Protruding Vulva Mutants Identify Novel Loci and Wnt Signaling Factors That Function During *Caenorhabditis elegans* Vulva Development

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ABSTRACT

The *Caenorhabditis elegans* vulva develops from the progeny of three vulval precursor cells (VPCs) induced to divide and differentiate by a signal from the somatic gonad. Evolutionarily conserved Ras and Notch extracellular signaling pathways are known to function during this process. To identify novel loci acting in vulval development, we carried out a genetic screen for mutants having a protruding-vulva (Pvl) mutant phenotype. Here we report the initial genetic characterization of several novel loci: *bar-1*, *pvl-4*, *pvl-5*, and *pvl-6*. In addition, on the basis of their Pvl phenotypes, we show that the previously identified genes *lin-26*, *mom-3/mig-14*, *egl-18*, and *sem-4* also function during vulval development. Our characterization indicates that (1) *pvl-4* and *pvl-5* are required for generation/survival of the VPCs; (2) *bar-1*, *mom-3/mig-14*, *egl-18*, and *sem-4* play a role in VPC fate specification; (3) *lin-26* is required for proper VPC fate execution; and (4) *pvl-6* acts during vulval morphogenesis. In addition, two of these genes, *bar-1* and *mom-3/mig-14*, are known to function in processes regulated by Wnt signaling, suggesting that a Wnt signaling pathway is acting during vulval development.

DURING development, polarized epithelial cells are exposed to signals from surrounding cells that cause them to modify their behavior or cellular fate. In responses to external signals, cells may undergo cell division and terminal differentiation or may undertake coordinated morphogenetic movements. One excellent model system for studying the processes of cell signaling, cell polarity, cell-fate determination, and morphogenesis is the development of the hermaphrodite vulva of the nematode *Caenorhabditis elegans* (reviewed in Greenwald 1997).

The formation of the vulval opening has been extensively studied, and this process has been divided into four stages (Figure 2; Ferguson *et al.* 1987; Greenwald 1997): (1) the "generation" stage in which the blast cells that will give rise to the vulva are born; (2) the "fate specification" stage in which these cells are induced to adopt distinct cell fates by extracellular signals; (3) the "execution" stage in which these cells divide and differentiate according to their cell fate and generate 22 vulval cells; and (4) the "morphogenesis" stage during which these 22 cells go through extensive morphogenetic movements to form the opening connecting the uterus to the outside.

During the generation stage, the 12 Pn.p cells, P1.p–P12.p, are born along the ventral midline of the animal in the early first larval stage (L1; Sulston and Horvitz 1977). The Pn.p cells are the posterior daughters of the 12 P cells, embryonic cells that migrate into the ventral midline from ventrolateral positions and divide to generate a Pn.a neuroblast and a Pn.p hypodermal cell (except P12, which follows a different fate). The 6 Pn.p cells in the mid-body region, P3.p–P8.p, express the Hox gene *lin-39* and form the Vulval Equivalence Group (Wang *et al.* 1993; Maloof and Kenyon 1998). The results of genetic and laser ablation experiments have shown that all 6 Pn.p cells in the Vulval Equivalence Group are competent to generate vulval tissue, and they are therefore referred to as the vulval precursor cells (VPCs; Sulston and Horvitz 1977; Sulston and White 1980; Sternberg and Horvitz 1986). The cells that do not express *lin-39* (P1.p, P2.p, P9.p–P12.p) do not form part of the Vulval Equivalence Group and become part of the large hypodermal syncytium of the animal.

The six VPCs are initially equivalent in developmental potential and are competent to adopt one of three distinct cell fates called 1°, 2°, and 3°. Cells adopting the 1° and 2° fates contribute to the vulva, while cells adopting the 3° do not, but rather contribute to the syncytial hypodermis (Sulston and White 1980; Sternberg and Horvitz 1986). Although all six VPCs are initially equivalent, the pattern of fates adopted in wild-type animals is always either 3° 3° 2° 1° 2° 3° or F 3° 2° 1° 2° 3° (in which P3.p adopts an alternate nonvulval fate described below). This invariant pattern of cell-fate specification is achieved through the use of multiple extracellular signals acting on the VPCs during the fate specification stage (reviewed in Greenwald 1997;

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Kornfeld 1997). First, in the late L2/early L3 stage, a single cell of the somatic gonad called the anchor cell sends an inductive signal to the VPCs that causes the vulval precursor cell closest to the anchor cell, P6.p, to adopt the 1° cell fate. Second, P6.p then sends a lateral signal that, perhaps combined with low levels of anchor cell signal, causes the adjacent VPCs, P5.p and P7.p, to adopt the 2° vulval fate (Sternberg 1988; Sternberg and Horvitz 1989; Koga and Ohshima 1995; Simske and Kim 1995). The remaining three VPCs, P3.p, P4.p, and P8.p, are not induced by either of these signals and consequently adopt the 3° cell fate, which is to divide once and fuse with the hypodermal syncytium. In addition, P3.p can also adopt a fourth cell fate called F, for fused, in which P3.p fuses with the hypodermal syncytium without dividing at all (Ferguson and Horvitz 1985; Sternberg and Horvitz 1986). The F fate is adopted by P3.p in ~50% of wild-type animals (Eisenmann *et al.* 1998). Finally, genetic evidence exists for a third signal, an inhibitory signal from the surrounding hypodermal syncytium that antagonizes the anchor cell inductive signal, ensuring that only cells receiving high amounts of inductive signal adopt vulval fates (Herman and Hedgecock 1990).

During the execution stage (L3) the three induced cells, P5.p, P6.p, and P7.p, will each divide three times to generate a total of 22 progeny cells. P6.p (1° fate) will divide to generate 8 cells that form the center of the developing vulva; P5.p and P7.p (2° fate) will each divide to generate 7 cells that form the sides of the developing vulva (Sulston and Horvitz 1977; Sternberg and Horvitz 1986). During the morphogenesis stage in the L4, these 22 cells will go through specific cell fusions and short-range migrations to generate a stack of 7 mono- or multinucleate toroidal cells that form the vulval opening (Newman and Sternberg 1996; Greenwald 1997; Sharma-Kishore *et al.* 1999).

Genetic analysis has identified a number of genes acting in vulval development (reviewed in Eisenmann and Kim 1994; Greenwald 1997; Kornfeld 1997). These genes were identified because when mutated they generally cause a multivulva (Muv) phenotype in which more than three Pn.p cells adopt vulval fates, a vulvaless (Vul) phenotype in which no Pn.p cells adopt vulval cell fates, or because they suppress other Muv or Vul mutations. Molecular characterization of these genes has shown that the reception and transduction of the anchor cell inductive signal in the Pn.p cells is mediated by a conserved receptor tyrosine kinase/RAS/mitogen-activated protein (MAP) kinase signaling pathway utilizing the products of the *let-23(RTK)* (Aroian *et al.* 1990), *let-60(Ras)* (Han and Sternberg 1990), and *mpk-1(MAPK)* (Lackner *et al.* 1994; Wu and Han 1994) genes, respectively. Two transcription factors, LIN-31 and LIN-1, are known downstream targets of the Ras/MAP kinase pathway in the VPCs (Miller *et al.* 1993; Beitel *et al.* 1995; Jacobs *et al.* 1998; Tan *et al.* 1998). In addition to the Ras pathway mediating the anchor cell inductive signal, the lateral signal acting between Pn.p cells to specify the 2° cell fate is mediated by LIN-12, a Notch-like transmembrane receptor (Yochem *et al.* 1988). Therefore, vulval cell-fate specification in *C. elegans* utilizes two evolutionarily conserved extracellular signaling pathways.

Previous genetic screens to identify mutations affecting vulval development relied extensively on the identification or suppression of the multivulva and vulvaless phenotypes (Horvitz and Sulston 1980; Sulston and Horvitz 1981; Ferguson and Horvitz 1985). We considered it likely that genes exist that function in vulval development but which may not mutate to a Muv or Vul phenotype or that may not strongly suppress such a phenotype. To identify novel loci acting in vulval development that may have been missed in previous screens, we performed a genetic screen that relied on a novel, more subtle mutant phenotype, the protruding vulva or Pvl phenotype. In this way we have identified at least 11 loci, representing both novel and previously identified genes, which appear to act at different stages during vulval development. Here we present the preliminary genetic characterization of these loci and their vulval mutant phenotypes.

MATERIALS AND METHODS

Genes, alleles, and general genetic procedures: Methods for culturing, handling, and genetic manipulation of *C. elegans* were as described (Brenner 1974). The animals described as wild type were *C. elegans*, variety Bristol, strain N2, which is the strain background in which all mutations were induced. The strain RW7000 was used for sequence-tagged site (STS) mapping experiments (Williams *et al.* 1992) and is *C. elegans*, variety Bergerac. All experiments were performed at 20° unless indicated. The genes, alleles, deficiencies, and STSs listed below were used in this work. The reference for all alleles is Riddle *et al.* (1997), unless noted. The allele *bar-1(sy324)* was identified in a screen for mutations capable of suppressing the Muv phenotype of *lin-1(e1777)* (A. Golden, unpublished results). The reference for all alleles designated "*gaxx*" is this work.

LGI: lin-17(n671, ga58, ga69, ga83), unc-11(e47), unc-73(e936), lin-44(n1792), unc-74(e883), egl-34(n171), dpy-5(e61), sem-2(n1343), sem-4(n1971, ga82), lin-28(n719, ga73), unc-29(e1072), unc-59(e261, ga77, ga78), unc-54(r323), sDf4, nDf24, stP124, hP4, TCbn2.

LGII: lin-31(n1053, n301, ga57, ga70), bli-2(e768), dpy-10(e128), unc-104(e1265), lin-26(n156, ga91), rol-6(e187), let-23(n1045), unc-4(e120), let-25(mn25), let-29/mix-1(mn29), let-243(mn226), let-244(mn97), let-245(mn185), let-268(mn189), lin-29(n333, ga93, ga94), mig-14(mu71, ga62), mom-3(or78) (Thorpe *et al.* 1997), *pvl-4(ga96), pvl-5(ga87), mnDf30, mnDF39, mnDf63, mnDf66, mnDf87, mnDf89, mnDf90, mnDf96, mnDf99, mnDf106, mnDf108, mnDf109, mnC1, stP100, stP196, stP101, stP50, stP36, stP98, maP1.*

LGIII: dpy-17(e164), dig-1(n1321, ga76), unc-32(e189), pvl(ga84), pvl(ga90), stP19, stP120, mgP21, stP127, stP17.

LGIV: dpy-9(e12), egl-4(n478), egl-18(n162, n475, ga97), pvl(ga79), lin-1(e1777, n304, ga56, ga68), unc-17(e245), dpy-13(e184), let-60(n1046, ga89), dpy-20(e1282, e1362), dpy-4(e1166), stP13, stP51, stP44, stP4, stP5, stP35.

LGV: pvl-6(ga81), unc-62(e644), unc-46(e177), unc-83(e1409, ga72), dpy-11(e224), pvl(ga88), unc-42(e270), lin-25(ga65, ga67), him-5(e1490), sDf20, stP192, bP1, stP6, stP18, stP108, stP105, stP23.

LGX: unc-20(e112), lin-18(e620, ga75), lon-2(e678), dpy-8(e130), unc-6(e78, n102), bar-1(ga80, sy324), dpy-7(e1324, e88), unc-18(e81), unc-10(e102), dpy-6(e14), lin-14(ga54), lin-2(n768, e1309, ga59, ga60, ga61), unc-9(e101), unc-84(n1325, ga55, ga71), unc-3(e151), uDf1, nDf19, szT1, stP41, stP156, stP33, stP103, stP129, stP61, stP72, stP2.

To construct double-mutant strains, *bar-1* was balanced by *dpy-7* or *unc-6*, *mom-3/mig-14* by *unc-52*, *egl-18* by *dpy-9* or *unc-17*, *sem-4* by *dpy-14*, and *let-60(n1046)* by *dpy-20*. Balanced double heterozygote animals were identified and allowed to self for two generations until Egl/Pvl animals that no longer segregated either balancing marker were found. Complementation tests were performed to verify the strain genotype.

