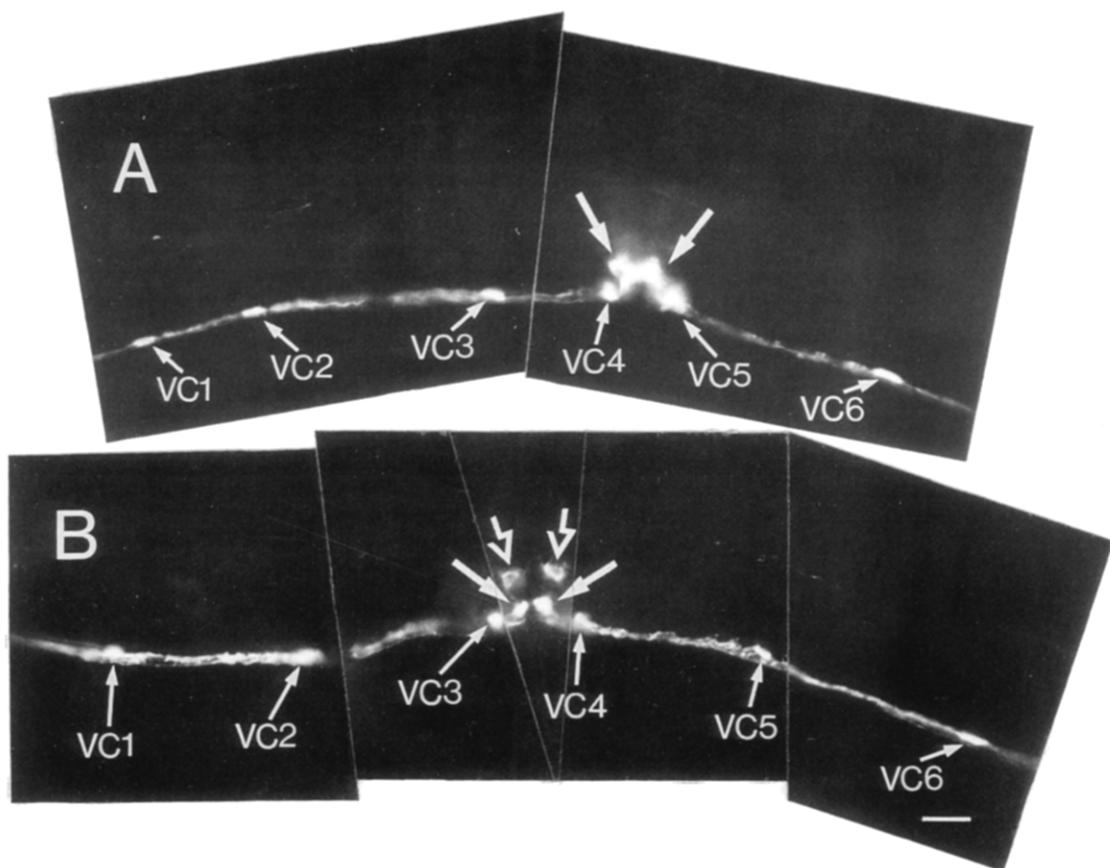


Figure 2. Displacement of the VC Branching to an Ectopic Vulval Position

Animals were stained with the anti-FMRFamide antiserum tagged with a rhodamine-conjugated secondary antibody. (A) In wild-type animals, the vulva lies between VC4 and VC5. The VC branches (unlabeled thick arrows) are visible at this position. The putative gonadal cells are out of the plane of focus. (B) In *dig-1* animals, the gonad, vulva, and vulval muscles are displaced anteriorly to a variable extent (J. Thomas and H. R. Horvitz, personal communication), while the positions of the VC and HSN cell bodies are unaffected. In this animal the vulva lies between VC3 and VC4, as determined by viewing the animal with Nomarski optics (not shown). The VC branching (thick arrows) and putative gonadal cells (open arrows) are similarly displaced anteriorly to the ectopic vulval position. Bar, 10  $\mu$ m.

#### Neither the Target Muscles nor Nearby Neurons Appear to Provide Guidance Cues to the VC Cells

Two possible mechanisms for how neurons find their appropriate partners are that the target cells (Bonhoeffer and Huf, 1980; Lumsden and Davies, 1986) or nearby neurons (Bate, 1976; Ho and Goodman, 1982; Bentley and Caudy, 1983; Walther and Chalfie, 1988) provide cues for guidance. The contribution of the target muscles to VC branching was assessed by examining animals in which the vulval muscles develop posterior to the vulva (*egl-15* [Trent et al., 1983]) or in which the M cell, the precursor cell of the vulval muscles (Sulston and Horvitz, 1977), is ablated, resulting in the absence of vulval muscles. Neither the mispositioning nor the absence of the vulval muscles has any detectable effect on VC branching (Table 1).

In addition to the VC cells, two other neuronal cell types branch into the vulval region. Only one of these cell types, the HSN neurons, is known to synapse onto the vulval muscles (White et al., 1986). In *egl-1*

mutants, which lack HSN neurons (Trent et al., 1983), the VC cells appear to branch normally (Table 1). Moreover, the VC cells branch normally when the M cell is ablated in *egl-1* mutants (Figure 3; Table 1), eliminating the possibility of redundant cues from the muscles and HSN neurons.

Although additional neuronal branches have been seen in the region of the vulva in electron micrographs, these branches do not make any apparent synapses in the region (White et al., 1986). White et al. (1986) could not determine whether the branches originate from the pair of PVN neurons or the unpaired PVT cell. In *lin-17(n671)* and *rh41* mutants, which lack the PVN neurons (Sternberg and Horvitz, 1988; E. Hedgecock, personal communication), the branching pattern of the VC cells appears unaffected (Table 1). Furthermore, in *lin-17; egl-1* double mutants, which lack both PVN and HSN cells, the VC cells also appear to branch normally (Table 1). Thus, neither the vulval muscles nor the HSN or PVN neurons appear to provide cues to the VC cells to branch.

Table 1. Absence of Target Muscles or Nearby Neurons Has No Detectable Effect on VC Branching

Mutant	Ablation	n	Staining Phenotype			
			HSNs	PVNs	Vulval Muscles	Branching
Wild-type		>75	+	+	+	+
<i>egl-15</i>		81	+	+	Misplaced <sup>a</sup>	+
Wild-type	M	5	+	+	-	+
<i>egl-1</i>		40	-	+	+	+
<i>lin-17(n671)</i> <sup>b</sup>		34	+	-	+	+
<i>lin-17(n671); egl-1</i>		47	-	-	+	+
<i>egl-1</i>	M	8	-	+	-	+

For this and all subsequent tables: the presence (+) or absence (-) of specific cell types as a result of mutations or laser ablations is indicated in the appropriate column; animals were scored for the presence (+) or absence (-) of VC branching under Staining Phenotype.

<sup>a</sup> The position of the vulval muscles in these mutants generally lies between VC5 and VC6, in contrast to their wild-type position of between VC4 and VC5.

<sup>b</sup> *lin-17(rh41)* mutants show a phenotype similar to that of *lin-17(n671)*, but animals were not tabulated.

