Control of Vulval Competence and Centering in the Nematode *Oscheius* sp. 1 CEW1

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Manuscript received August 27, 2002
Accepted for publication October 10, 2002

ABSTRACT

To compare vulva development mechanisms in the nematode *Oscheius* sp. 1 to those known in *Caenorhabditis elegans*, we performed a genetic screen for vulva mutants in *Oscheius* sp. 1 CEW1. Here we present one large category of mutations that we call *cov*, which affect the specification of the P*n*.p ventral epidermal cells along the antero-posterior axis. The P*n*.p cells are numbered from 1 to 12 from anterior to posterior. In wild-type *Oscheius* sp. 1 CEW1, the P(4–8).p cells are competent to form the vulva and the progeny of P(5–7).p actually form the vulva, with the descendants of P6.p adopting a central vulval fate. Among the 17 mutations (defining 13 genes) that we characterize here, group 1 mutations completely or partially abolish P(4–8).p competence, and this correlates with early fusion of the P*n*.p cells to the epidermal syncytium. In this group, we found a putative null mutation in the *lin-39* HOM-C homolog, the associated phenotype of which could be weakly mimicked by injection of a morpholino against *Osp1-lin-39* in the mother's germ line. Using cell ablation in a partially penetrant competence mutant, we show that vulval competence is partially controlled by a gonadal signal. Most other mutants found in the screen display phenotypes unknown in *C. elegans*. Group 2 mutants show a partial penetrance of P*n*.p competence loss and an abnormal centering of the vulva on P5.p, suggesting that these two processes are coregulated by the same pathway in *Oscheius* sp. 1. Group 3 mutants display an enlarged competence group that includes P3.p, thus demonstrating the existence of a specific mechanism inhibiting P3.p competence. Group 4 mutants display an abnormal centering of the vulval pattern on P7.p and suggest that a specific mechanism centers the vulval pattern on a single P*n*.p cell.

THE phenotypic analysis of mutants obtained in genetic screens after mutagenesis provides information at two levels: (i) on the mechanism of the process under study, as used, for example, in developmental genetics, and (ii) on the evolutionary potential of the population, by exploring the mutational phenotypic neighborhood. The comparison of phenotypes obtained by a similar genetic screen in different species therefore reveals both the evolution of the underlying mechanisms and the difference in evolutionary potential between the two populations.

The nematode *Caenorhabditis elegans* is used as a genetic model system; one of its best-studied developmental features is the formation of its egg-laying organ, the vulva (Wang and Sternberg 2001). We previously introduced *Oscheius* sp. 1 CEW1 as another nematode genetic model system (Félix *et al.* 2000b; Dichtel *et al.* 2001) that displays a different vulval precursor cell patterning mechanism (Félix and Sternberg 1997) and is extremely common in the soil (Delattre and Félix 2001c; Félix *et al.* 2001).

The nematode vulva is formed during postembryonic development from vulval precursor cells in the ventral epidermis, called P*n*.p cells (the posterior daughters of P*n* cells; Figure 1). In *C. elegans*, the six cells P3.p–P8.p are competent to form vulval tissue, as a result of their expression of the HOM-C gene *lin-39* (Clark *et al.* 1993; Wang *et al.* 1993). The vulva is normally formed by the progeny of only three of these cells, with P6.p adopting a central (1°) fate and P5.p and P7.p adopting lateral (2°) fates; P(3,4,8).p adopt a nonvulval (3°) fate. The 1°/2°/3° terminology of fates corresponds to their hierarchy of replacement within the competence group (Horvitz and Sternberg 1991). Each fate can be distinguished by several features, in particular the division pattern of the P*n*.p cell. The nonvulval 3° fate can be distinguished from that of more anterior or posterior P*n*.p cells because P(3,4,8).p divide once in the L3 stage and then fuse with the large epidermal syncytium that surrounds the animal (called hyp7), whereas P(1,2,9–11).p do not divide and already fuse in the L1 stage with hyp7. Within the vulval competence group, the three fates are patterned by a combination of a graded induction through epidermal growth factor/Ras signaling from the gonadal anchor cell and of *lin-12*/Notch

Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under accession no. AJ431730.