EMS mutagenesis, identification, and mapping of Pvl mutants: EMS mutagenesis of N2 hermaphrodites was carried out as described (Brenner 1974; Riddle *et al.* 1997). Mutagenized P0 animals were allowed to self-fertilize, F_1 progeny from these animals were picked, seven animals to a 60-mm plate, and their F_2 progeny were screened for fertile animals having a protruding vulval phenotype (Pvl). Only a single Pvl mutant animal was taken from each plate of F_2 animals. A total of 38,300 mutagenized haploid genomes were screened and 230 Pvl mutant strains were identified.To distinguish animals that displayed a Pvl phenotype due to defects in VPC generation, fate determination, or fate execution from those that were Pvl due to defects in vulval morphogenesis, we examined 30–50 L4 stage hermaphrodites from each mutant line by Nomarski differential interference contrast microscopy on a Zeiss Axioplan2 microscope. In wild-type animals at this stage, the 22 vulval cell nuclei occupy stereotyped positions, and the developing vulval structure forms a "Christmas tree"-shaped structure (Figure 2D and Figure 3C). We screened for those strains with animals that had fewer than 22 vulval nuclei, more than 22 vulval nuclei, or 22 vulval nuclei in incorrect positions. At this stage, 55 mutant strains had such defects, and of these, 36 had high or moderately penetrant Pvl or Egl (egg-laying-defective) phenotypes and were characterized further (for most Pvl mutants the Egl phenotype is more penetrant than the Pvl phenotype). At this stage, 175 mutant strains were wild type, but went on to be Pvl; therefore many of these mutants may have defects in the process of vulval morphogenesis. This class of mutants will not be described further here.

Each of the 36 mutant strains was subjected to a PCR-based STS mapping strategy that relies on differences in transposon number and location between N2 and another *C. elegans* strain, RW7000. Briefly, for each strain *pvl/+* males were crossed to RW7000 hermaphrodites and F_2 Pvl mutant animals were isolated. Two rounds of PCR were performed on individual F_2 Pvl animals to localize the mutation to one of the six chromosomes and then to localize the mutation to a smaller region of that chromosome (data not shown). The STS markers and oligonucleotides used are described in Williams *et al.* (1992). Traditional three-factor mapping (Table 1) and deficiency mapping were also performed. Once each of the mutations was localized, standard complementation tests were performed with any loci in the same genetic interval that were known to perturb vulval development or egg laying when mutated (*lin, let, egl, sar, sur, mig, unc*, etc., genes; see Greenwald 1997 and Kornfeld 1997). Complementation tests were not performed for new alleles of *lin-14*, *lin-25*, *lin-28*, and *lin-29*, but gene assignments were made based on phenotypes and map position and were subsequently confirmed by other investigators (Tuck and Greenwald 1995; Bettinger *et al.* 1996; S. Euling, personal communication). On the basis of complementation data and other criteria, we identified mutations in the following known genes: *dig-1(ga76)* (subsequently lost), *egl-18(ga97)*, *let-60(ga89)*, *lin-1(ga56, ga68)*, *lin-2(ga59, ga60, ga61)*, *lin-14(ga54)*, *lin-17(ga58, ga69, ga83)*, *lin-18(ga75)*, *lin-25(ga65, ga67)*, *lin-26(ga91)*, *lin-28(ga73)*, *lin-29(ga93, ga94)*, *lin-31(ga57, ga70)*, *mom-3/mig-14(ga62)*, *sem-4(ga82)*, *unc-59(ga77, ga78)*, *unc-83(ga72)*, and *unc-84(ga55, ga71)*.

Several of the Pvl mutants may identify novel loci on the basis of their phenotypes, map positions, and complementation of mutations in known genes in the same genetic intervals. These mutants identify eight complementation groups each represented by a single allele isolated in this screen: *bar-1(ga80)*, *pvl-4(ga96)*, *pvl-5(ga87)*, *pvl-6(ga81)*, *pvl(ga79)*, *pvl(ga84)*, *pvl(ga88)*, and *pvl(ga90)* (Figure 1). Those alleles designated *pvl(gaxx)* have been less well characterized and have not yet been given specific gene designations. *pvl(ga90)* is likely to be a heterochronic mutant and has not been studied further. All Pvl phenotypes are recessive and not affected by temperature, except that caused by *let-60(ga89)*, which causes a partially dominant, temperature-sensitive Muv phenotype as described in Eisenmann and Kim (1997).

Additional genetic data: *pvl-4:* The deficiencies *mnDf83* and *mnDf66*, but not *mnD87*, fail to complement *pvl-4(ga96)* for the Pvl phenotype. *pvl-4(ga96)* complements *let-25(mn25)*, *let-29(mn29)*, *let-243(mn226)*, *let-244(mn97)*, *let-245(mn185)*, and *let-268(mn189)*.

pvl-5: The deficiencies *mnDf30*, *mnDf39*, and *mnDf96* fail to complement *pvl-5(ga87)* for the Pvl phenotype.

ga62/mom-3(or78) complementation test: N2 males were mated with *rol-1(e91) mom-3(or78)/mnC1* hermaphrodites, and cross-progeny males from this cross were mated with *unc-4(e120) ga62* hermaphrodites. Cross-progeny from this mating carrying the wild-type chromosome II, the *mnC1* chromosome, and recombined *rol-1 mom-3* chromosomes were found, but no *rol-1 mom-3(or78)/unc-4 ga62* progeny could be definitively identified. A few sickly, Pvl cross-progeny were found that gave no live progeny. Examination of progeny embryos from known *or78/ga62* mothers showed that while endoderm induction appeared normal, all embryos failed to hatch and exhibited morphogenesis defects similar to the ~30% of embryos from *or78/or78* mothers that make endoderm but also fail to hatch (A. Schlesinger and B. Bowerman, personal communication). Therefore, on the basis of their failure to complement for the zygotic Pvl phenotype and the maternal effect embryonic lethal phenotype, we believe *ga62* and *or78* are allelic.

egl-18: For *egl-18* strains, a low percentage of animals had a strong Roller phenotype: *ga97* = 8% (n = 240); *n475* = 10% (n = 154); *n162* = 10% (n = 283).

Characterization of Pvl mutants: Following multiple backcrosses, each of the 12 Pvl loci in Figure 1 was characterized as follows. First, each strain was checked for embryonic and larval lethality by picking eggs to a petri plate (>200) and observing their development over sequential days. The mutants with >5% dead eggs were *mom-3/mig-14(ga62)* (20%), *egl-18(ga97)* (8%), *pvl-4(ga96)* (13%), *pvl-5(ga87)* (17%), and *pvl(ga79)* (7%). The only mutant displaying significant larval lethality was *pvl-4(ga96)* (14%). Second, the number of large hypodermal nuclei in the ventral midline of early L2 stage animals was determined to look for defects in Pn.p cell generation. Third, late L3 and early L4 stage animals were examined by Nomarski microscopy to determine the number of animals containing an abnormal vulval structure. P12.p to P11.p cell-fate transformations and defects in gonad migration were also noted. Finally, for several strains, the division patterns of P3.p–P8.p were directly determined by following vulval development in several living hermaphrodites starting in the L2 stage,

TABLE 1

Three-factor genetic mapping data

Gene (allele)	Genotype of heterozygote	Phenotype of recombinant	Genotype of selected recombinant
	LGII		
mom-3/mig-14(ga62)	*rol-6 unc-4 +/ + + ga62*	Rol	10/10 *ga62/+*
		Unc	0/3 *ga62/+*
	unc-4 rol-1 +/ + + ga62	Rol	0/15 *ga62/+*
		Unc	13/13 *ga62/+*
pvl-4(ga96)	*dpy-10 unc-4 +/ + + ga96*	Dpy	27/27 *ga96/+*
		Unc	0/28 *ga96/+*
	unc-4 rol-1 +/ + + ga96	Rol	13/13 *ga96/+*
		Unc	0/1 *ga96/+*
pvl-5(ga87)	*dpy-10 + unc-4/ + ga87 +*	Dpy	0/27 *ga87/+*
		Unc	21/24 *ga87/+*
	LGIII		
pvl(ga84)	*dpy-18 unc-25 +/ + + ga84*	Dpy	0/7 *ga84/+*
		Unc	9/9 *ga84/+*
	dpy-17 unc-32 +/+ + ga84	Dpy	4/4 *ga84/+*
		Unc	0/7 *ga84/+*
	LGIV		
egl-18(ga97)	*dpy-9 + unc-17/ + ga97 +*	Dpy	39/81 *ga97/+*
		Unc	41/80 *ga97/+*
	+ lin-1 dpy-13/ga97 + +	Dpy	20/20 *ga97/+*
pvl(ga79)	*dpy-9 + unc-17/ + ga79 +*	Dpy	4/17 *ga79/+*
		Unc	4/15 *ga79/+*
	LGV		
pvl-6(ga81)	*+ unc-46 dpy-11/ga81 + +*	Dpy	15/15 *ga81/+*
		Unc	0/15 *ga81/+*
	+ unc-62 dpy-11/ga81 + +	Dpy	9/9 *ga81/+*
pvl(ga88)	*unc-46 dpy-11 +/ + + ga88*	Dpy	0/11 *ga88/+*
	dpy-11 + unc-42/+ ga88 +	Dpy	16/21 *ga88/+*
		Unc	2/8 *ga88/+*
	LGX		
bar-1(ga80)	*+ dpy-7 unc-18/ga80 + +*	Dpy	0/13 *ga80/+*
		Unc	6/6 *ga80/+*
	dpy-8 unc-6 +/ + + ga80	Dpy	11/11 *ga80/+*
		Unc	0/21 *ga80/+*
	unc-6 + dpy-7/ + ga80 +	Dpy	9/23 *ga80/+*
		Unc	2/2 *ga80/+*

Three-factor genetic crosses for the indicated *pvl* loci were performed utilizing heterozygotes with the genotype shown (column 2). Progeny with the given recombinant phenotypes were picked (column 3) and analyzed for the presence of the third marker in their progeny. The number of recombination events in which the third marker was also present is shown in the last column.

using Nomarski optics. The criteria for designation of cell fate were as described in Sternberg and Horvitz (1986). Cells were assigned a fate of either 1°, 2°, 3°, or F.

The F fate: We have adopted the designation F (or fused) for the fate of P3.p in 50% of wild-type animals and for the fate adopted by other vulval precursor cells in *mom-3/mig-14(ga62), bar-1(ga80), sem-4(ga82)*, and *egl-18(ga97)* mutants. A cell adopting this fate initially joins the Vulval Equivalence Group in the L1, unlike P1.p, P2.p, and P9.p–P11.p, which fuse with the hypodermal syncytium at this time. However, a vulval precursor cell adopting the F fate then fuses without dividing in the L3 stage at the same time as P4.p–P8.p begin their first round of cell division, as judged by MH27 staining (Eisenmann *et al.* 1998 and data not shown).

Q_L migration: To determine if *mom-3/mig-14(ga62), bar-1(ga80), egl-18(ga97)*, and *sem-4(ga82)* mutants exhibited defects in the migration of the progeny of the neuroblast Q_L, we used *muIs35* (gift of C. Kenyon), an integrated array containing a *mec-7::GFP* reporter fusion gene that is expressed in the touch receptor neurons, including AVM (Q_R.paa) and PVM (Q_L.paa) (Chalfie *et al.* 1994). We observed the position of green fluorescent protein (GFP) expressing cells in animals on a Zeiss Axioplan 2 using fluorescence microscopy.

RESULTS

Identification of protruding vulva (Pvl) mutants: To identify genes that function in vulval development but