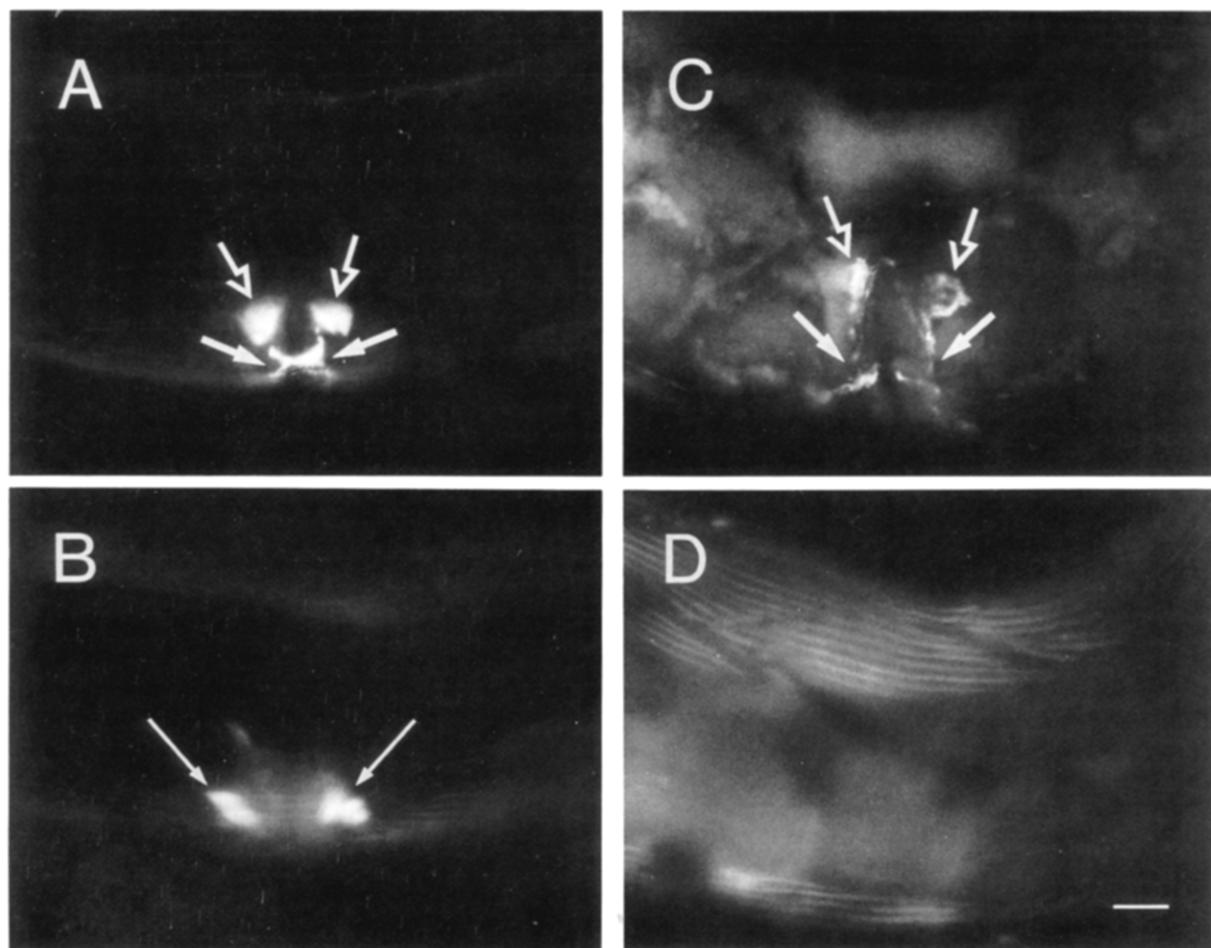


Figure 3. Absence of Target Muscles and HSN Neurons Has No Detectable Effect on VC Branching

Animals were double-labeled with anti-FMRFamide (A and C) and anti-myosin (B and D) antisera to show the VC branching and vulval muscles, respectively.

(A and B) A wild-type animal showing the VC branches (arrows in [A]) and vulval muscles (arrows in [B]), respectively. (C and D) An *egl-1* mutant, which lacks HSN cells (Trent et al., 1983), in which the M cell was ablated. The VC branching pattern (filled arrows in [C]) is normal despite the absence of nearby HSN neurons and target vulval muscles (only body wall muscle is visible in [D]). The putative gonadal cells are indicated by open arrows in (A) and (C). Bar, 10  $\mu$ m.

We have not excluded a possible role of the PVT neurons in VC guidance; however, our results suggest that this possibility is not likely. The PVT cell appears to be FMRFamide-like immunoreactive. In the absence of the VC neurons (through laser ablations of the VC precursor cells), however, only a few thin branches, which appear to arise from the putative gonadal cells (see below), are seen. Thus, the PVT neuron probably does not branch in the vulval region.

#### Vulval Cells Appear Capable of Inducing VC Branching

The 18 cells of the vulva (J. White and H. R. Horvitz, personal communication) arise from 3 of 6 potential vulval precursor cells (P(3-8).p) that undergo either of two lineages, termed primary ( $1^{\circ}$ ) and secondary ( $2^{\circ}$ ) (Sulston and Horvitz, 1977; Sulston and White, 1980). In wild-type animals one cell, P6.p, undergoes a  $1^{\circ}$  lineage and gives rise to the vulval cells nearest the gonad, and 2 cells, P5.p and P7.p, undergo  $2^{\circ}$  lineages to produce the vulval cells nearest the hypodermis (Sulston and Horvitz, 1977). Mutants that do not express these lineages have no vulval cells and are therefore considered Vulvaless (Horvitz and Sulston, 1980; Sulston and Horvitz, 1981; Ferguson and Horvitz, 1985; Ferguson et al., 1987). In most Multivulva mutants all 6 potential vulval precursor cells express either a  $1^{\circ}$  or  $2^{\circ}$  lineage (Sulston and Horvitz, 1981; Greenwald et al., 1983; Ferguson and Horvitz, 1985; Ferguson et al., 1987). The ectopic clusters of vulval cells produced by these lineages form one or more protrusions, called pseudovulvae, along the ventral surface of the animal.

To determine whether the vulva was involved in guiding the neurons into the vulval region, we examined Vulvaless and Multivulva mutants. Despite the presence of VC cell bodies and processes and the vulval muscles (see below) in the Vulvaless mutants *lin-12(n302* and *n676*) and *n300*, the VC cells rarely (<10% of the animals) branch into the vulval region (Figures 4A and 4B; Table 2). These data suggest that the presence of vulval cells is necessary for either directing or stabilizing VC branches in the vulval region. Although in the Vulvaless mutants the VC processes could have initially branched into the presumptive vulval region and later retracted, we have not observed such branching in larval animals, suggesting that the role of the vulval cells is probably not for the stabilization of branches. The vulval cells could direct the VC branches from the ventral cord into the vulval region by inducing branches or by guiding previously formed branches. The former possibility, induction of neuronal branching by the vulval cells, is supported by experiments with Multivulva mutants.

The Multivulva mutants *lin-1(e1777* and *e1275*), *lin-8*; *lin-9*, *lin-8*; *lin-37*, *lin-12(n137*, *n950*, and *n952*), and *lin-15(n309)* generally have a normal vulva (except for *lin-12*) and multiple pseudovulvae (Sulston and Horvitz, 1981; Greenwald et al., 1983; Ferguson and Horvitz, 1985; Ferguson et al., 1987). In *lin-12* mutants the vul-

val cells between VC4 and VC5 form a protruding structure, which we refer to as a protruding vulva. In the Multivulva mutants examined, the VC cells not only branch at the true vulva, but also branch ectopically to project into multiple pseudovulvae (Figures 4C and 4D; Table 2); in many instances, these ectopic branches can be seen emerging from the VC cell body. Thus, vulval cells, even in ectopic positions, appear capable of inducing VC branching.

Vulval cells derived from either  $1^{\circ}$  or  $2^{\circ}$  lineages are capable of inducing branching. Animals whose pseudovulvae are composed of vulval cells derived from  $1^{\circ}$  and  $2^{\circ}$  lineages (*lin-1*, *lin-8*; *lin-9*, *lin-8*; *lin-37*, and *lin-15(n309)*), however, had more pseudovulvae with branches (>75%) than animals whose pseudovulvae are composed of vulval cells derived from only  $2^{\circ}$  lineages (about 35%; *lin-12(n137*, *n950*, and *n952*) and *lin-17(n671)*). Thus, vulval cells derived from  $1^{\circ}$  lineages appear capable of inducing more VC branching than vulval cells from  $2^{\circ}$  lineages.

#### Gonadal Cells Are Not Necessary for VC Branching

The gonad was also examined for its role in VC branching. The gonadal primordium of newly hatched animals consists of 4 cells: 2 (Z1 and Z4) that give rise to the somatic gonad and 2 (Z2 and Z3) that give rise to the germline (Kimble and Hirsh, 1979). Because the gonad normally induces the vulva (Sulston and White, 1980; Kimble, 1981), the effect of gonadal ablations was tested in *lin-15(n309)* Multivulva mutants, which can express vulval lineages independent of the gonad (Ferguson et al., 1987; Sternberg, 1988; Sternberg and Horvitz, 1989); such ablated animals have a protruding vulva. Ablation of the gonadal primordium in newly hatched animals ( $n = 7$ ) results in animals in which VC branches are visible at the vulva and in multiple pseudovulvae (data not shown). Thus, induction of VC branching can occur independent of the gonad.