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signaling between the P*n*.p cells (Wang and Sternberg 2001).

Vulval development in *Oscheius* sp. 1 CEW1 differs in several ways from that of *C. elegans*: (i) The vulval competence group is reduced to P(4–8).p; (ii) the same spatial pattern of vulval precursor fates is obtained by two successive inductions from the anchor cell, the first specifying a vulval *vs.* a nonvulval fate in P(5–7).p and the second an inner *vs.* an outer (1° *vs.* 2°) vulval sublineage in P6.p daughters; and (iii) the cell division pattern corresponding to the 3° and 2° sublineages differs from those of *C. elegans* (Sommer and Sternberg 1995; Félix and Sternberg 1997; Figure 1).

Because of these differences in vulval development between the two species, we performed a screen for egg-laying (Egl)-defective mutants in *Oscheius* sp. 1. We previously described a large class of mutations that specifically affect the number of divisions of the vulval precursor cells and not their overall fate (Dichtel *et al.* 2001); such mutants were not found in similar *C. elegans* screens (Ferguson *et al.* 1987; Wang and Sternberg 2001). Here we describe a second large class of mutations found in the screen, which affect the antero-posterior specification of the P*n*.p cells, namely their vulval competence and/or the centering of the vulval pattern (a third smaller class of mutants, which affect the level of vulval induction, will be described elsewhere; M.-L. Dichtel and M.-A. Félix, unpublished data). We call this class of *Oscheius* sp. 1 mutants *cov* for *c*ompetence and/or centering *o*f the *v*ulva.

Within this large class of mutants, we further distinguish four groups of mutants (Figure 2): (1) mutants with a reduced vulval competence, among which is an *Osp1-lin-39* mutant; (2) mutants with a reduced vulval competence as well as centering of the vulva pattern (1° fate) on P5.p; (3) mutants with a vulval competence group enlarged to P3.p; and (4) mutants with a vulval pattern centered on P7.p. The phenotypes that we found in *Oscheius* sp. 1 show interesting similarities to and differences from those found in *C. elegans*. Moreover, we show that a gonadal signal can maintain competence of the P*n*.p cells in *Oscheius* sp. 1.

MATERIALS AND METHODS

Nematode culture: The wild-type reference strain of *Oscheius* sp. 1 (Félix *et al.* 2001) used in these studies is the strain CEW1. The nematodes were cultured on *Escherichia coli* OP50 as described for *C. elegans* (Wood 1988). Cultures were usually grown at 25°, except for matings, which were performed at 23° (Dichtel *et al.* 2001).

Genetic screen and crosses: The mutagenesis of *Oscheius* sp. 1 CEW1 and the screen for egg-laying mutants were described in Dichtel *et al.* (2001). The *mf89* mutant was subsequently isolated by M. Delattre in a F_1 clonal screen. All mutations were obtained after ethyl methanesulfonate mutagenesis, except for *mf79*, *mf80*, *mf85*, and *mf89*, which were obtained after trimethylpsoralen-ultraviolet irradiation (TMP-UV) mutagenesis.

We previously named the *Oscheius* sp. 1 vulva mutants that are affected in vulval divisions *dov-1* to *dov-16* (Dichtel *et al.* 2001). To distinguish phenotypic categories of vulva mutants in *Oscheius* sp. 1, we call the mutants that are affected in the competence or centering of the vulva *cov* mutants and reserve *dov* for those affected in the *d*ivisions *o*f the *v*ulva (instead of *d*evelopment *o*f the *v*ulva as mentioned in Dichtel *et al.* 2001).

Genetic complementation analyses were performed as described in Dichtel *et al.* (2001). Complementation tests could not be performed with *mf70* because its vulva defect is so strong that it could not be mated into and we could not get males to mate; we therefore did not assign a gene number to it (however, after its molecular characterization, we could check that it defines a different locus than other mutants of its group; see RESULTS). All mutations appear recessive, with the exception of two mutants with a vulval centering on P7.p: *cov-10(mf55)* is semidominant (heterozygotes show ~12% of centering on P7.p) and *cov-11(mf71)* either is semidominant (to a similar degree) or shows a maternal effect (these two possibilities could not be distinguished in this mutant because heterozygous males are sterile).