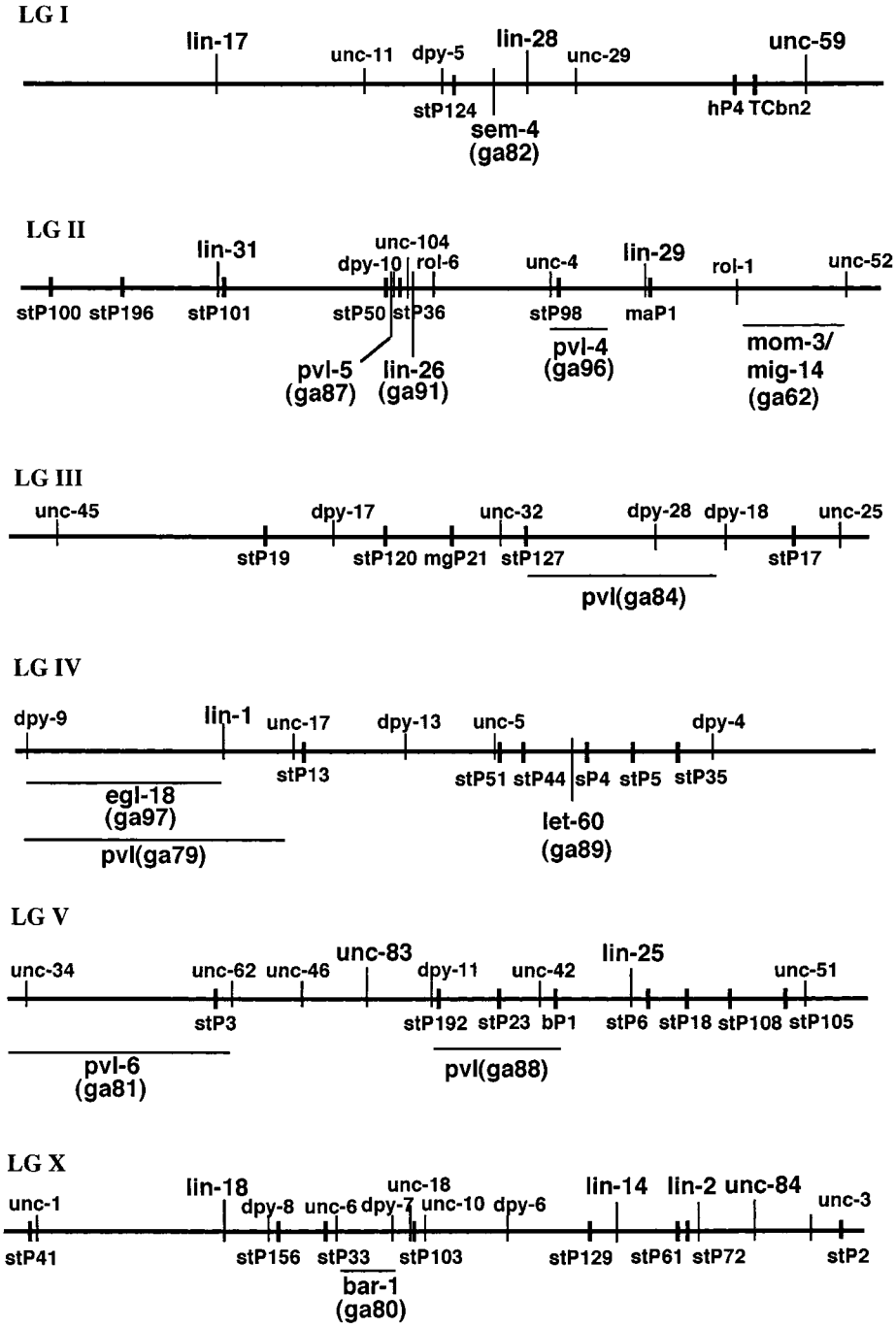

FIGURE 1.—Genetic map locations of the *pvl* loci. Each of the six *C. elegans* linkage groups is shown with the map positions of visible markers (above the line) and Tc1-linked STS markers (below the line) used in the mapping of the Pvl mutants. The three-factor mapping data upon which this figure is based are given in Table 2. Some mapping intervals are defined by deficiency mapping data, described in MATERIALS AND METHODS (deficiencies are not shown).

which may have been missed in previous genetic screens that relied on the multivulva and vulvaless mutant phenotypes, we mutagenized wild-type worms with EMS and screened for mutations causing a protruding vulva or Pvl phenotype. The Pvl phenotype (Figure 3B) is characterized by the production of some vulval tissue (as opposed to a vulvaless mutant), but the inability to form a wild-type vulval structure, which results in eversion of vulval tissue and the formation of a single protrusion at the site of the vulva (as opposed to a multivulva mutant that has several ventral protrusions). Mutants exhibiting a similar mutant phenotype (Evl, for defective *e*version of the *vul*va) have been previously described (SEYDOUX *et al.* 1993). We screened the equivalent of 38,300 mutagenized haploid genomes at 20° and identified 230 fertile mutants that display a Pvl phenotype at a penetrance >20%. Most of these mutants are also egg-laying defective (Egl) and can be either Pvl, Egl Pvl, or Egl non-Pvl. For many of the mutants, the Egl phenotype is more penetrant than the Pvl phenotype (Table 3).

In wild-type animals, the three ventral hypodermal cells P5.p, P6.p, and P7.p adopt vulval fates and divide to generate the 22 cells that make up the vulva

TABLE 2

Cell lineages of selected Pvl mutants

Strain	P3.p	P4.p	P5.p	P6.p	P7.p	P8.p
Wild type	S S	S S	LLTN	TTTT	NTLL	S S
	F	S S	LLTN	TTTT	NTLL	S S
bar-1(ga80)	F	F	LLOX	S S	OLLL	S S
	F	F	TTOT	F	NTLL	S S
	F	F	S S	TLTT	OOON	S S
	F	F	LLON	TOTT	NOLL	S S
	F	F	S S	OOOO	S S	F
	F	F	S S	LTTT	NTLN	S S
	F	F	LTTT	NTLL	F	S S
	F	F	LLTN	LTTT	NTLL	S S
	F	F	LLTN	TTTT	NTLL	S S
	F	F	F	OTTO	LLLL	S S
	F	S S	LLLO	NOLL	S S	S S
	F	F	LTTT	NOLL	S S	S S
	F	F	LLTN	TOOT	OOLL	F
mig-14(ga62)	F	F	F	TTTT	NTLL	S S
	F	F	F	TTTT	NTLO	S S
	F	F	LLTN	TTTT	NTLO	S S
	F	F	LTTO	F	S S	S S
	F	F	LLTN	TTTT	NTLO	S S
	F	F	F	TTTT	NTOL	S S
	F	F	OTTO	TTLL	S S	S S
	F	F	LLTT	TTTT	NTLL	S S
	F	F	F	TTTT	OTLL	S S
	F	F	OOTN	TTTT	NTLL	S S
egl-18(ga97)	F	S S	S S	S S	TLOT	S S
	F	F	LLON	OTOO	S S	F
	F	F	NNNN	LTTL	S SNN	S S
	F	S S	LLTN	TOLT	NLLL	F
	F	F	NNNN	OTTT	NLLL	F
	F	F	NNNN	OOOO	TOLL	S S
	F	F	LLTN	TTTT	NOLL	F
	F	F	LLTN	TTTT	NTLL	F
	F	F	NLO	TOTL	S S	F
	F	F	NNNN	TLOT	F	F
egl-18(n475)	F	S S	LLTN	TTTT	F	F
	F	F	LLTN	TTTT	NLLL	F
	F	S S	LOON	TTTT	LLNN	S S
	F	S S	LLTN	TTTT	LLLL	F
egl-18(n162)	F	LLTN	TTTT	NTLL	S S	F
	F	S S	LLTN	TTTT	NTLL	F
	F	F	LLTN	TTTT	NTLL	F
sem-4(ga82)	F	S S	LLTN	TTTO	S S	S S
	F	S S	NNNL	TTTT	NTLL	S S
	F	S S	LLTN	TTTT	NTLL	S S
	F	F	S S	TTTT	S S	S S
	F	F	F	OOOO	S S	S S
sem-4(n1971)	F	F	LLTN	TTTT	S S	S S
	F	F	LLTN	TTTT	NTLL	S S
	F	F	S S	TTTT	S S	S S
	F	F	S S	TTTT	S S	S S
	F	F	LLTN	TTTT	S S	S S

(*continued*)

TABLE 2

(Continued)

Strain	P3.p	P4.p	P5.p	P6.p	P7.p	P8.p
lin-26(ga91)	S S	F	LLLN	TOTT	NLLL	S S
	S S	F	LLLN	TTTT	NLLL	S S
	S S	S S	LLON	TTTO	NOLL	S S
	F	S S	LLLN	TTTT	NOLL	S S
	S S	S S	LLLN	TTTT	NTLL	S S
	F	S S	LLTN	TTTT	NTLL	S S
	S S	S S	LLLN	TTTT	NLLL	S S
	S S	S S	LLLN	LTTT	NLLL	S S
	S S	S S	LLLN	TTTT	NTLL	S S
pvl(ga88)	F	LLLL	TOTO	NTLL	S S	S S
	F	S S	LLON	OOOT	NOLL	S S
	S S	S S	LLON	TTTT	NTLL	S S
	F	S S	LLTN	TOOO	NTLL	S S

Each line shows the final pattern of division or cellular fate for the cells P3.p–P8.p or their descendants in an individual live animal observed with Nomarski differential interference contrast microscopy. The criteria for defining these patterns are described in Sternberg and Horvitz (1986) and Ferguson *et al.* (1987) and in materials and methods. Letters represent the type of final cell division or cell fate adopted by Pn.p descendants: S S, cell divided along the anterior-posterior axis and the two daughters fuse with the hypodermal syncytium; L, cell divided along the longitudinal (anterior-posterior) axis and daughters contributed to vulva formation; T, cell divided along the transverse (left-right) axis and daughters contributed to vulva formation; N, cell did not divide (nondividing); O, cell divided along an oblique axis and daughters contributed to vulva formation; F (Fused), cell did not divide and appeared to fuse with the hypodermal syncytium. TTTT is a wild-type 1° fate, LLTN or NTLL is a wild-type 2° fate, and S S is a wild-type 3° fate.

(Greenwald 1997; Kornfeld 1997). The nuclei of these 22 cells occupy stereotyped positions in the early L4 stage (Figure 2D and Figure 3C). We examined the 230 Pvl mutant strains at this stage using Nomarski differential interference contrast microscopy, and on the basis of the positions of the vulval cell nuclei in these mutants we classified them into two groups: (1) those with altered number or position of the vulval cell nuclei, suggesting defects in generation, fate specification, or fate execution by the VPCs and (2) those with 22 vulval cell nuclei in the proper positions, but which still become Pvl, suggesting defects in later processes such as vulval morphogenesis. Of the 230 mutants, 55 fell into the first class and we continued the characterization of 36 of these mutants that had highly penetrant mutant phenotypes.

On the basis of genetic mapping and complementation tests we have identified 26 mutations in the following previously identified genes: *dig-1, egl-18, let-60, lin-1, lin-2, lin-14, lin-17, lin-18, lin-25, lin-26, lin-28, lin-29, lin-31, mom-3/mig-14, sem-4, unc-59, unc-83*, and *unc-84*. Most of these genes were already known to function in vulval development in some manner. We have further characterized mutations in several of these genes because (1) their role in vulval development was previously not known (*egl-18*, *mom-3/mig-14*), (2) we discovered an additional role for the gene in vulval development distinct from that previously characterized (*lin-26*, *sem-4*), or (3) we identified an interesting allele of a gene (*let-60(ga89)*). *let-60(ga89)* creates a novel temperature-dependent activated Ras protein and is described in Eisenmann and Kim (1997). Our characterization of the vulval defects in *egl-18*, *lin-26*, *mom-3/mig-14*, and *sem-4* mutants is described below.

The remaining mutants appear to identify novel loci based on their map positions, phenotypes, and complementation of mutations in known vulval mutant loci (Figure 1). These mutants identify seven complementation groups, each represented by only a single recessive allele isolated in this screen: *bar-1(ga80)*, *pvl-4(ga96)*, *pvl-5(ga87)*, *pvl-6(ga81)*, *pvl(ga79)*, *pvl(ga84)*, and *pvl(ga88)*. We have continued to characterize these *pvl* loci and below we present our analysis of four of them: *bar-1*, *pvl-4*, *pvl-5*, and *pvl-6*.

***bar-1*, *mom-3/mig-14*, *egl-18*, and *sem-4* mutants have defects in vulval precursor cell fate determination:** During wild-type development P6.p adopts the 1° fate, P5.p and P7.p adopt the 2° fate, and P3.p, P4.p, and P8.p adopt the 3°, nonvulval cell fate. In addition, only P3.p can adopt another fate, the F fate (also called the 4° fate; Clandinin *et al.* 1997), in which P3.p fuses with the hypodermal syncytium in the L3 stage without dividing (Sternberg and Horvitz 1986; Ferguson *et al.* 1987; Eisenmann *et al.* 1998). Cell lineage analysis of animals carrying mutations in the genes *bar-1*, *mom-3/mig-14*, *egl-18*, and *sem-4* shows that in these mutant animals vulval precursor cells other than P3.p can adopt the F fate (Table 2 and Figure 3). This F fate phenotype is most penetrant for the cells P3.p and P4.p in these mutants (Table 2). In addition, in these mutants cells that should

TABLE 3

Phenotypes of strains containing *pvl* mutations

Gene	% WT	% Egl	% Egl/Pvl	% Pvl	% Spew	*N*	% abnormal vulva	% P12.p	% gonad migration	*N*
N2	100	0	0	0	0	Many	0	0	0	Many
bar-1(ga80)	50	20	16	11	3	781	68	90	2	330
bar-1(sy324)	74	5	7	9	5	396	37	89	0	144
mom-3/mig-14(ga62)	14	58	10	0	20	604	75	43	53	110
mig-14(mu71)	71	24	4	1	0	459	11	6	2	104
egl-18(ga97)	5	55	25	5	9	418	62	0	4	115
egl-18(n162)	22	46	27	4	16	434	44	2	6	100
egl-18(n475)	9	31	37	12	11	441	27	0	6	100
sem-4(ga82)	1	97	1	0	1	390	55	12	3	109
sem-4(n1971)	3	89	7	0	1	360	77	11	4	101
pvl-4(ga96)	14	38	18	9	20	317	ND	ND	ND	
pvl-5(ga87)	12	57	5	19	4	317	63	0	0	115
pvl-6(ga81)	42	23	13	15	7	670	54	0	0	100
sem-4(ga82); bar-1(ga80)	5	88	0	0	7	328	99/70	97	10	200
sem-4(ga82); mig-14(ga62)	0	92	2	0	7	419	99/84	90	71	100
sem-4(ga82); egl-18(ga97)	1	75	17	1	5	495	99/23	25	12	100
mig-14(ga62); bar-1(ga80)	9	30	21	5	34	673	87/35	65	66	100
egl-18(ga97); bar-1(ga80)	3	2	13	19	63	551	93/10	80	ND	30