#### Vulval Cells May Also Induce HSN Branching

Because both the VC and HSN neurons synapse onto the vulval muscles, the two cell types may use some of the same cues for branching into the vulval region. The HSN process normally extends to the ventral nerve cord, where it runs anteriorly to the head region; a few short branches leave the main process in the vulval region to innervate the vulval muscles (White et al., 1986). In an experiment analogous to that with the VC cells, HSN cells were stained with an anti-serotonin antibody (Desai et al., 1988), which can visualize the HSN processes and branches. In *lin-15(n765)(20°)* ( $n = 7$ ) and *lin-15(n309)* ( $n = 12$ ) Multivulva mutants, we observed ectopic branching into the pseudovulvae. This ectopic branching has also been noted by G. Garriga and H. R. Horvitz (personal communication); in addition, these researchers have found that in the absence of vulval cells, such as in the Vulvaless mutants, HSN cells do not branch appropriately in the vulval region. Therefore, the HSN neurons may, like the VC cells, receive cues from the vulval

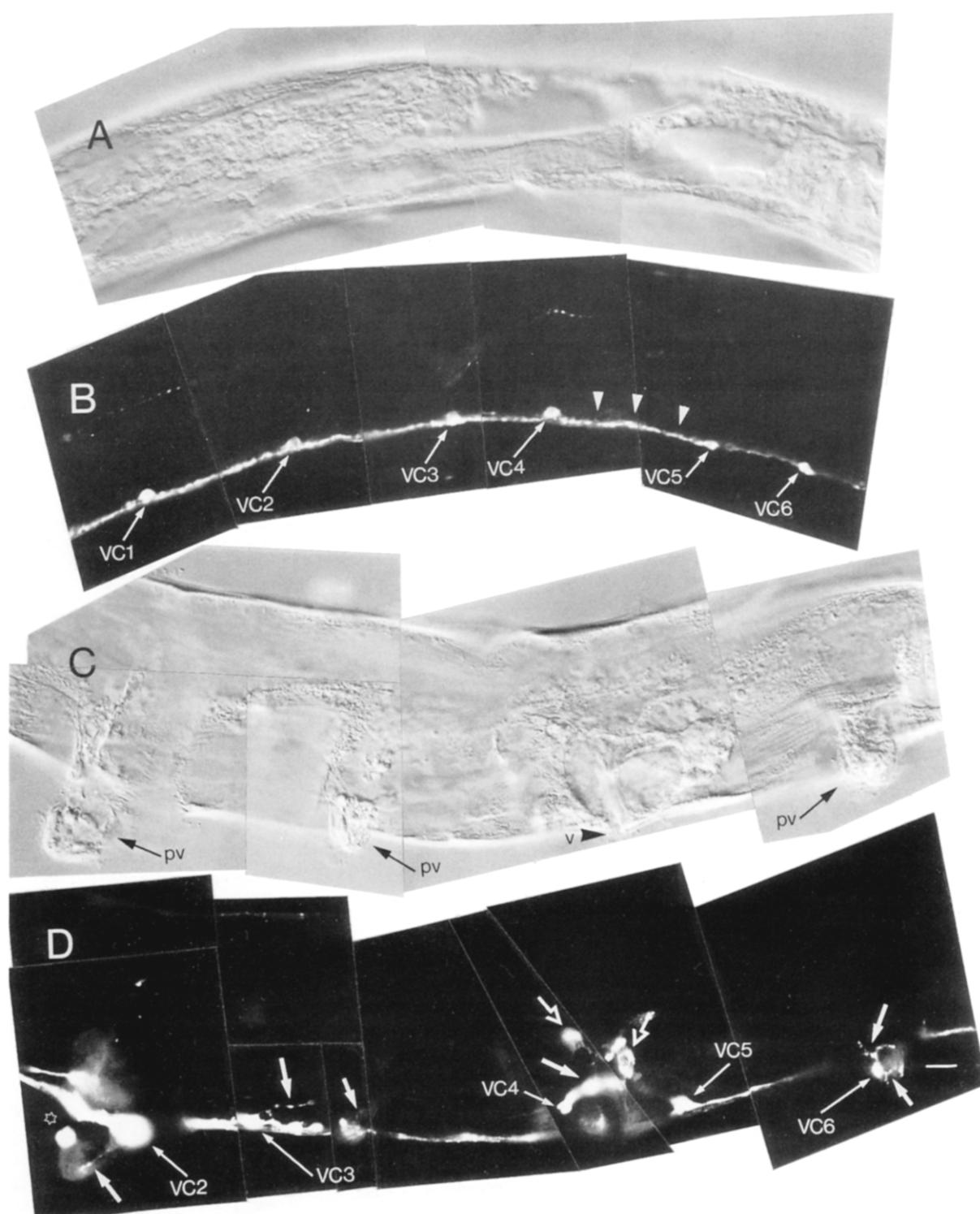


Figure 4. Vulval Cells Appear Capable of Inducing VC Branching

Animals are shown with Nomarski (A and C) and fluorescence (B and D) optics and are stained with the anti-FMRFamide antiserum tagged with a rhodamine-conjugated secondary antibody. (A and B) The *n300* mutant has no vulval cells and is Vulvaless ([A]; Ferguson, 1985). No branching is detected between VC4 and VC5 (arrowheads in [B]), the normal position of the vulva, although the VC cell bodies and processes stain normally. (C and D) In *lin-15* Multivulva animals, ectopic clusters of vulval cells, which form pseudovulvae along the ventral surface of the animal (pv in [C]; Horvitz and Sulston, 1980), induce VC branching (thick arrows in [D]) from the ventral nerve cord into the pseudovulvae. The putative gonadal cells (open arrows) are indicated in (D). Ectopic branches can be seen emerging from the cell bodies of VC2, VC3, and VC6 and projecting toward or into nearby pseudovulvae in (D). VC2 and a varicosity in a pseudovulva (open star in [D]) are out of the plane of focus. The position of the true vulva (v) is indicated in (C). Bar, 10  $\mu$ m.

Table 2. Vulval Cells Are Required for VC Branching

Mutant	n	Vulval Cells		Staining Phenotype	
		1°	2°	Vulva Branching	Pseudovulvae Branching
Wild-type	>75	+	+	+	
Vulvaless mutants <sup>a</sup>					
<i>lin-12(n302 and n676), lin-39(n1760), n300</i>	26–179	–	–	–	
Multivulva mutants <sup>b</sup>					
<i>lin-8; lin-9, lin-8; lin-37, lin-15<sup>c</sup>, lin-12(n137, n950, and n952), lin-17(n671)</i>	26–66 47–60 34	++ – +	++ ++ +/abn. <sup>e</sup>	+ + <sup>d</sup> +	+ +(35%) +(33%)

<sup>a</sup> Vulvaless mutants were scored for the presence or absence of branches from any position along the ventral cord. The Vulvaless mutations *lin-2*, *lin-3*, *lin-4*, *lin-7*, *lin-10*, and *unc-84(25°)* have a lower penetrance than the Vulvaless mutations listed. In these animals, branching was detected in about 25%–30% of the animals. When such staining was seen, a vulva or partial vulva was usually present.

<sup>b</sup> Multivulva mutants with extra vulval cells are denoted as (++) . Multivulva mutants were scored for the presence (+) of VC branches at either the true vulva or the pseudovulvae. Except where noted parenthetically, percentage of occurrence is greater than 70% under Staining Phenotype.

<sup>c</sup> The Multivulva phenotype in two strains, *lin-8; lin-9* and *lin-8; lin-37*, results from interactions between two mutations (Ferguson, 1985; Ferguson and Horvitz, 1989). *lin-1(e1275 and e1777)* Multivulva mutants show a phenotype similar to that of these Multivulva mutants, but animals were not tabulated.

<sup>d</sup> Incidence of branching at the vulva and pseudovulvae was not calculated separately; the combined incidence is indicated parenthetically under Pseudovulvae.

<sup>e</sup> In *lin-17* mutants, P5.p and P7.p may undergo an abnormal 2° lineages, resulting in pseudovulvae generally posterior to the vulva.

cells to branch, or alternatively, the HSN branches may be guided into the vulval region by the VC neurons.