Nomarski microscopy and laser ablations: The worms were mounted on agar pads as described in Wood (1988) and observed by Nomarski optics with a ×100 objective on a Zeiss Axioskop. Cells were ablated as described in Epstein and Shakes (1995), using a Photonic Instruments laser system.

Permeabilization, fixation, and immunofluorescence staining of worms: To fix and permeabilize the worms, the Finney-Ruvkun protocol (Finney and Ruvkun 1990) was used with paraformaldehyde (Fluka) at a final concentration of 2%. The procedures were followed in 1.5-ml eppendorf tubes. The immunofluorescence staining of the epithelial cell adherens junctions was performed with the monoclonal antibody MH27 (Francis and Waterston 1991) and nuclei were stained with propidium iodide (Podbilewicz and White 1994; Podbilewicz 1996; Sharma-Kishore *et al.* 1999).

The stained worms were analyzed using an MRC-1024 laser confocal scanning microscope (Bio-Rad, Hempstead, UK) with the objective Nikon Plan Apo 60×/1.40 (Sharma-Kishore *et al.* 1999; Shemer *et al.* 2000). The projection pictures were obtained from 10–25 serial optical sections, collected every 0.4–0.5 mm, in the *z*-axis. For a more precise visualization of the details, tilt and rotation projections were performed using the Laser Sharp Processing program (Bio-Rad). We relate to the cell contacts and cell fusions according to the appearance or vanishing of the adherens junctions boundaries marked by MH27 (Shemer and Podbilewicz 2000).

Genomic library: A genomic library of *Oscheius* sp. 1 CEW1 was constructed in the phage λFIX II/*Xho*I (Stratagene, La Jolla, CA) according to the manufacturer's instructions, after partial *Sau*3A digestion of CEW1 genomic DNA. A total of 150,000 plaques from the primary library were further amplified to yield the genomic library.

Molecular cloning of the *Oscheius* sp. 1 CEW1 *lin-39* gene: The homeodomain of the *Oscheius* sp. 1 CEW1 *lin-39* gene was amplified by RT-PCR. Total RNA from the CEW1 strain was first prepared using the RNAXEL kit (Eurobio). Reverse transcription was performed for 1 hr at 42° using the MMLV reverse transcriptase (GIBCO BRL, Gaithersburg, MD) and oligo(dT) as a primer. The homeodomain was then amplified by PCR using the degenerate primers 5′-CGTCAGMGTACTG CNTAYAC-3′ and 5′-CATGCKACKRTTYTGRAACCA-3′ (as in Eizinger and Sommer 1997), subcloned into pGEM-T (Promega, Madison, WI), sequenced, and used as a probe for screening the *Oscheius* sp. 1 genomic library, according to standard procedures (Maniatis *et al.* 1989). A positive phage was purified and digested with *Not*I and the insert (~20 kb in size) was purified and subcloned into pBluescript, yielding plasmid pSL1. The insert starts 5.8 kb upstream of the translation start

of the *Osp1-lin-39* gene and was sequenced on one strand until 2.6 kb downstream of the stop codon. All exons and the last three introns were then sequenced on both strands.

The intron-exon structure was first analyzed using PCR on an *Oscheius* sp. 1 mixed-stage cDNA library (Félix *et al.* 2000b). The 5′ part of the cDNA was amplified using the M13 reverse primer in the cloning vector and a primer on the reverse strand in the homeodomain (in exon 4). The 3′ part was amplified with a forward primer at the start of exon 2 and a reverse primer downstream of the stop codon in the 3′ untranslated region. From the few clones sequenced, alternative splicing was apparent at the 5′ ends of exons 3 and 6. We therefore directly sequenced (without cloning) a RT-PCR product that was amplified from poly(A)-RNA and that covered the last five exons. The major splice form is presented in Figure 5, but the two other forms at the 5′ ends of exons 3 and 6 could be found in the sequence in the form of double and then triple peaks. First, the alternatively spliced form at the beginning of exon 3 adds a CAG, transforming the protein sequence into SNSTGPR (instead of SNSSPR). Second, the alternatively spliced form at the beginning of exon 6 removes the nine nucleotides (GACTTTCAG) that correspond to amino acids DFQ after the end of the homeodomain. Interestingly, in both cases, the alternatively spliced form can be well aligned with the *C. elegans* sequence. The alignment of the protein sequence to *C. elegans* and *Pristionchus pacificus* was initially performed using Clustal W and further refined by hand.