The first column indicates the genotype of each strain analyzed. The next five columns indicate the percentage of animals observed under a dissecting microscope to have the indicated phenotype, and the seventh column shows the number of animals observed. WT, wild type; Egl, egg-laying defective (animals accumulate eggs and/or larvae internally); Pvl, protruding vulva; Spew, animals that have ruptured at the site of the vulva. The next three columns indicate the percentage of animals observed at high power by Nomarski differential interference microscopy to have the indicated mutant phenotype, and the last column shows the number of animals observed. Abnormal vulva, animals with an abnormally shaped vulval invagination at the L4 stage (Figure 3); P12.p, animals in which two large P11.p-like hypodermal nuclei are found immediately anterior to the anus, indicating a P12 to P11 cell-fate transformation (Figure 4, A and B); gonad migration, animals in which one or both arms of the gonad failed to migrate correctly (Figure 4, C–E). For the last five strains (double mutants), in the "% abnormal vulva" column, the first number indicates the percentage of animals with an abnormal vulva and the second number indicates the percentage of animals with no vulval invagination at all (vulvaless). ND, no data.

adopt the 1° and 2° cell fates, P5.p–P7.p, can also adopt the F fate or the 3° nonvulval cell fate ("S S" in Table 2). The result of these two defects is that fewer than 22 vulval cells are formed, leading to the Pvl and/or Egl mutant phenotypes. Three of these loci were identified previously in different genetic screens: *sem-4* (Desai *et al.* 1988; Basson and Horvitz 1996), *egl-18* (Trent *et al.* 1983), and *mom-3/mig-14* (Harris *et al.* 1996; Thorpe *et al.* 1997; Nishiwaki 1999), but their effects on vulval development were not characterized. The fourth gene, *bar-1*, was initially identified in this screen and has subsequently been further characterized (Eisenmann *et al.* 1998; Maloof *et al.* 1999).

***bar-1(ga80)* X:** A single mutation in the *bar-1* gene, *ga80*, was identified in the screen for Pvl mutants. Roughly half of *bar-1(ga80)* mutant animals display an Egl and/or Pvl mutant phenotype (Table 3 and Figure 3B), and analysis of individual *ga80* mutant animals by Nomarski microscopy shows that this is due to defects in cell-fate specification by the cells P5.p, P6.p, or P7.p (Tables 3 and 4, Figure 3F). In most animals, only one of these three cells is affected, although animals in which two or all three of P5.p, P6.p, and P7.p adopted an uninduced cell fate (3° or F) were seen (the latter are true vulvaless animals and are ~10% of *ga80* hermaphrodites; data not shown). In addition, this analysis showed that in *ga80* mutants, the cells P3.p and P4.p adopt the Fused fate in the majority of animals, while the cells P5.p–P8.p adopted this fate less often (Table 2 and Eisenmann *et al.* 1998). Therefore, in *bar-1(ga80)* mutant animals all six vulval precursor cells can exhibit defects in adoption of their proper cell fate, indicating this gene must be required for cell-fate specification by all of the cells of the Vulval Equivalence Group. Additional alleles of *bar-1* have been identified in other genetic screens (*mu38*, *mu349*, Maloof *et al.* 1999; *sy324*, A. Golden, unpublished results).

STS mapping and three-factor crosses indicate that the *bar-1* locus is located between *unc-6* and *dpy-7* on linkage group X. The deficiency *uDf1* fails to complement *bar-1(ga80)*, and the Egl mutant phenotype of these animals is not substantially different from that of *ga80* homozygotes (data not shown), suggesting that *ga80* may be a null mutation. This result was substantiated by the cloning and sequencing of the *bar-1* gene and the determination that the *ga80* mutation causes a premature stop codon early in the predicted open reading frame (Eisenmann *et al.* 1998). The locus identified

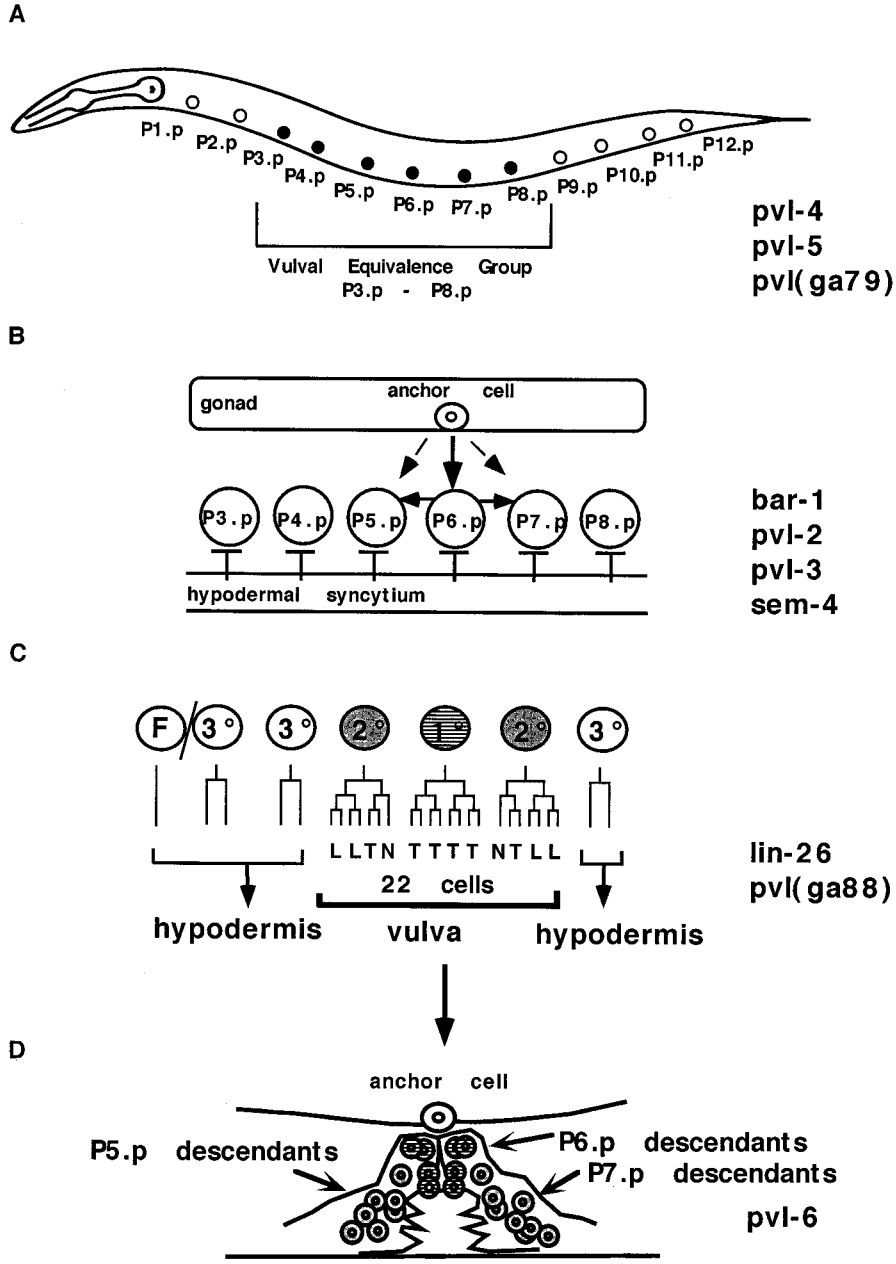

FIGURE 2.—Four stages of *C. elegans* vulval development. (A) Generation of P1.p–P12.p during the L1 stage. P3.p–P8.p make up the Vulval Equivalence Group. The remaining six cells fuse with the hypodermis of the animal during the L1 stage. (B) In the late L2 stage, the fates of the six vulval precursor cells (P3.p–P8.p) are determined in response to several extracellular signals: an inductive signal from the anchor cell, a lateral signal acting between Pn.p cells, and a general inhibitory signal acting between the Pn.p cells and the surrounding hypodermal syncytium. The outcome of these signaling events is that P6.p adopts the 1° vulval cell fate, its neighbors, P5.p and P7.p, adopt the 2° vulval cell fate, and P3.p, P4.p, and P8.p adopt the 3°, nonvulval cell fate. Note that P3.p also adopts the F fate 50% of the time in wild-type animals. (C) The vulval precursor cells execute their cell fates in the L3 stage. Shown are P3.p–P8.p with their patterns of cell division and the fate of their progeny cells (vulval or hypodermal). For P5.p–P7.p, the letters indicate the plane of the axis of the last cell division (see Table 2). (D) The 22 vulval cells go through morphogenetic movements during the L4 larval stage to form the vulval opening. A diagram of an intermediate stage in that process, referred to as the "Christmas tree" stage, is shown here. The 8 cells descended from P6.p occupy the center of the developing vulva, and the 7 cells each descended from P5.p and P7.p occupy the sides of the developing vulva. To the right of each diagram are indicated the genes examined in this work that are likely to function at that stage in vulval development.

by the *ga80* mutation was named *bar-1*, after the determination that the protein product is related to the β-catenin/Armadillo family of proteins, which are known to function in Wnt signaling pathways. We have previously reported that the defect in vulval cell-fate specification in *bar-1* mutants may be due to a defect in maintenance of expression of the Hox gene *lin-39* in these cells (EISENMANN *et al.* 1998).

In addition to the Egl/Pvl phenotype, *bar-1* mutant animals have several other phenotypes. First, *bar-1* mutants have a defect in cell-fate specification by the posterior ectodermal cell P12 (Table 3). In wild-type animals, the most posterior Pn.p cell, P12.p, divides during the L1 stage to give rise to a posterior daughter, P12.pp, which undergoes a programmed cell death, and an anterior daughter P12.pa, which becomes the hypodermal cell hyp12 (SULSTON and HORVITZ 1977). When observed in the L2 stage, the nucleus of P12.pa is smaller than that of the other undivided Pn.p cells, which all have a large nucleus (Figure 4A). In *bar-1* mutants a cell corresponding in nuclear morphology to P12.pa is not found and a second, large nucleus resembling P11.p is found in the same location (Figure 4B). This phenotype is due to a transformation of cell fate in the cell P12 to that of the cell P11 (FIXSEN *et al.* 1985). Mutations in components of a Ras signaling pathway, components of a Wnt signaling pathway, and the Hox genes *mab-5* and *egl-5* all affect cell-fate determination by this cell (see DISCUSSION; JIANG and STERNBERG 1998, and references therein). Second, *bar-1* mutant animals have a defect in

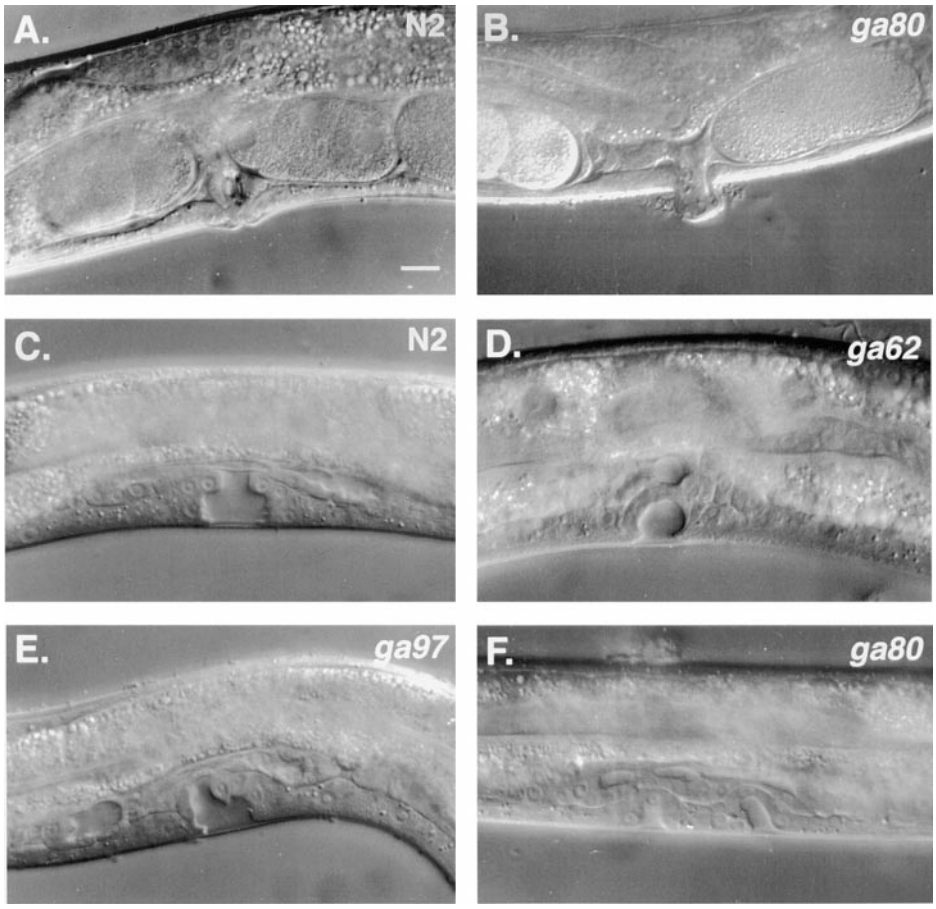

Figure 3.—Vulval phenotypes of Pvl mutants. Wild-type adult hermaphrodite (A) and Pvl adult hermaphrodite (B) showing the ventral mid-body region with the wild-type vulval opening (A) and the protruding vulva (Pvl) phenotype (B). Wild-type L4 stage animal showing the Christmas tree stage of vulva development (C) and similarly staged animals showing defective vulval induction in *mom-3/mig-14* (D), *egl-18* (E), and *bar-1* (F) mutants. In these photos anterior is to the left and dorsal is to the top. Bar, 20 μm. In these mutant animals fewer than three Pn.p cells adopted induced cell fates only P6.p in D, P6.p and P7.p in E, and P5.p and P7.p in F.