#### The Positioning of the Vulval Muscles Also Requires Cues from the Vulval and Gonadal Cells

We examined whether the targets of the VC cells, the vulval muscles, also use cues from tissues in the vulval region for their positioning around the vulva. The vulval muscles arise from 2 descendants of the M cell, the sex myoblasts, which migrate anteriorly during the second larval stage. This migration can be separated into at least two components (J. Thomas and H. R. Horvitz, personal communication): an initial anterior migration that places the myoblasts within a small interval (30–60 µm) of their correct position (this migration appears to be independent of the gonad and vulva) and a subsequent migration that places the myoblasts into their correct position (this migration appears to be regulated by the somatic gonad). During the third larval stage, the sex myoblasts divide to give rise to four pairs of vulval muscles and four pairs of uterine muscles (Sulston and Horvitz, 1977). These muscles assume their final positions, attaching to the vulval and uterine cells and to the body wall (White et al., 1986), during the fourth larval stage (Sulston and Horvitz, 1977). We have focused on identifying the cues used in the positioning of the muscles. The position of the vulval muscles between VC4 and VC5 and the characteristic V-shaped orientation of the vulval muscles around the vulva in a lateral view of the animal (see Figure 5A) can be visualized immunocytochemically with an anti-myosin antibody (see Experi-

mental Procedures). We have not been able to detect the uterine muscles in wild-type animals with the antibody.

Because the initial migration of the sex myoblasts appears to be independent of the gonad and vulva, we examined animals that lack these structures to determine the effect of their absence on the position of the vulval muscles. Wild-type animals in which the precursors of the somatic gonad are ablated lack both the gonad and the vulva (Sulston and White, 1980). In such ablated animals, one or more bundles of muscle fibers are detected randomly distributed along the length of the animal as far anterior as VC2 and as far posterior as halfway between VC6 and the anus (Table 3; Figure 5B). These muscle bundles resemble vulval muscles and will be referred to as vulval-like muscles, although they may also contain some uterine muscle cells and, more unlikely, some body wall muscle (body wall muscle, however, is normally striated and does not have the same appearance as these vulval-like muscles). The number of vulval-like muscles does not appear to be greater than the normal complement of vulval and uterine muscles in wild-type animals. Vulval-like muscles were also observed dorsally. In many animals the muscle fibers are oriented randomly and do not align in roughly parallel bundles, as in wild-type unablated animals. The absence of the gonad and vulva therefore appears to result in errors in both the position and the orientation of the vulval muscles.

To determine whether the vulva is necessary for the positioning of the vulval muscles, we examined animals in which the gonad is presumably normal, but

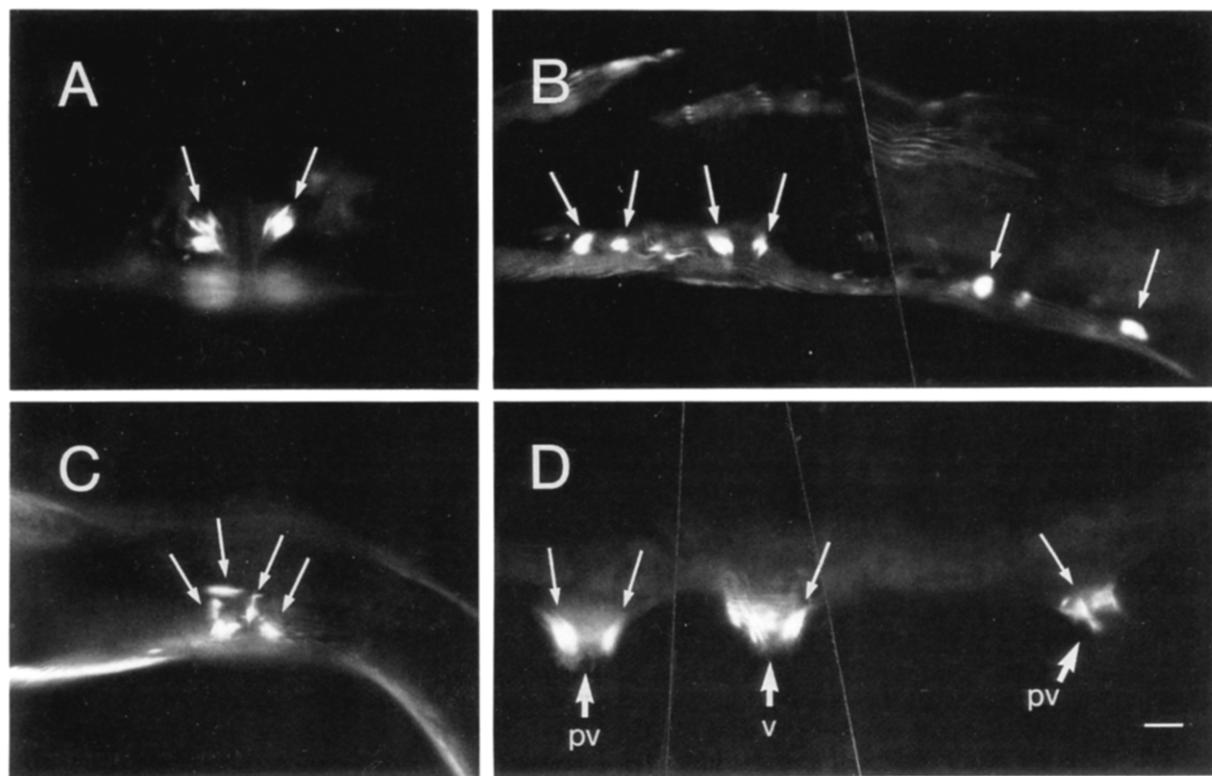


Figure 5. The Position and Orientation of the Vulval Muscles Appear Dependent on the Vulva and Gonad

The muscles are stained with an anti-myosin antibody, as in Figure 3.

(A) The vulval muscles (arrows) form a characteristic V-shaped pattern in wild-type animals. Only some of the vulval muscles are visible in this plane of focus. Generally, body wall muscle, which is striated, is not in the same plane of focus as the vulval muscles.

(B) When the somatic gonad precursors Z1 and Z4 are ablated in wild-type animals, resulting in the absence of the gonad and vulva (Sulston and White, 1980), vulval-like muscles (arrows), sometimes randomly oriented, are observed scattered throughout the length of the animal. Some body wall muscle is also visible in this field.

(C) In the absence of vulval cells, as in the *n300* Vulvaless mutant (Ferguson, 1985) shown here, vulval-like muscles are observed essentially in their correct position between VC4 and VC5, but the muscles are randomly oriented (arrows).

(D) When the somatic gonad precursors Z1 and Z4 are ablated in the *lin-15* Multivulva mutant, vulval-like muscles (arrows) in their correct orientation are observed associated with the protruding vulva (v) and with multiple pseudovulvae (pv). Bar, 10  $\mu$ m.

the vulva is absent (Vulvaless) or extra vulval cells are present (Multivulva). In wild-type animals in which the vulval cell precursors P(3-8).p are ablated, producing experimentally a Vulvaless animal, vulval-like muscles appear in their correct position between VC4 and VC5, but are randomly oriented (Table 3). The number of vulval-like muscles in the ablated or mutant animals does not appear to be greater than the normal complement of vulval and uterine muscles in wild-type animals. A similar result is seen in *lin-12(n302* and *n676*), *lin-10*, *lin-2*, *lin-26*, and *n300* Vulvaless mutants (Table 3; Figure 5C). The muscles, therefore, can be correctly positioned when the gonad is present and the vulva is absent. The loss of vulval muscle orientation may result from the lack of vulval attachment sites or from the loss of vulval cues.

The presence of extra vulval cells, and hence extra attachment sites for vulval muscles, in the Multivulva mutant *lin-15(n309)* has little or no effect on muscle position when the gonad is present (Table 3). The vulval muscles usually appear only at the true vulva, al-

though in a few instances, vulval-like muscles are detected at the pseudovulvae.