Morpholino injection: A morpholino against the *Osp1-lin-39* gene (sequence GACAAAGTCAGTCGGAGAAGTCATC spanning the translational start site; Gene Tools, Philomath, OR) was injected into the syncytial germ line of *Oscheius* sp. 1 CEW1 adult hermaphrodites at a concentration in the injection needle of 2 mM or 0.2 mM. The injected animals were transferred to new plates at several time points after injection. At 2 mM, the morpholino caused sterility of the injected adult after 1 day, as did injection of the standard control morpholino (CCTCTTACCTCAGTTACAATTTATA). The vulval phenotypes were scored on progeny that were laid 5–20 hr after injection.

Double-stranded RNA against *Osp1-lin-39* was synthesized *in vitro* from the T7 and Sp6 transcription sites of a plasmid (pMA44) containing a RT-PCR clone of *Osp1-lin-39* exons 2–5 into the pGEM-T vector (Promega). Injection of this double-stranded RNA (1 mg/ml) had no effect (as for other genes in *Oscheius* sp. 1 CEW1; Delattre 2001). Injection of siRNAs against *Osp1-lin-39* (6 mg/ml of the sense oligo GGACGACA GUGACAAAGAGACCGUU annealed with the antisense oligo AACGGUCUCUUUGUCACUGUCGUCC; Xeragon, Huntsville, AL) also did not result in any phenotype.

Polymorphism in the *Osp1-lin-39* gene: A molecular polymorphism in the *Osp1-lin-39* gene was found by amplifying intron 2 in four different *O.* sp. 1 wild strains (Delattre and Félix 2001c; Félix *et al.* 2001) and digesting it with several restriction enzymes. A *Pvu*I site was present in the reference strain CEW1 and absent in the strain JU149 (from Madagascar). For analysis of genetic linkage of the *cov-1(mf53)* and *cov-2(mf57)* mutations to *Osp1-lin-39*, this polymorphic region was amplified using primers 5′-AGTGGGTCAGTTGCTCCAC-3′ and 5′-GCTCAT GAGTCTAACTTTCT-3′ on single F_2 progeny of a cross between the mutant and JU149 and then subjected to *Pvu*I digestion.

RESULTS

Mutants with a reduced vulval competence group: The vulval competence group is formed by the P(4–8).p cells in *Oscheius* sp. 1. In the wild type, P(5–7).p adopt a vulval fate whereas P4.p and P8.p adopt a nonvulval fate and become part of the epidermal syncytium after two divisions (noted "ssss" for syncytial; Figure 1). By contrast, the noncompetent cells, P(1–3).p and P(9–11).p, do not divide and fuse with the epidermal syncytium (noted "S"; Table 1). Therefore, a change in division number may correspond to a change in cell competence. We found several mutants in which several or all of P(4–8).p adopt the S fate (groups 1 and 2 in Figure 2).

The strongest phenotype is shown in group 1 mutants by *cov-(mf70)*, in which all P(4–8).p adopt the S fate and