the migration of the progeny of the neuroblast Q_L (Figure 4G). This *bar-1* phenotype, in which the progeny of Q_L migrate incorrectly and behave like the progeny of Q_R, the sister cell of Q_L, has been described in detail (Maloof *et al.* 1999). Mutations affecting Wnt signaling pathway components and the Hox gene *mab-5* cause the same type of Q_L progeny migration defect as that seen in *bar-1(ga80)* (Harris *et al.* 1996; Maloof *et al.* 1999). Third, *bar-1* animals exhibit an incompletely penetrant uncoordinated phenotype (Unc). Most *bar-1(ga80)* animals move forward well but do not move backward as well as wild-type animals and often coil upon themselves (data not shown). Finally, *bar-1(ga80)* mutant males have a reduced efficiency of mating, as many more *bar-1(ga80)* males than wild-type males are routinely required to successfully sire cross-progeny (data not shown). Many mutations that affect cell-fate determination during vulval formation in the hermaphrodite are also known to affect cell-fate determination during formation of the male tail, leading to defects in male mating (Emmons and Sternberg 1997).

***mom-3/mig-14(ga62)* II:** Mutant animals carrying the mutation *ga62* display a range of phenotypes similar to those of *bar-1(ga80)* mutants. The majority of *ga62* mutant animals have an Egl or Egl Pvl phenotype due to defects in vulval precursor cell-fate determination (Tables 2 and 3 and Figure 3D). Specifically, the cells that give rise to the vulva can adopt either the F fate, the 3° fate, or abnormal fates causing too few vulval cells to be generated, and the cells P3.p and P4.p usually adopt the F fate. *ga62* mutants also have defects in P12 cell-fate specification (Table 3) and Q_L progeny migration (Figure 4H), and *ga62* males mate poorly (data not shown). In addition, *ga62* animals exhibit a defect in gonad migration only rarely seen in *bar-1* mutants (Table 3 and Figure 4, D and E).

Molecular and genetic mapping data indicate that the locus identified by *ga62* is located on the right arm of linkage group II. Two previously identified loci, *mig-14* and *mom-3*, map to the same region. A single mutation in *mig-14*, *mu71*, was identified in a screen for mutants with misplaced Q descendants (Harris *et al.* 1996). Our observation that *mu71* mutants have a weak Egl phenotype and show defects in vulval cell and P12 fate determination (Table 3), coupled with the observation that *ga62* mutants have defects in the migration of the progeny of Q_L, suggested that *mu71* and *ga62* might be allelic. Indeed, we found that these two mutations fail to complement for the Egl phenotype (data not shown), suggesting that *ga62* is a new allele of *mig-14*.

Mutations in the gene *mom-3* cause a maternal-effect embryonic lethal phenotype characterized by a conversion of endoderm to mesoderm (Thorpe *et al.* 1997). Although the *mom-3* gene product has not been identified, loss of activity of the genes *mom-2* (Wnt), *mom-5* (Frizzled-type receptor), *wrm-1* (β-catenin), and *pop-1*

TABLE 4

pvl* mutations suppress *let-60(n1046)

Genotype	% Muv	% Pvl	% WT	*N*
N2	0	0	100	Many
let-60(n1046)	93	0	7	300
let-60(n1046); bar-1(ga80)	8	46	46	456
mig-14(ga62); let-60(n1046)	4	46	50	315
egl-18(ga97) let-60(n1046)	47	20	33	463
sem-4(ga82); let-60(n1046)	25	14	61	405

The first column indicates the genotype of each strain examined. The next three columns indicate the percentage of animals having either a multivulva (Muv, multiple ectopic ventral protrusions), protruding vulva (Pvl), or wild-type (WT) phenotype. The last column shows the number of animals examined by dissecting microscope. Only viable adult animals were scored.

(HMG box/LEF factor) also disrupts this cell-fate decision, indicating a Wnt signaling pathway is functioning in this process (Rocheleau *et al.* 1997; Thorpe *et al.* 1997). Homozygous *mom-3* mutant animals, in addition to producing dead embryos due to maternal requirements for endoderm induction, also display a protruding vulva phenotype, consistent with a zygotic requirement for *mom-3* during larval development (Thorpe *et al.* 1997). On the basis of the observations that 20% of *ga62* homozygous animals die during embryogenesis (data not shown) and that *ga62* causes phenotypes similar to those caused by mutation of the β-catenin homolog, BAR-1, we performed a complementation test to determine whether *ga62* might be a reduction-of-function mutation in *mom-3*. We found that rare *ga62/mom-3(or78)* animals were very sick, Unc, and Pvl and gave few or no viable progeny, indicating that *ga62* and *or78* fail to complement for both the Pvl phenotype and a maternal-effect embryonic lethal phenotype (see materials and methods). Therefore *ga62* is also likely to be a viable allele of *mom-3*. Since no deficiency exists in the *ga62/mom-3/mig-14* genetic interval, we have been unable to test whether *ga62*/deficiency animals have more severe phenotypes, perhaps resembling those of *ga62/mom-3(or78)*. However, our interpretation of the complementation data is that *mu71*, *ga62*, and *or78* are likely to be mutations in a single locus and that *mu71* and *ga62* retain more wild-type function than *or78*. Another allele of this gene, *k124*, was recently identified in a screen for mutations causing a gonad migration defect (Nishiwaki 1999). We refer to this gene as *mom-3/mig-14* and conclude that *mom-3/mig-14* gene function is necessary for the vulval precursor cells and other cells to specify their fates correctly.

***egl-18(ga97)* IV:** The majority of animals homozygous for the *ga97* mutation display an Egl, Pvl, or Egl Pvl phenotype (Table 3). Cell lineage analysis of *ga97* hermaphrodites showed that the basis for these phenotypes was similar to that for *bar-1(ga80)* and *mom-3/mig-14(ga62)*; P5.p–P7.p can adopt the Fused or 3° cell fates instead of the 1° or 2° cell fate (Table 2 and Figure 3). In addition, P3.p, P4.p, and P8.p adopted the Fused fate most of the time. *ga97* mutants also show another phenotype affecting the vulval precursor cells: in this strain P5.p (and less frequently P7.p) began to divide but did not complete three rounds of cell division (Table 2; *N* = nondivided). The cells that were generated did not participate in vulval formation and began to lose their characteristic "hypodermal" nuclear morphology (data not shown). Unlike *bar-1(ga80)* and *mom-3/mig-14(ga62)* mutants, *egl-18(ga97)* mutants do not display a highly penetrant defect in cell-fate determination by P12 (Table 3) or in migration of the Q_L progeny (data not shown). However, like these other mutants, *ga97* males mate poorly (data not shown). In addition, *ga97* mutant animals display a low penetrance Roller phenotype (Rol) at all stages (8%, *n* = 250). Observation of *egl-18(ga97)* L4 stage larvae by Nomarski microscopy showed that those animals that display the Rol phenotype have an abnormally twisted head region, suggesting a defect in the cuticle or hypodermis in this region (data not shown).

The *ga97* mutation was mapped to the left arm of linkage group IV between *dpy-9* and *lin-1*. The gene *egl-18*, which was identified in a screen for mutants exhibiting an Egl phenotype, also maps in this region (Trent *et al.* 1983). Previous analysis noted that *egl-18* mutants sometimes adopt a "bag of worms" phenotype; however, the nature of the vulval defect in *egl-18* mutants was not determined. We found that the existing *egl-18* alleles *n162* and *n475* fail to complement *ga97* for the Egl and Rol phenotypes, and *egl-18(n162)* and *egl-18(n475)* mutant animals show the same types of defects in vulval cell-fate specification as observed for *ga97* mutants (Table 2). These results indicate that *ga97* is a new allele of *egl-18* and that the *egl-18* locus is required for vulval precursor cells to undergo proper cell-fate specification. Since no deficiency exists for the genetic interval containing *egl-18*, we do not know if the defects in vulval induction we have observed in *ga97*, *n162*, and *n475* represent the null phenotype for this locus.

***sem-4(ga82)* I:** The mutation *ga82* causes a highly penetrant Egl phenotype and very rarely causes a Pvl phenotype (Table 3). Cell lineage analysis of *ga82* mutant

animals shows that P5.p and P7.p sometimes adopted the 3° fate inappropriately, leading to fewer than three Pn.p cells adopting induced fates, and that P3.p and P4.p often adopted the F fate (Table 2). Finally, *ga82* mutants also display a P12 to P11 cell-fate transformation, but at much lower penetrance than that seen in *bar-1* or *mom-3/mig-14* mutants (12%; Table 4). *sem-4(ga82)* mutants do not display an obvious Q_L descendant migration defect (data not shown).

Genetic and physical mapping placed the *ga82* mutation on linkage group I between *dpy-5* and *unc-13*, a location containing the locus *sem-4*, which encodes a putative zinc-finger transcription factor (Basson and Horvitz 1996). *sem-4* mutant animals were identified by their sex muscle migration-defective (Sem) phenotype, but also have defects in several other cell-fate specification processes. The lack of appropriately placed sex muscles leads to a highly penetrant Egl phenotype in *sem-4* mutants. In addition, it was noted that *sem-4* mutants display precocious divisions of Pn.p cells, resulting in extra hypodermal cells in the ventral mid-body region (Basson and Horvitz 1996). The defect in vulval cell-fate specification described here was not noticed in the previous analysis; however, we observed the same type of defect in animals containing *sem-4(n1971)* (Table 2), a mutation believed to be a null mutation on the basis of genetic and molecular criteria (Basson and Horvitz 1996). We found that *sem-4(n1971)* and *ga82* fail to complement for the egg-laying defective phenotype, indicating that *ga82* is a new allele of *sem-4*. This suggests that *sem-4* activity is required for the vulval precursor cells to adopt their cell fates correctly.

Double-mutant analysis: We constructed several double-mutant strains containing mutations in two of the loci *bar-1*, *mom-3/mig-14*, *egl-18*, and *sem-4* (Table 3). Since these mutations cause the same type of phenotype the utility of this type of analysis is limited; however, two observations are worth noting.

First, in double mutants containing the *sem-4(ga82)* mutation and either *bar-1(ga80)* or *mom-3/mig-14(ga62)*, a synthetic vulvaless phenotype was observed when the double-mutant animals were examined by Nomarski microscopy. In general, animals singly mutant for *bar-1*, *mom-3/mig-14*, or *sem-4* show some vulval invagination at the L4 stage because at least one cell adopts an induced fate (Table 2 and Figure 3). For each of these single mutants the percentage of animals in which none of the vulval precursor cells adopts an induced fate (a vulvaless phenotype) is <10% (Table 2 and data not shown). However, most animals display no vulval invagination in the *sem-4(ga82); mom-3/mig-14(ga62)* and *sem-4(ga82); bar-1(ga80)* double-mutant strains when observed by Nomarski microscopy (70% for *sem-4; bar-1* and 84% for *sem-4; mom-3/mig-14*; $n = 100$), and most animals display the "bag of worms" phenotype characteristic of a vulvaless mutant phenotype.

Second, we also observed that each of these mutations causes a low penetrant "spewed gonad" phenotype in which bodies are found on the plate containing their gonads everted out through the vulval opening (20%; Table 3). These animals are often smaller than the living adults, suggesting that this defect may have manifested at the L4 to adult molt. We found that in the *egl-18(ga97); bar-1(ga80)* double mutant the penetrance of the spewed gonad phenotype was higher (63%) than expected from addition of the single-mutant phenotypes. We believe that this synergistic spewed gonad phenotype also prevented us from isolating a double-mutant strain containing *egl-18(ga97)* and *mom-3/mig-14(ga62)* (data not shown). The cellular basis for this mutant phenotype is currently not known, although it may indicate a role for these loci in later steps in vulval development.