Ablation of the somatic gonad precursors in *lin-15(n309)* Multivulva animals, however, reveals that the vulval cells may also play a significant role in the positioning of the vulval muscles (Table 3). In such ablated animals, vulval-like muscles in their correct orientation are visible at the protruding vulva and at multiple (two to three) pseudovulvae (Figure 5D). No vulval-like muscles are detected except at the vulva or pseudovulvae. The number of vulval-like muscles does not appear to be greater than the normal complement of vulval muscles in wild-type animals. *lin-15* animals in which the germline precursors were ablated had correctly positioned vulval muscles and no ectopic vulval-like muscles.

To determine whether ectopic VC branches in the pseudovulvae of *lin-15* Multivulva gonad-minus animals were responsible for or contributed to the positioning of the vulval-like muscles, we examined pseudovulvae for the presence of both ectopic VC branches

Table 3. Vulval Muscle Position Is Dependent on Vulval and Gonadal Cues

Mutant	Ablation	n	Vulval Muscles	Vulval Cells	Gonad	Staining Phenotype		
						Muscle Position		Muscle Orientation
						Vulva	Ectopic	
Wild-type		>75	+	+	+	+	+	+
Wild-type	Z1, Z4	9	+	-	-	+/-	++	+/-
Vulvaless animals								
<i>lin-12(n302 and n676), lin-39(n1760), n309<sup>a</sup></i>		37–52	+	-	+	+	-	-
Wild-type	P(3-8).p	12	+	-	+	+	-	-
Multivulva mutants <sup>b</sup>								
<i>lin-15</i>		66	+	++	+	+	-	+
<i>lin-15</i>	Z1, Z4	14	+	++	-	+	++	+
<i>lin-15<sup>c</sup></i>	Z1, Z4, M	2	-	++	-	-	-	
<i>egl-1; lin-15</i>	Z1, Z4	6	+	++	-	+	++	+

Animals were scored for the position and orientation of vulval-like muscles under Staining Phenotype. The wild-type position of the muscles is at the vulva. Under Staining Phenotype: (+/-) indicates that vulval-like muscles were sometimes seen at that position, but not always; (++) indicates that more than one set of muscles were observed.

<sup>a</sup> *lin-2*, *lin-10*, and *lin-26* mutants display a phenotype similar to that of the mutants listed, but only 10–19 Vulvaless animals were examined for these mutations.

<sup>b</sup> The presence of extra vulval cells in Multivulva animals is indicated as (++).

<sup>c</sup> The vulval-like muscles seen in the *lin-15* animals where Z1 and Z4 have been ablated are likely to be equivalent to the vulval muscles; in addition to ablation of Z1 and Z4, ablation of a precursor to the vulval muscles, the M cell, in *lin-15* animals results in the absence of any vulval-like muscles.

and vulval-like muscles. There is no apparent correlation between the position of the ectopic vulval-like muscles and the position of the ectopic VC branches. Although ectopic vulval muscles and VC branches are usually present in the same pseudovulvae, vulval-like muscles are detected at pseudovulvae that have no ectopic VC branching (Figures 6A and 6B), and conversely, ectopic VC branches project into pseudovulvae that have no vulval-like muscles (Figures 6C and 6D). These observations suggest that the VC cells do not provide positional cues to the muscles, nor do the muscles provide cues to the VC cells, which supports our previous results. Furthermore, the VC cells and vulval muscles do not receive positional cues from the HSN neurons. The somatic gonad precursors were ablated in the double mutant *egl-1; lin-15(n309)*, which has no HSN cells because of the *egl-1* mutation (Trent et al., 1983). As in similarly ablated *lin-15(n309)* animals, vulval-like muscles are associated with pseudovulvae independently of the presence of VC ectopic branches (Table 3).

We examined the position of the vulval muscles in *dig-1* animals, in which the gonad is formed dorsally rather than ventrally, to determine the structure with which the vulval muscles would be associated. Both the gonad and the vulva are anteriorly displaced in these animals, and the sex myoblasts migrate anteriorly and usually dorsally, following the gonad (J. Thomas and H. R. Horvitz, personal communication). In these animals ( $n = 24$ ) vulval-like muscles, which are sometimes oriented correctly and sometimes randomly, are generally ( $n = 17$ ) associated with the ventrally situated vulval cells (data not shown). In 4 animals

vulval-like muscles were detected both ventrally and dorsally, and in 3 animals muscles were detected ventrally and centrally. This result suggests that the ectopic position of the gonad may be interfering with its signaling to the vulval muscles or that the vulval cues may be used preferentially over the gonadal cues in this instance.

#### The Vulva Also Appears to Provide Cues to Other Cells

Four nonneuronal cells in the vulval region also stain with the anti-FMRFamide antibody (Figure 1C). These cells appear to be gonadal by their position and may represent the uterine cells directly adjacent to the vulva. The presence of the FMRFamide-like peptide in these cells suggests that they may function in a neurosecretory role in egg-laying. The cells appear to have a few thin, ventrally oriented processes, which run in the ventral cord (J. White, personal communication).

Staining in these putative gonadal cells first occurs either late during the fourth larval stage or in early adulthood and is independent of the presence of vulval muscles (Figure 3C), HSN cells (Figure 3C), or VC neurons (data not shown). The staining is absent in Multivulva *lin-15* mutants lacking the somatic gonad (see above), although the VC cells in such animals still branch.

Our data suggest that vulval cells must be present for the expression of the FMRFamide-like peptide in these putative gonadal cells. When the vulval cell precursors P(3-8).p are ablated in wild-type animals, producing a Vulvaless animal, these putative gonadal cells are not detected ( $n = 12$ ). Furthermore, in *lin-2*,

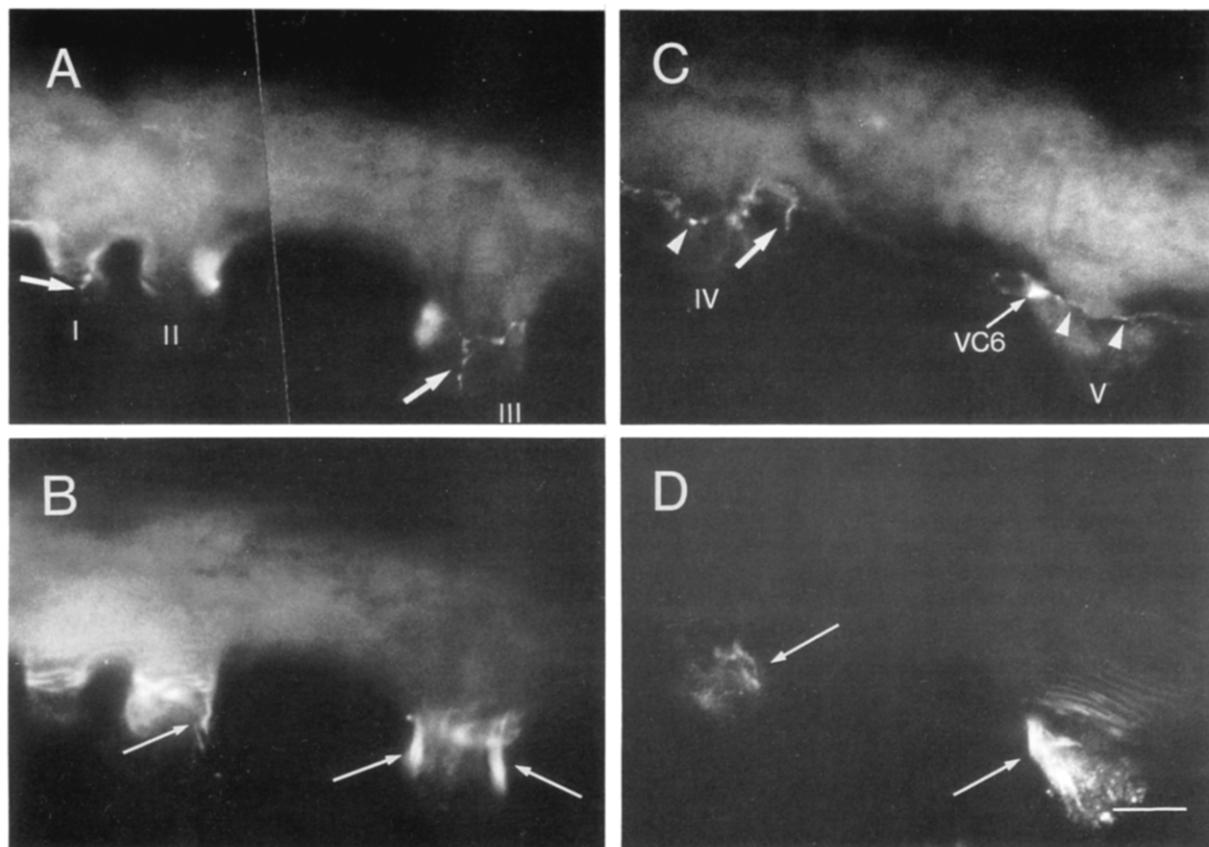


Figure 6. The Positioning of the Vulval Muscles Appears Independent of Neuronal Input