Mutations in *bar-1*, *mom-3/mig-14*, *egl-18*, and *sem-4* suppress the multivulva phenotype caused by an activated Ras mutation: Since the mutations described above cause defects in cell-fate specification by the vulval precursor cells, a process known to be regulated by a Ras signaling pathway, we determined whether the activity of these genes was necessary for cell-fate specification by the Ras pathway. To address this we built double-mutant strains containing each of the four mutations in combination with an activated Ras mutation, *let-60(n1046)* (Ferguson and Horvitz 1985; Beitel *et al.* 1990). *let-60(n1046)* causes all six vulval precursor cells to adopt the 1° and 2° induced cell fates, leading to a highly penetrant Muv phenotype (93%; Table 4). If signaling through the Ras pathway requires the activity of these four genes, then the Muv phenotype caused by the ectopic activation of that pathway by *let-60(n1046)* should be decreased when the *pvl* mutations are introduced. This was the case (Table 4), since both *bar-1* and *mom-3/mig-14* mutations strongly reduced the penetrance of the Muv phenotype caused by *let-60(n1046)* (from 93 to <10%), and *egl-18* and *sem-4* mutations also reduced the Muv phenotype to <50%. Since activation of *let-60* Ras cannot bypass the requirement for these four genes, this suggests that *bar-1*, *mom-3/mig-14*, *egl-18*, and *sem-4* are required for cells to respond to activation of the Ras pathway and adopt induced cell fates.

***lin-26(ga91) II* mutants have defects in adoption of the 2° vulval cell fate:** During the execution stage Pn.p cells divide to generate progeny cells in a manner indicative of the fate they adopted during the cell-fate specification stage (Greenwald 1997). For example, a cell adopting the 2° fate divides in the manner LLTN (or NTLL), where L refers to a longitudinal, or anterior-posterior, cell division, T indicates a transverse, or left/right, axis of division, and N indicates a nondividing cell. We identified a mutation, *ga91*, which causes a defect in the execution stage of vulval development.

ga91 mutant animals have an almost completely penetrant protruding vulva phenotype (>98%, $n = 400$). Cell lineage analysis of *ga91* mutant animals showed that generally in these animals the correct number of

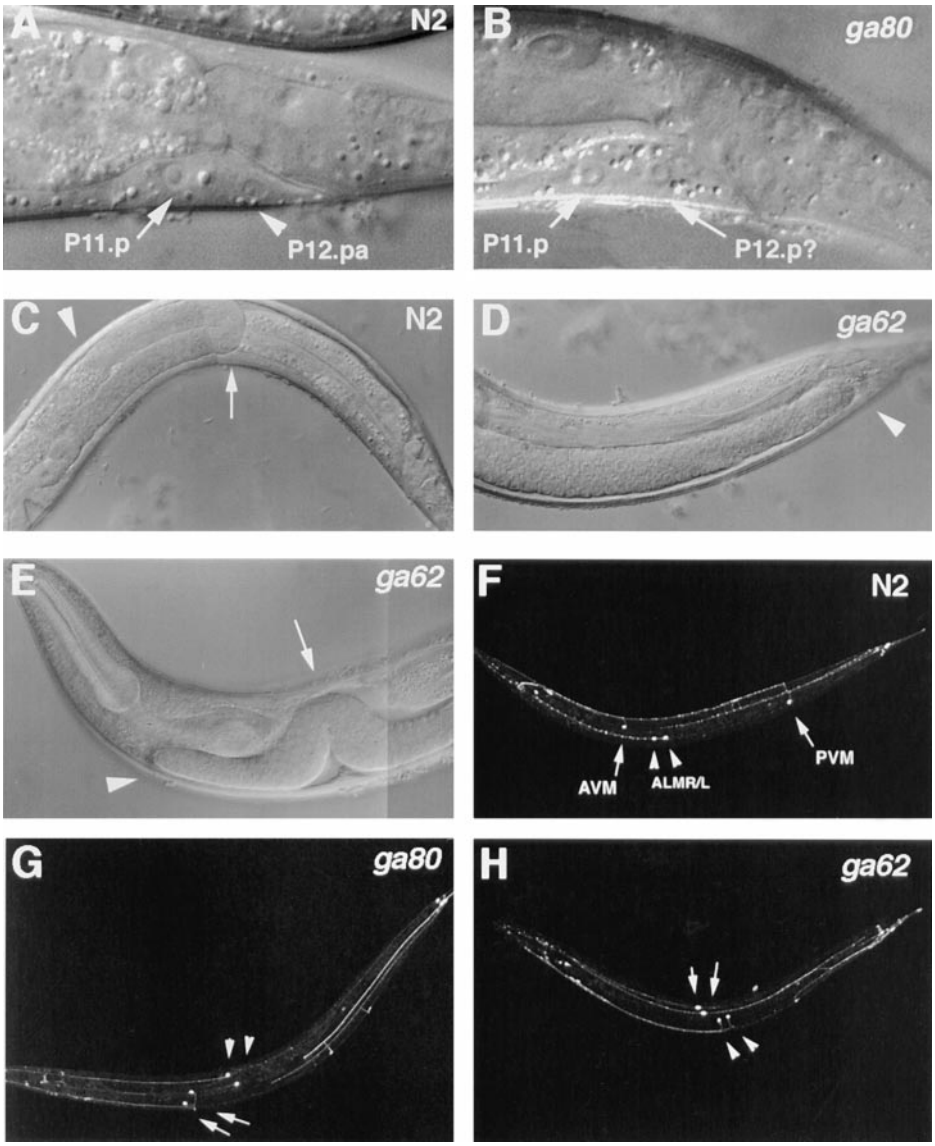

FIGURE 4.—P12, gonad migration, and Q_L migration phenotypes of *bar-1(ga80)* and *mom-3/mig-14(ga62)* mutants. (A–B) Posterior hypodermal cells in wild-type (A) and *bar-1* mutant animals (B). (A) In wild type, P11.p has a large nucleus with a large nucleolus visible inside it, a nuclear morphology characteristic of the cells P1.p–P11.p. The cell P12.pa (anterior daughter of P12.p) has a noticeably smaller nucleus and nucleolus. (B) In *bar-1(ga80)* and *mom-3/mig-14(ga62)* mutants (not shown) two large nuclei are seen, characteristic of a transformation of the fate of the cell P12.p to that of P11.p. (C) Migration of the posterior arm of the gonad in a wild-type hermaphrodite at the L4 stage. The arrowhead points to the leading edge of the migrating gonad arm (the distal tip cell). This gonad arm began by migrating toward the posterior, then turned dorsally (indicated by the arrow), and finally turned back toward the anterior. (D) Defective posterior gonad arm migration in a *ga62* mutant animal. In this animal the posterior gonad arm did not reflex dorsally and continued to migrate into the tail of the animal. (E) Defective migration of the anterior gonad arm into the head in a *mom-3/mig-14(ga62)* mutant animal. (F–H) Expression of the touch receptor reporter construct *mec-7::GFP* in wild type, *bar-1*, and *mom-3/mig-14* mutants. The Q_L neuroblast is born in the posterior of the animal and migrates further posteriorly, dividing to generate three progeny neurons. One of these cells, PVM (Q_L.paa), is a touch receptor neuron in the posterior of the animal that expresses the *mec-7::GFP* fusion protein (CHALFIE *et al.* 1994). The sister of Q_L, the neuroblast Q_R, divides in the same pattern but migrates toward the anterior. Q_R generates the touch receptor neuron AVM in the anterior, which expresses *mec-7::GFP*. (F) GFP expression pattern of a wild-type animal carrying the *mec-7::GFP* transgene (*muIs35*). The cell bodies of Q_R- and Q_L-derived touch receptor neurons AVM and PVM are indicated (arrows). Also shown are ALMR and ALML (not distinguished), two other touch receptor neurons in the mid-body that also express the *mec-7::GFP* fusion at this time. In *bar-1* (G) and *mom-3/mig-14* (H) mutants at this stage, there is no large PVM-like cell body expressing GFP in the posterior, but there are now two GFP-expressing cell bodies (arrows) anterior to AVML/R (arrowheads), consistent with Q_L and its progeny having migrated toward the anterior as Q_R does.

Pn.p cells adopted induced cell fates, but that the most animals displayed a subtle defect in the execution of the 2° cell fate by P5.p and/or P7.p. Only 4 of 18 cells adopting the 2° fate divided in the correct pattern. Instead P5.p and P7.p most often divided with the pattern LLLN (P5.p) or NLLL (P7.p) (Table 2 and Figure 5). This indicates that the fate of the cells that would normally adopt the 2° T fate (P5.ppa and P7.pap) has been altered in these animals. The P6.p descendant cells that divide transversely were not usually affected (Table 2). It is not clear whether this 2° cell-fate defect is the cause of the strong Pvl phenotype of these animals; however, *ga91* animals have no other obvious defects.

We were surprised to find that *ga91*, which maps to the *dpy-10-unc-4* interval on LG II, failed to complement *lin-26(n156)*. The *ga91/n156* transheterozygote has a Pvl/Egl phenotype like that of *ga91* homozygous animals (data not shown). Sequence analysis has verified that *ga91* represents a missense mutation in the *lin-26* open reading frame (DUFOURCQ *et al.* 1999). *lin-26* encodes a putative zinc-finger transcription factor that is expressed in the nuclei of all hypodermal cells and is believed to be a general factor required for hypodermal differentiation. The phenotype of the previously isolated *lin-26* alleles (such as *n156*) precluded the identification of this phenotype because these mutations

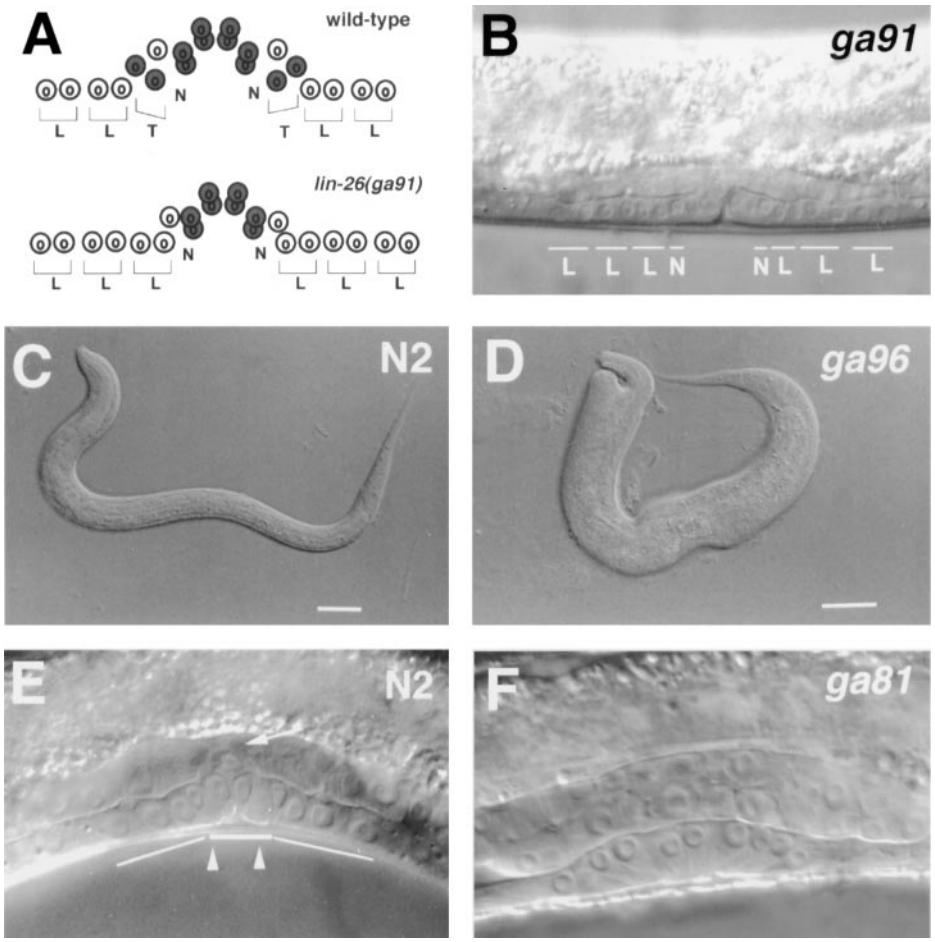

FIGURE 5.—Phenotypes of *lin-26(ga91)*, *pvl-4(ga96)*, and *pvl-6(ga81)* mutants. (A) Diagram showing the location of the progeny of P5.p–P7.p after their third rounds of division in wild-type and *lin-26(ga91)* mutants. Cells located along the anterior-posterior midline [the result of a longitudinal division (L)] are in white; cells located to left or right of the midline [the result of a transverse division (T)] are shown shaded. (B) Nomarski photomicrograph of a *lin-26(ga91)* mutant in which both P5.p and P7.p executed the 2° cell fate incorrectly. (C) Wild-type L1 stage larva. (D) L1 stage *pvl-4(ga96)* larva. Note the defects in morphology at the head and in the mid-body (Vab phenotype). (E) Nomarski photomicrograph of a wild-type animal after the second round of divisions of the cells P5.p–P7.p (L3 stage). White lines indicate the four progeny of each vulval precursor cell. The cells P6.paa and P6.ppp have begun dividing, and their nuclei are no longer distinct (arrowheads). The anchor cell of the somatic gonad is visible (arrow) and has crossed the basement membrane separating the gonad from the hypodermis and is contacting the inner two daughters of P6.p, which have begun to invaginate upward. (F) *pvl-6(ga81)* mutant at a slightly later stage of development than the animal in E. In this animal the anchor cell has not descended and is not obvious, and the progeny of P5.p, P6.p, and P7.p have recently finished their divisions. Although 22 vulval cells are present, only one focal plane is shown. Although this animal is at a later stage than the animal in E, the VPCs have not detached from the cuticle and begun to migrate dorsally. In all photos, anterior is to the left and dorsal is to the top.

cause the absence of Pn.p cells (LABOUESSE *et al.* 1994; FERGUSON and HORVITZ 1985). This result indicates that *lin-26*, which is required early in development for the generation of the Pn.p cells, is also likely to play a later role in vulval development during the execution of the 2° fate.

***pvl(ga88)*:** Another mutation, *pvl(ga88)*, also shows a defect in Pn.p cell-fate execution and appears to affect predominantly cells dividing along the T axis. In *ga88* mutant animals, the cells that would normally divide along the transverse (left-right) axis during the third divisions for the cells P5.p–P7.p are seen to divide along the longitudinal axis or in an oblique manner (not along any of the three defined axes; Table 2).

***pvl-4 II* and *pvl-5 II* mutants have defects in the generation of the Pn.p cells:** In wild-type hermaphrodites at the L2 stage there are 11 large hypodermal nuclei along the ventral midline from anterior to posterior (the nuclei of P1.p–P11.p) and one smaller hypodermal nucleus (the nucleus of P12.pa; SULSTON and HORVITZ 1977). Animals carrying recessive mutations in the genes *pvl-4* and *pvl-5* have too few Pn.p cells present in the ventral hypodermal region at this time. *pvl-4(ga96)* mutants have an average of 7.7 Pn.p nuclei in the ventral cord ($N = 50$, range of 6–11) and *pvl-5(ga87)* mutants have an average of 7.4 Pn.p nuclei ($N = 50$, range of 4–10). Often in these mutants, fewer than three of the Pn.p cells that are generated adopt vulval fates, leading to the Pvl/Egl phenotype. To determine if the decrease in Pn.p cell numbers seen in *pvl-4(ga96)* and *pvl-5(ga87)* animals represents the null phenotype for both of these loci, we placed each mutation over a chromosomal deficiency. We found that in *pvl-5(ga87)/mnDf30* animals, Pn.p cells are still generated, and the number of Pn.p nuclei in these animals is similar to that in *pvl-5(ga87)* animals (P. JOSHI and D. EISENMANN, unpublished results). This suggests that either loss of *pvl-5* does not completely abolish Pn.p cell generation and that *ga87* represents a null mutation in *pvl-5* or that the *ga87* mutation is a non-null mutation that, when hemizygous, still provides enough *pvl-5* activity to allow the process to occur. The isolation of additional *pvl-5* mutations and the molecular characterization of the *pvl-5* gene should allow us to distinguish between these possibili-

ties. We have not determined the number of Pn.p cells in *pvl-4(ga96)/Df* animals because these animals generally do not develop to the L2 stage (see below), suggesting that *ga96* is a hypomorphic allele of *pvl-4*.

We do not know the reason for the decrease in Pn.p cell nuclei in *pvl-4* and *pvl-5* mutants. In neither of these mutants are there Pn.p-like hypodermal nuclei present in the dorsal region as in *unc-83* and *unc-84* mutants (Ferguson and Horvitz 1985). In addition, staining with antibodies to the transcription factor LIN-31, which is present in the nuclei of P1.p–P11.p, shows that the only LIN-31-expressing nuclei are present in the ventral hypodermal region, and the number of LIN-31-expressing nuclei is comparable to the number of hypodermal nuclei observed by microscopy (data not shown).

In addition to the defect in Pn.p cell number, *pvl-4(ga96)* animals also have defects in head and body morphology. In particular, 52% ($N = 418$) of *pvl-4* L1 and L2 larvae have a bent or notched head phenotype similar to that described for Vab mutants (Chisholm and Horvitz 1995; Figure 5D). In addition, some *pvl-4(ga96)* early L1 larvae have a more severe phenotype characterized by a general misshapen or lumpy appearance. These larvae generally fail to thrive (data not shown). The specific nature of these defects is not known, but may indicate defects in other hypodermal cells besides the Pn.p cells in *pvl-4* mutants. Further, when *pvl-4(ga96)* is placed *in trans* to the deficiency *mnDf83*, only a few lumpy or misshapen *ga96/mnDf83* larvae are found, which die as young L1 animals (data not shown). This suggests that the null mutant phenotype of *pvl-4* may be embryonic and/or larval lethality and that *ga96* is a hypomorphic allele. *pvl-4(ga96)* complements the known *let* genes located in the same genetic interval, suggesting *ga96* is likely to identify a novel locus.

***pvl(ga79) IV*:** Unlike *pvl-4* and *pvl-5* mutants, *pvl(ga79)* animals have too many Pn.p cell nuclei present in the ventral midline region. *pvl(ga79)* animals have an average of 12.8 Pn.p-like nuclei in the L2 ($N = 20$; range 12–14), suggesting that the extra cell(s) arise prior to this stage, perhaps from the precocious division of one or more of P1.p–P11.p during the L1 or L2, as is seen in *lin-25*, *lin-31*, and *sem-4* mutants (Miller *et al.* 1993; Tuck and Greenwald 1995; Basson and Horvitz 1996). In addition to this defect in Pn.p cell number *pvl(ga79)* animals also often have a misshapen vulval structure (data not shown); however, the exact nature of the vulval defect for *pvl(ga79)* has not been determined.

***pvl-6 V* mutants display an altered interaction between the anchor cell and the descendants of P6.p:** During wild-type vulval development, the first morphogenetic movements begin in the L3 stage when the cells P5.p, P6.p, and P7.p have each divided twice to generate a total of 12 Pn.pxx cells (Sulston and Horvitz 1977; Greenwald 1997). At this time, the anchor cell, which is centered over the four P6.p descendants, moves ventrally and appears to cross over the basement membrane separating the gonad from the hypodermis. At the same time, the inner two P6.p descendants, P6.pap and P6.ppa, move dorsally toward the descending anchor cell (Figure 5E; Sulston and Horvitz 1977; Sharma-Kishore *et al.* 1999). As these two cells move inward from the ventral midline, the remaining progeny of P6.p and some P5.p and P7.p progeny follow. At the same time, these cells begin their third round of divisions (Figure 5E), such that at the end of this process the 22 vulval cells have been generated and the Christmas tree-shaped vulval structure is formed (Figure 3C).

In animals carrying the *pvl-6(ga81)* mutation, the interaction between the anchor cell and the descendants of P6.p is often abnormal, presumably leading to the protruding vulva phenotype. We directly observed vulval development in eight *pvl-6(ga81)* animals and found that at the time during wild-type development when the anchor cell makes contact with P6.pap and P6.ppa, in six of eight *ga81* animals the anchor cell had not descended to make contacts with these cells and these cells did not move dorsally toward the somatic gonad. These cells and the other Pn.pxx cells went on to divide and generate 22 cells; however, in the absence of morphogenetic movements by the P6.p descendants no vulval invagination was seen (Figure 5F). Subsequent observation of these *ga81* mutants showed that the anchor cell did descend ventrally in these animals, sometimes making the correct contacts with the P6.pxxx cells and forming a wild-type-looking vulva (data not shown). In the remaining two of eight animals we observed that the anchor cell descended ventrally at the correct time, but did not make contact with P6.pap and P6.ppa and instead was displaced anteriorly or posteriorly and made contacts with other Pn.pxx cells at the 12-cell stage. In these animals the vulval structure that was formed was often misshapen (data not shown). Therefore, in *pvl-6* mutants the defect is not in the generation of the vulval precursor cells, or in the adoption and execution of cell fates by those cells, but in the later process of vulval morphogenesis.

DISCUSSION

Here we describe the results of a genetic screen designed to identify novel loci functioning during *C. elegans* vulval development. We chose the Pvl phenotype as the basis for a new screen in an attempt to identify genes functioning at any stage of vulval development that may have been missed in previous genetic screens. Two reasons validate the choice of Pvl as a useful phenotype for this approach. First, the 36 mutations we isolated that have defects in the number or placement of the 22 vulval cell nuclei were either mutations in previously identified genes known to function in vulval development or identified novel loci. Second, preliminary characterization of the mutants described here shows that

mutations affecting all four stages in vulval development can lead to a Pvl phenotype. Additionally, the fact that we isolated only a single allele for each novel locus suggests that this screen was not saturated and that additional loci may be found by continuing to identify mutants exhibiting this phenotype. We have initially concentrated our attention on several loci identified in this screen, some of which represent known loci not previously shown to function in vulval development and others that represent loci described here for the first time.

Genes affecting generation of the Pn.p cells: During the L1 stage, the embryonically derived P cells divide to generate the 12 Pn.p cells, P1.p–P12.p. Six of the 12 Pn.p cells, P3.p–P8.p, constitute the VPCs, so mutations affecting the generation of the Pn.p cells can lead to defects in vulval development. Previous genetic analysis has identified several genes that affect the generation of the proper number of VPCs in the ventral midline when mutated. These include mutations that affect the migration of the P cells or their nuclei (*unc-83*, *unc-84*, *unc-40*; Ferguson and Horvitz 1985; Chan *et al.* 1996; Malone *et al.* 1999), the division of the P cells (*lin-26*; Ferguson and Horvitz 1985; Labouesse *et al.* 1994), and the specification of Pn.p cells as VPCs (*lin-39*; Clark *et al.* 1993; Wang *et al.* 1993). In addition, mutations in *lin-25*, *lin-31*, and *sem-4* result in too many Pn.p-like nuclei in the ventral hypodermal region because some Pn.p cells divide precociously during the L1 stage (*lin-25*) or L2 stage (*lin-31* and *sem-4*; Miller *et al.* 1993; Tuck and Greenwald 1995; Basson and Horvitz 1996).

We describe here two mutations, *pvl-4(ga96)* and *pvl-5(ga87)*, that cause too few Pn.p nuclei to be present in the ventral midline at the L2 stage. We currently do not know the process that is defective in either *pvl-4* or *pvl-5* mutants. For example, defects in P cell survival, migration, or division or in Pn.p cell survival or differentiation could all result in too few Pn.p-like nuclei being present. However, in neither *pvl-4(ga96)* nor *pvl-5(ga87)* animals are obviously mislocalized P cell nuclei seen, and the only cells that express a transcription factor found in P1.p–P11.p (Tan *et al.* 1998) are in the ventral cord in these mutants, suggesting that Pn.p cells are not born in other locations in these mutants. Preliminary data suggest that the egg-laying defect of *pvl-5(ga87)* mutants is partially suppressed by a mutation in the programmed cell death gene *ced-3*, suggesting that abnormal cell death of P or Pn.p cells may occur in *pvl-5* mutants (P. Joshi and D. Eisenmann, unpublished results). This suggests a possible function for *pvl-5* in keeping the Pn.p cells from undergoing inappropriate cell death.

It is likely that the vulval phenotype seen in *pvl-4(ga96)* animals does not represent the *pvl-4* null phenotype, since *pvl-4(ga96)/mnDf83* animals do not survive and have severe defects in body morphology. The defects in head and body morphology seen in *pvl-4(ga96)* animals [the Vab phenotype (Brenner 1974)] are similar to those seen in *vab-1* and *vab-2* mutants, which encode an Ephrin receptor and an Ephrin ligand, respectively (George *et al.* 1998; Chin-Sang *et al.* 1999). *pvl-4(ga96)* complements known lethal mutations in the genetic region where it maps and does not map near other predicted Ephrin homologs (Wang *et al.* 1999), suggesting that *pvl-4* may represent a novel locus that is required for the structure, function, or differentiation of hypodermal cells, including the P and/or Pn.p cells. Future experiments will help determine how these two mutants function in Pn.p cell generation.