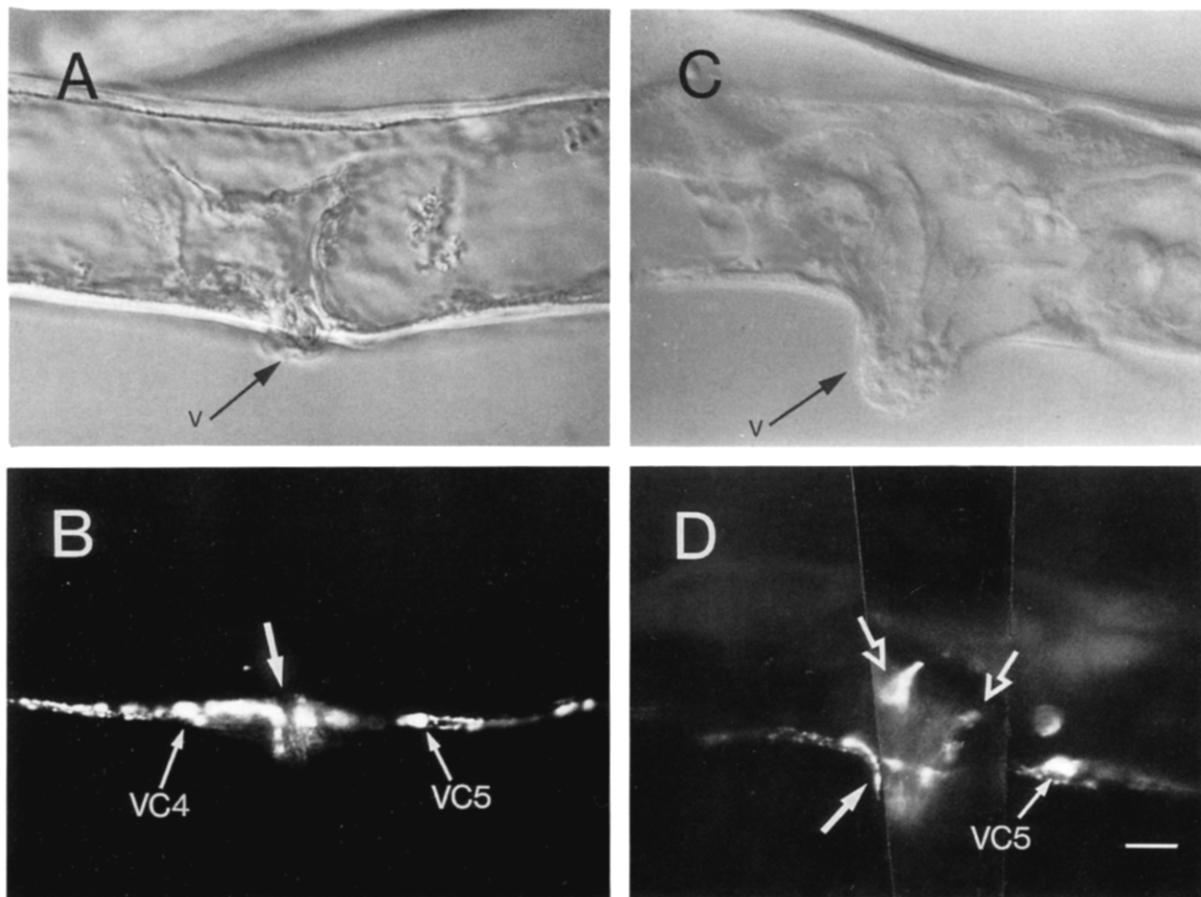
*egl-1; lin-15(n309)* Multivulva mutants in which Z1 and Z4 have been ablated were double-labeled with anti-FMRFamide (A and C) and anti-myosin (B and D) antisera to show the VC branching and vulval muscles, respectively. (In some places, body wall muscle, which is striated and readily distinguishable from the vulval-like muscles, is visible.) (A) and (B) show a more anterior region than (C) and (D). These mutants have pseudovulvae (labeled with Roman numerals), but lack gonads and HSN neurons. Although most pseudovulvae or protruding vulvae contain both ectopic VC branches (thick arrows) and vulval-like muscles (thin arrows), some have only one of these. Pseudovulva II and III and the protruding vulva IV contain both VC branches and vulval-like muscles (the VC branches to pseudovulva II are not visible in the plane of focus of the figure). Pseudovulva I has an ectopic VC branch, but no vulval-like muscles, whereas pseudovulva V has vulval-like muscles, but no VC branch (the VC process is indicated by arrowheads). Bar, 10  $\mu$ m.

*lin-3, lin-7, lin-10, lin-12(n302 and n676), lin-26, and n300* Vulvaless mutants, these cells are not detected (Figures 4A and 4B), supporting the observations seen with the laser-ablated animals.

The presence of vulval cells derived from a 1° lineage is necessary for the expression of the peptide in these putative gonadal cells. Certain *lin-12* dominant mutations cause all 6 vulval precursor cells to undergo 2° lineages, resulting in a Multivulva animal with a protruding vulva (Greenwald et al., 1983). Although VC cell branches are observed at the vulva and pseudovulvae, the putative gonadal cells are not detected in animals carrying the *lin-12* dominant alleles *n137, n950, or n952* (Figures 7A and 7B). The presence of vulval cells derived only from a 2° lineage is therefore not sufficient for the expression of the peptide. The cells stain, however, in 34% of the *lin-12; lin-15* mutants examined ( $n = 110$ ). In these animals the *lin-15* mutation partially suppresses the vulval lineage defect resulting from the *lin-12* dominant mutation,

allowing some vulval precursors to produce a 1° lineage (Sternberg, 1988; Sternberg and Horvitz, 1989).

Observations on two mutants suggest that cells derived from a 2° lineage are not necessary for the expression of the FMRFamide-like peptide. In mutants with the loss-of-function *lin-12(n941)* mutation, vulval cells are derived only from 1° lineages (Greenwald et al., 1983). These animals are sterile, grow slowly, and stain poorly. In 14 of the 21 animals in which staining was seen, the putative gonadal cells were present. This observation suggests that only vulval cells derived from a 1° lineage are necessary for the expression of the peptide. This conclusion is further supported by examination of the Vulvaless mutant *lin-3*. In *lin-3* animals, the Vulvaless phenotype is about 90% penetrant by functional criteria and is incompletely expressed (Ferguson, 1985; Sternberg and Horvitz, 1989). In about 20%–60% of the *lin-3* animals, the vulval precursor cell P6.p undergoes a 1° lineage and forms a functional “mini-vulva” that has no 2° vulval



**Figure 7. Expression of the FMRFamide-like Peptide in the Putative Gonadal Cells Appears Dependent on Vulval Cues**  
Animals are shown with Nomarski (A and C) and fluorescence (B and D) optics and are stained with the anti-FMRFamide antiserum tagged with a rhodamine-conjugated secondary antibody.  
(A and B) The *lin-12(d)* mutation causes a protruding vulva (v in [A]). In the *lin-12(n952)* Multivulva mutant, vulval cells are derived only from 2° lineages (Greenwald et al., 1983). In these mutants no putative gonadal cells are stained, although VC branching still occurs (thick arrow in [B]).  
(C and D) In *lin-12(n952); lin-15(n309)* mutants, vulval cells derived from 1° lineages are also present and VC branches (thick arrow in [D]) can be seen in the protruding vulva (v). The putative gonadal cells (open arrows) also stain in 34% of these animals. Bar, 10  $\mu$ m.

cells (Sulston and Horvitz, 1981; Sternberg and Horvitz, 1989). Although we cannot differentiate between normal and mini-vulvae in fixed animals, we have observed that about 30% of the *lin-3* animals ( $n = 179$ ) appear to have a vulva, and in these animals, the putative gonadal cells are present. This high percentage of *lin-3* animals with a vulva suggests that many of these animals actually have a mini-vulva and that the presence of vulval cells derived from a 1° lineage alone are necessary and sufficient for the expression of the FMRFamide-like peptide in the putative gonadal cells.