Genes acting during vulval precursor cell-fate specification: The majority of genes identified in previous genetic screens for vulval mutants appear to act during the stage of vulval precursor cell fate specification (reviewed in Greenwald 1997; Kornfeld 1997). Mutations in these genes generally cause either a multivulva phenotype or a vulvaless phenotype or suppress other mutations causing a Muv or Vul phenotype. We describe here four loci, *bar-1*, *mom-3/mig-14*, *egl-18*, and *sem-4*, that have a vulval cell fate specification defect that is distinct from those described previously. In these mutants the six VPCs can adopt a fourth cell fate, called the F or fused fate, which is a fate normally adopted by only P3.p in wild-type animals (Sulston and Horvitz 1977; Sternberg and Horvitz 1986). It is unclear what causes P3.p to sometimes fall out of the Vulval Equivalence Group and adopt the F fate instead of remaining as a vulval precursor cell during wild-type development. Whatever the mechanism, the mutant phenotypes of *bar-1*, *mom-3/mig-14*, *egl-18*, and *sem-4* suggest that the activity of these genes is required to keep P4.p–P8.p from adopting a similar F fate in wild-type animals and that these four gene products normally function to ensure that proper vulval precursor cell fate specification occurs.

A Wnt signaling pathway is likely to be acting during vulval induction: *bar-1* encodes a *C. elegans* homolog of vertebrate β-catenin and Drosophila Armadillo proteins (Eisenmann *et al.* 1998). Proteins of this family are known to function in both epithelial cell adhesion and in signal transduction by wingless/Wnt signaling pathways (Gumbiner 1995; Miller and Moon 1996). We have previously proposed, on the basis of the identity of the BAR-1 product, the *bar-1* mutant phenotype, and the regulation of *lin-39* by *bar-1*, that a Wnt signaling pathway acts in the VPCs during cell fate specification (Eisenmann *et al.* 1998). Several lines of evidence support the model that a Wnt signaling pathway is acting in the VPCs during induction and that *bar-1* and *mom-3/mig-14* act in that pathway. First, *bar-1(ga80)* and *mom-3/mig-14(ga62)* mutants have a defect in cell-fate specification by the posterior ectodermal cell P12 like that caused by mutations in *lin-44*, which encodes a Wnt protein (Herman and Horvitz 1994; Herman *et al.*

1995), and *lin-17*, which encodes a Frizzled (Wnt receptor) homolog (Sawa *et al.* 1996; Jiang and Sternberg 1998), suggesting that *bar-1* and *mom-3/mig-14* are functioning in a Wnt-mediated developmental process in P12. Second, *bar-1(ga80)* and *mom-3/mig-14(ga62)* mutants have a defect in the migration of the progeny of the neuroblast Q_L that is like that caused by mutations in *egl-20*, which encodes a Wnt protein (Maloof *et al.* 1999) and *lin-17*, suggesting that *bar-1* and *mom-3/mig-14* function in a Wnt-mediated process in Q_L and/or its progeny. Third, in both of those processes, a mutation in the Hox gene expressed in that cell (*mab-5* in Q_L and *egl-5* in P12) causes a defect like that caused by mutations in *bar-1*, *mom-3/mig-14*, or other Wnt pathway component genes (Chisholm 1991; Jiang and Sternberg 1998; Maloof *et al.* 1999). Consistent with this, *bar-1* and *mom-3/mig-14* are required for the expression of *mab-5* in Q_L (Harris *et al.* 1996; Maloof *et al.* 1999), and *bar*-1 is required for the expression of *lin-39* in the VPCs (Eisenmann *et al.* 1998). Wnt signaling pathways are known to regulate Hox gene expression in Drosophila and Xenopus (Brannon *et al.* 1997; Riese *et al.* 1997). Finally, disrupting the activities of three other *C. elegans* homologs of Wnt pathway components can cause a Pvl phenotype. Reduction of *mig-5 disheveled* (Antebi *et al.* 1997) activity by RNA-interference technology (J. Wagmaister and D. Eisenmann, unpublished results), production of an antisense message to *apr-1 APC* in the VPCs (Hoier *et al.* 2000), and mutation of the *mom-1 porcupine* gene (Thorpe *et al.* 1997) all cause a Pvl phenotype, suggesting that these genes may function in vulval development in a manner similar to *bar-1* and *mom-3/mig-14*.

Therefore, since the *bar-1* and *mom-3/mig-14* mutant phenotypes in Q_L and P12 development are identical to those caused by mutations in known Wnt pathway components, we believe the most likely hypothesis is that *bar-1* and *mom-3/mig-14* are acting in a Wnt pathway during vulval precursor cell development as well. Mutations affecting this pathway were not identified in previous genetic screens for vulval mutants, most likely due to their incompletely penetrant vulval defects. In fact, only when Wnt signaling and Ras signaling are both compromised do most VPCs adopt the F fate (Eisenmann *et al.* 1998, and data not shown). This indicates that in the process of cell-fate specification by the six VPCs, evolutionarily conserved Ras, Notch, and Wnt pathways all play a role. Also, Wnt signaling mediated by *bar-1* has been shown to regulate expression of the Hox genes *lin-39*, *mab-5*, and *egl-5* in three different postembryonic developmental events (cell-fate specification by the VPCs, Q_L, and P12), which suggests that Wnt signaling may be a general mechanism used to maintain expression of Hox genes in cells that adopt their fates during *C. elegans* postembryonic life.

Finally, it should be noted that *mom-3/mig-14* appears to be involved in almost all developmental processes known or suggested to be controlled by Wnt signaling in *C. elegans*: (1) specification of the VPCs (this work and Eisenmann *et al.* 1998), (2) migration of Q_L (Harris *et al.* 1996), (3) specification of P12 (this work and Jiang and Sternberg 1998), (4) asymmetric division of the embryonic blastomere EMS (Thorpe *et al.* 1997), (5) proper orientation of the EMS mitotic spindle (Schlesinger *et al.* 1999), and (6) asymmetric division of P7.p (A. Schlesinger and B. Bowerman, personal communication). Experiments on the role of *mom-3/mig-14* in EMS fate determination in the embryo have shown that *mom-3/mig-14* activity is required in P2, the cell believed to secrete the Wnt ligand, and is not required in EMS itself (Thorpe *et al.* 1997). This suggests a role for *mom-3/mig-14* activity in expressing, processing, or secreting the Wnt ligand. In Drosophila, mutations in the *porcupine* gene, which encodes a transmembrane protein associated with the endoplasmic reticulum (Kadowaki *et al.* 1996), and in several genes encoding factors involved in proteoglycan biosynthesis can lead to decreased signaling through the wingless pathway, presumably through a decrease in the amount of Wnt ligand available to interact with its receptor on receiving cells (van den Heuvel *et al.* 1993; Binari *et al.* 1997; Hacker *et al.* 1997; Haerry *et al.* 1997; Lin and Perrimon 1999). Although the *mom-3/mig-14* gene has not been identified, examination of the *C. elegans* genomic DNA sequence in the interval to which it localizes shows that there are no genes homologous to known Wnt/wingless pathway components or regulators, suggesting that *mom-3/mig-14* may encode a novel, general factor involved in Wnt signaling in *C. elegans*.

sem-4 is a previously identified gene that encodes a putative transcription factor containing seven C_2H_2-class zinc fingers similar to those in the Drosophila gene *spalt* and the human transcription factor PRDII-BFI (Basson and Horvitz 1996). *sem-4* mutants have defects in cell-fate specification for several mesodermal and neuronal cell types generated during larval life. Here we show that *sem-4* mutants display defects in VPC fate choices like those in *bar-1* and *mom-3/mig-14* mutants. Together this analysis suggests that *sem-4* may be a transcription factor functioning in the differentiation of several postembryonically derived cells, including the VPCs. On the basis of the result that the combination of *sem-4(ga82)* with either *bar-1(ga80)* [a likely null mutation (Eisenmann *et al.* 1998)], *mom-3/mig-14(ga62)*, or *egl-18(ga97)* leads to a highly penetrant synthetic vulvaless phenotype (Table 3), it is possible that *sem-4* may function in a distinct, redundant pathway from these other loci and that only when both pathways or functions are compromised is vulval induction strongly inhibited. It will be interesting to determine whether *sem-4* is a downstream target of the Ras pathway or the Wnt pathway or whether *sem-4* regulates *lin-39* expression.

***lin-26* acts during the execution of the 2° cell fate:** During the "fate execution" stage of vulval development,

the vulval precursor cells execute the cell fate they adopted during the previous stage by dividing and differentiating in a fate-specific manner. Although there is much pattern formation going on at this stage, only a few mutations have been identified that perturb cell-fate execution. For example, *lin-11* mutations cause cells adopting the 2° fate to divide in the pattern LLLL rather than LLTN/NTLL (Ferguson and Horvitz 1985; Freyd *et al.* 1990). The *lin-11* gene encodes a LIM domain transcription factor that is expressed in the cells in the "TN" branch of the 2° lineage. Also, in *lin-17* and *lin-18* mutants, P7.p, which should execute the NTLL fate, executes either a reversed asymmetric LLTN fate (like P5.p) or the symmetric LLLL fate, as in *lin-11* (Ferguson and Horvitz 1985; Sternberg and Horvitz 1988). *lin-17* encodes a member of the Frizzled family of seven-transmembrane proteins that function as receptors in Wnt signal transduction pathways, suggesting that the polarity of the P7.p lineage may be controlled by an extracellular Wnt signal (Sawa *et al.* 1996).

lin-26 encodes a zinc-finger transcription factor expressed in the nuclei of all hypodermal cells that is believed to be a general factor required for hypodermal differentiation (Ferguson and Horvitz 1985; Labouesse *et al.* 1994). Our analysis shows that *lin-26* also functions in a more specific manner, being required for the proper differentiation of two of the 12 Pn.pxx cells that generate the vulva. Consistent with the more subtle effect of this *lin-26* mutation, it has been found that the *lin-26(ga91)* mutation represents a missense mutation in one of the two zinc fingers of LIN-26 (Dufourcq *et al.* 1999).

It is possible that the 2° lineage may be defined by the function of specific transcription factors in specific sublineages. For example, the presence of the LIN-11 factor in the TN half of the lineage causes those two cells to develop differently from the LL half of the lineage. The asymmetric segregation of *lin-11* activity may be regulated by a Wnt signal, mediated by *lin-17*. The activity of *lin-26* might then serve to make the T cell different from the N cell. However, this model presumes that the activity of *lin-26* is required in the vulval precursor cell descendants for proper execution of the 2° lineage. Since *lin-26* is expressed in all hypodermal cells, *lin-26* could be acting in a nonautonomous manner to affect the division of the cells of the 2° T sublineage. Experiments designed to determine in which cells of a *lin-26(ga91)* mutant the wild-type *lin-26* gene must be expressed for wild-type vulval induction to occur could resolve this issue.

***pvl-6* acts at an early step in vulval morphogenesis:** After the 22 vulval cells have been generated by the divisions of P5.p–P7.p, the cells go through a series of short-range migrations and cell fusions to form the vulval opening (Newman and Sternberg 1996; Sharma-Kishore *et al.* 1999). During this process the 22 cells fuse into 7 mono- and multinucleate toroidal-shaped cells that make attachments to the surrounding hypodermal syncytium, the lateral hypodermal seam cells, and uterine cells in the somatic gonad. We know little about the genes required for the morphogenetic movements of the vulval cells. Mutations in eight *sqv* genes caused defects in morphology of the vulval opening, and three of these genes encode enzymes involved in glycosylation (Herman and Horvitz 1999; Herman *et al.* 1999). Also, mutations in the genes *cog-2* (encodes a Sox domain transcription factor; Hanna-Rose and Han 1999), *egl-38* (encodes a Pax transcription factor; Chamberlin *et al.* 1997), and *lin-11* (encodes a LIM-domain transcription factor; Freyd *et al.* 1990; Bellloch *et al.* 1999) cause defects in the creation of a functional connection between the vulval cells and the uterine cells. We show here that a mutation in the gene *pvl-6* causes defects in an early step in vulval morphogenesis, the interaction of the anchor cell with the descendants of P6.p.

In many *pvl-6* mutants, the anchor cell does not descend ventrally at the time normally observed in wild type, and after eventually descending, it sometimes interacts with P6.paa and P6.pap, or P6.ppa and P6.ppp, instead of the central two P6.p descendants P6.ppa and P6.pap. We believe these "off-center" contacts are at least one cause of the Egl and Pvl phenotypes in these mutant animals. We do not know the identity of the *pvl-6* gene product, nor do we know in what cell *pvl-6* functions. Given the behavior of the anchor cell in *pvl-6* mutants, it is possible that there is signaling between the anchor cell and P6.pap/P6.ppa to ensure that the proper cell-cell contact is made. *pvl-6* could function in such a signaling process in either the anchor cell or P6.pap/P6ppa. Alternatively, *pvl-6* could function in the migration of the anchor cell. Both of these models would explain why the behavior of the anchor cell appears temporally slower than in wild type, yet often the cell ends up making a correct interaction. Further genetic and molecular analysis of *pvl-6*, including the determination of whether this phenotype represents the *pvl-6* null phenotype, will help clarify the role of *pvl-6* in this early morphogenetic process.

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