The vulval signal to the putative gonadal cells appears to be spatially constrained. In *dig-1* animals with dorsally positioned gonads, the cells are not stained ( $n = 24$ ). The vulval cells must therefore be in close apposition to the putative gonadal cells to exert their effect. In the presence of extra vulval cells, as in the Multivulva mutants *lin-1, lin-8; lin-9, lin-8; lin-37, lin-12; lin-15*, and *lin-15* described previously, only the puta-

tive gonadal cells at the true vulva are seen staining. No additional putative gonadal cells are detected, indicating that no new peptide expression is induced.

## Discussion

During the development of the egg-laying motor system, neurons and their target muscles must be brought together to form a functional neuromuscular unit. Neither the neurons nor the muscles appear to be the principal organizers of this process. Instead, the neurons and muscles appear dependent on the gonad and vulva to organize and bring them together in the vulval region.

### VC Innervation of the Vulval Muscles

Many mechanisms, such as interactions between axons and nearby neurons, target cells, substratum, or extracellular matrix (for reviews see Purves and Licht-

man, 1985; Gilbert, 1988), have been proposed to explain directed process outgrowth in the developing nervous system. We have found that in the innervation of the vulval muscles by the VC neurons, neither neuron-target nor neuron-neuron interactions appear necessary to guide the VC processes into the vulval region and to their target cells. Instead, branching of the VC cells appears dependent on cues from nearby nonneuronal and nonmuscle cells.

Initial axonal outgrowth of the VC cells along the ventral cord probably occurs late during the third larval stage (J. White, personal communication). This outgrowth appears to be independent of vulval cues, since VC processes are still seen in the Vulvaless mutants. Process outgrowth in the VC cells can therefore be separated into vulva-independent and vulva-dependent stages.

During this latter stage, the VC neurons appear to receive cues from the vulval cells to branch into the vulval region. These cues are probably sufficient to determine, at least grossly, the VC branching pattern and to direct the VC branches to the vulval muscles. The vulval cues may also provide the "stop" commands for process outgrowth, or alternatively, the VC branches may have a limited capacity for outgrowth.

We do not totally exclude contributions from the vulval muscles or HSN neurons to the VC branching pattern. Their roles in this process, however, are likely to be confined to modifying the branching pattern, structural changes that would be difficult to detect immunocytochemically. In addition, synaptogenesis presumably depends on cell interactions between the vulval muscles and VC neurons.

#### Positioning and Attachment of the Vulval Muscles

The positioning and attachment of the vulval and possibly uterine muscles (collectively referred to as the sex muscles) appear to depend on the presence of the somatic gonad and vulva. One mechanism by which the gonad and vulva may position the muscles is to constrain the subsequent divisions of the sex myoblast that produce the sex muscles to occur in the region between P5.p and P7.p, which is roughly the vulval region. Consistent with this hypothesis are our observations on Vulvaless animals in which the vulval muscles appear correctly positioned. In the absence of a signal from the somatic gonad, as in the Z1, Z4 ablated wild-type animals, vulval-like muscles are found broadly distributed in the animal. This broadening of the region populated by the muscles may be due to the sex muscle precursors moving or being shifted away from the vulval region before, during, or after their divisions. A broader distribution of sex muscle precursors has been seen in similarly ablated animals (M. Stern, personal communication); the distribution of the muscles themselves, however, seems more extensive than that of their precursors, suggesting that other factors affect muscle positioning. The gonadal signal could be the same signal that precisely positions the sex myoblasts (J. Thomas and H. R. Horvitz,

personal communication), which would imply that the gonadal signal is active in the second and third larval stages, or alternatively, an additional signal could be used.

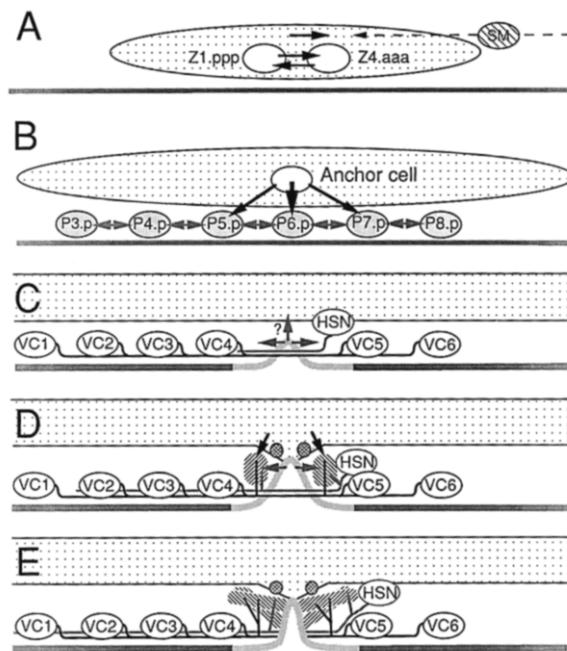
The attachment of the vulval muscles appears to depend primarily on the finding of an appropriate vulval site, and secondarily on the position of the muscles after division. In the Z1, Z4 ablated *lin-15* Multivulva animals we expected broadly distributed muscles, including a few muscles located dorsally, but all vulval-like muscles are associated with pseudovulvae. Proper attachment can therefore occur without any previous muscle positioning and is independent of the gonad. Attachment to a vulval site may occur through random sampling for an appropriate site by the muscles, by the vulval muscles receiving a signal from the vulval cells, or by a combination of the two. In the absence of a vulva, the muscles appear to attach randomly, as in the Z1, Z4, or P(3-8).p ablated wild-type animals or the Vulvaless mutants. These results also suggest that there is low specificity or affinity for the normal distal body wall attachment sites.

Since the VC and HSN cells extend branches into the vulval region, these neurons might also direct the positioning of the vulval muscles. Since muscles can be positioned in pseudovulvae that do not contain either VC or HSN branches, however, any guidance cues contributed by the neurons are small, if present at all.

#### Development of the Reproductive System

The cell interactions required in the assembly of the egg-laying motor circuit are part of a larger series of cell interactions needed to form a functional egg-laying system (Figure 8). This series of interactions includes interactions between 2 cells of the somatic gonad to determine which becomes the anchor cell (Kimble, 1981; Sternberg and Horvitz, 1986); induction of the vulval lineages by the anchor cell (Kimble, 1981); interactions among the vulval precursor cells to determine which cells will express the 1° or 2° lineages (Sternberg, 1988); signaling of the vulva to cells presumably in the gonad to induce expression of a FMRFamide-like peptide; interactions between the somatic gonad and the sex myoblasts to determine the final position of the myoblasts (J. Thomas and H. R. Horvitz, personal communications); guidance cues from the vulval cells to the VC and, perhaps, HSN neurons to induce branching from the ventral cord into the vulval region; cues from gonadal cells to the sex muscle precursors perhaps to limit their movements during division and cues from the vulval cells to the vulval (and possibly uterine) muscles for attachment and proper orientation; and interactions presumably between the neurons and muscles to form synaptic connections.

In the development of the egg-laying system there appears to be a hierarchy of regulating and responding cells. The primary organizer in this developmental pathway is the somatic gonad; without the gonad, none of the subsequent development occurs (except



**Figure 8.** Summary of Cell Interactions Needed to Form a Functional Egg-Laying System, Presented in Chronological Sequence (A) The final position of the sex myoblasts (SM) is dependent on interactions between the sex myoblasts and the gonad (J. Thomas and H. R. Horvitz, personal communication). Interactions between 2 cells in the somatic gonad, Z1.aaa and Z4.hpp, determine which cell becomes the anchor cell (Kimble, 1981). (B) The lineages of the vulval precursor cells P(3-8).p are induced by the anchor cell (Kimble, 1981). Interactions among the vulval precursor cells also determine which cells will express which lineage (Sulston and White, 1980; Sternberg, 1988). (C) The VC, and perhaps HSN, neurons receive cues from the vulva to branch into the vulval region. Vulval cells may also induce peptide expression in the putative (?) gonadal cells. (D) The sex muscle precursors receive cues from the gonad perhaps to limit their region of division, and the vulval muscles receive cues from the vulva for attachment. The cross-hatched cells indicate the putative gonadal cells that stain with the anti-FMRFamide antiserum. (E) The final organization of the egg-laying system. Additional interactions, presumably between the neurons and muscles, are necessary during synaptogenesis.

for the initial anterior migration of the sex myoblasts). Other components of the system, such as the vulva and vulval muscles, depend at least in part on cell interactions with the somatic gonad for their development. The secondary organizer of the egg-laying system is the vulva. After its induction by the gonad, the vulva in turn helps organize the motor system. The vulval cells induce branching of the VC and, perhaps, HSN neurons. In conjunction with the gonad, the vulva cells also assist in the positioning and attachment of the vulval muscles. In addition, the vulval cells may induce peptide expression in the gonad, suggesting that there are also feedback signals in the development of the egg-laying system. Finally, interactions presumably between neurons and muscles are necessary to establish functional synapses.

By controlling the development of each component of the reproductive system, the somatic gonad may be able to regulate the timing of the various cell interactions. For example, the HSN and VC neurons arise relatively early in development. Their differentiation with respect to their egg-laying functions appears to be delayed until the fourth larval stage. During this larval stage, vulval morphogenesis occurs. As the neurons are dependent on cues from the vulva, the onset of their differentiation may be delayed until the production of the vulva. Thus, the temporal and spatial components of pattern formation in the developing reproductive system may be closely linked. The egg-laying system in *C. elegans* provides a simple, assessible system in which the events that occur during the development of the reproductive system can be dissected genetically and the genes that are responsible for directing these events identified.

#### Experimental Procedures

##### Growth and Maintenance of Nematode Strains

*C. elegans* strains were maintained according to Brenner (1974), with modifications described by Way and Chalfie (1988). Most experiments were done at 20°C; for strains that were cold (cs)- and heat (ts)-sensitive, experiments were performed at 15°C and 25°C, respectively. Animals that had undergone laser microsurgery were grown at 15°C.

In addition to the wild-type *C. elegans* strain (variety Bristol, N2 [Brenner, 1974]), strains with the following mutations were used.

- LGI: *lin-10(e1439)*, *lin-17(n671)* and *rh41* (*rh41*, E. Hedgecock, personal communication).
- LGII: *lin-8(n111)*, *lin-31(n301)*, *lin-4(e912)*, *lin-26(n156)*, *let-23(n1045cs)*, *lin-7(e1413)*.
- LGIII: *lin-37(n758)*, *lin-39(n709)* and *n1760* (*n1760*, S. Clark and H. R. Horvitz, personal communication), *lin-9(n112)*, *unc-32(e189)*, *lin-12(n137)*, *n302*, *n676*, *n950*, *n952*, and *n941*, *dig-1(n1321*) (J. Thomas and H. R. Horvitz, personal communication)
- LGIV: *lin-1(e1777)* and *e1275*, *lin-3(e1417)*, *lin-24(n432)*.
- LGV: *him-5(e1467)* and *e1490*, *egl-1(n487)*.
- LGX: *egl-15(n484)*, *lin-2(e1309)*, *unc-84(e1410ts)*, *lin-15(n309)* and *n765*.

Except where noted, these mutations have been previously characterized: *lin-12* by Greenwald et al. (1983); other *lin* mutations and *let-23* have been characterized by Horvitz and Sulston (1980), Sulston and Horvitz (1981), Ferguson and Horvitz (1985), and Ferguson et al., (1987); *him-5* by Hodgkin et al. (1979); *egl* mutations by Trent et al. (1983); and the remaining mutations by Brenner (1974). The alleles used are either the reference alleles listed in the above publications or alleles that result in similar phenotypes. The translocations *n71* (*I/V*), which carries the *n300* mutation, a mutation that results in a Vulvaless phenotype (Ferguson and Horvitz, 1985), and *e71* (*III;V*) (Rosenbluth and Baillie, 1981) were also used. Strains were kindly provided by the Caenorhabditis Genetics Center and I. Greenwald, E. Hedgecock, H. R. Horvitz, and their colleagues. *lin-12(n941)* was derived from *lin-12(n941)/e71*. Strains with *lin-12* alleles *n137* and *n950* also carried the *him-5* mutations *e1467* and *e1490*, respectively.

An *unc-32 lin-12(n952); lin-15(n309)* strain was constructed by mating males from a cross of *unc-32 lin-12(n952)/lin-12(n137n720)* hermaphrodites and N2 males to *lin-15(n309)* hermaphrodites. *lin-12(n952)* animals are egg-laying defective (Egl); homozygotes could be distinguished from heterozygotes by the degree of penetrance of the Egl phenotype. F1 progeny that were Egl were individually plated and allowed to self. Among these progeny, animals that had large, irregularly sized pseudovulvae, indicating that they were homozygous for *lin-15*, and that were Egl and

uncoordinated, indicating that they were homozygous for *lin-12* and *unc-32*, were selected. The *unc-32* mutation was not removed from the *lin-12* strains and did not affect the VC staining pattern (data not shown).

*egl-1; lin-15(n309)* and *lin-17(n671); egl-1* strains were constructed by mating *egl-1* males with *lin-15* or *lin-17* hermaphrodites. F1 progeny were allowed to self; among these progeny, Egl animals that had pseudovulvae were individually plated and allowed to self. Since *egl-1* is semidominant, each F2 clone was followed for three generations to ensure that the clone was homozygous for *egl-1*.

#### Laser Microsurgery

Two laser microbeam systems, a Candela flashlamp-pumped dye laser (Walthal and Chalfie, 1988) and a Laser Science fixed wavelength dye laser (Avery and Horvitz, 1987), were used. Ablations followed the procedure of Sulston and White (1980), except that animals were anesthetized with 10 mM sodium azide (Avery and Horvitz, 1987). Animals were examined 1–2 hr and 1 day later to verify that the desired cells were killed and to check for surrounding damage. In addition, since animals lacking either the vulval cell precursors P(3–8).p or the M cell do not lay eggs (Kimble, 1981; Trent et al., 1983), egg retention was used as further confirmation of the killing of these cells. The absence of vulval muscles derived from the M cell was also verified by staining with an anti-myosin antibody. To determine whether any somatic gonad cells remained after ablation of Z1 and Z4, we stained animals with diamidinophenylindole (Sigma).

#### Immunocytochemistry

Adult hermaphrodites were fixed for at least 20 hr at 4°C with 4% paraformaldehyde (Fisher) in 0.1 M phosphate buffer (pH 7.4). In cases where large number of animals could be grown, fixed animals were broken by gentle homogenization in a Corning 2 ml tissue grinder. The degree of breakage was monitored by examining animals under a dissecting microscope; optimal preparations for immunocytochemistry contained whole shells of animals whose intestine and gonad were extruded. When fewer than 1000 animals were available (e.g., animals that had undergone laser microsurgery or *lin-12(n941)* mutants) or when animals were too small to homogenize (e.g., larval animals), fixed animals were placed in microtiter wells or Eppendorf tubes and were treated with enzyme, as modified from the procedure of Desai et al. (1988). Animals were incubated with gentle agitation in 5% β-mercaptoethanol (Sigma), 0.5% Triton X-100 (Sigma) in 100 mM Tris buffer (pH 7.2) for 20–30 hr at 37°C. The animals were rinsed in 100 mM phosphate buffer (pH 7.4) and then incubated with gentle agitation with Type IV collagenase (Sigma) at 900 U/ml 100 mM Tris buffer (pH 7.7) for 10–14 hr at 37°C.

Immunocytochemical procedures were performed as described in Li and Calabrese (1985). The following primary antibodies were used: a rabbit polyclonal antiserum against synthetic FMRFamide conjugated to succinylated bovine thyroglobulin (Marder et al., 1987), a mouse monoclonal antibody (5–6) against nematode myosin (Miller et al., 1983; Ardizzi and Epstein, 1987), and a rabbit polyclonal against serotonin (Immunonuclear). Antibodies were visualized with either rhodamine-, fluorescein-, or peroxidase-conjugated IgG antibodies (Cooper Biochemical). Diamidinophenylindole at 1 mg/ml was added to the final washes.

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