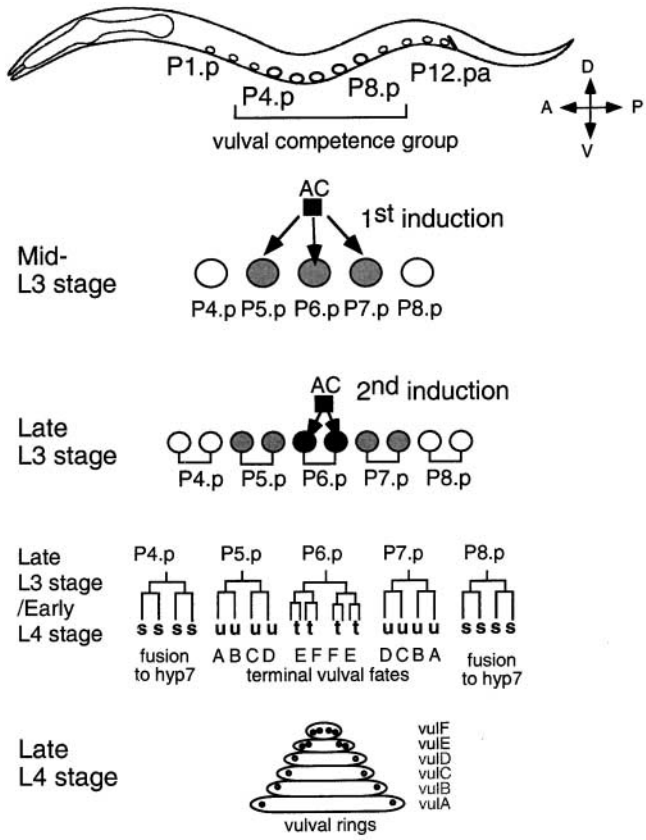

Figure 1.—Vulval formation in *Oscheius* sp. 1 CEW1. The vulva is formed by the progeny of the ventral epidermal cells P5.p, P6.p, and P7.p. In *Oscheius* sp. 1 CEW1 as in *C. elegans*, P6.p adopts a central 1° vulval sublineage (solid symbols), whereas P5.p and P7.p adopt a lateral 2° vulval sublineage (shaded symbols). P4.p and P8.p usually adopt a nonvulval fate (open) and their progeny fuse with the epidermal syncytium hyp7 that surrounds most of the animal; however, they are able to replace P(5–7).p and to adopt a vulval fate. P(4–8).p thus form the vulval competence group in *Oscheius* sp. 1 CEW1. The three different P*n*.p sublineages within the vulval competence group are specified by two successive inductions by the gonadal anchor cell (AC) on the P*n*.p cells and their daughters. The subsequent division pattern of each cell is given at the bottom. The sublineages of P*n*.p granddaughters are abbreviated as follows: s, fuses with the epidermal syncytium; u, remains undivided; t, divides transversally (left-right). After fusion of cells with the same terminal fate, the progeny of P(5–7).p give rise to six vulval rings, lettered vulA to vulF (I. Kolotuev and B. Podbilewicz, unpublished results).

TABLE 1

Mutants with reduced P*n*.p competence

Genotype	P4.p	P5.p	P6.p	P7.p	P8.p	*n*
Wild type	ssss	uuuu	tttt	uuuu	ssss	95/100
	S	uuuu	tttt	uuuu	ssss	4/100
	S S	uuuu	tttt	uuuu	ssss	1/100
cov-(mf70)	S	S	S	S	S	25/25
cov-1(mf53)	S	S	S	S	S	40/83
	S	S	tttt	S	S	34/83
	S	uuuu	tttt	uuuu	S	3/83
	S	U U	tttt	uuuu	S	1/83
	S	uuuu	tttt	S	S	1/83
	ssss	S	tttt	uuuu	S	1/83
	S	S	tttt	uuuu	S	1/83
	S	S	uttt	S	S	1/83
	S	S	ttss	S	S	1/83
cov-2(mf57)	S	uuuu	tttt	uuuu	S	5/33
	S	S	tttt	S	S	4/33
	S	uuuu	tttt	S	S	4/33
	S	S	tttt	uuuu	S	3/33
	S	ssuu	tttt	uuuu	S	2/33
	S	uuuu	tttt	uuss	S	2/33
	S	ssss	tttt	S	S	1/33
	S	S S	tttt	uuuu	S	1/33
	S	ssuu	tttt	S	S	1/33
	S	S ss	tttt	uu S	S	1/33
	S	U uu	tttt	ssss	S	1/33
	S	uuuu	tttt	uuuu	S	1/33
	S	S	tttt	S	ssss	1/33
	S	suuu	tttt	uuuu	ssss	1/33
	S	uuuu	tttt	uuuu	ssss	1/33
	S ss	uuuu	tttt	S	S	1/33
	ssss	suuu	tttt	uuus	S	1/33
	S	S	S	S	S	1/33
	S	ssuu	uuss	S	S	1/33#
cov-3(mf79)	S	tttt	uuuu	ssss	ssss	12/50
	uuuu	tttt	uuuu	ssss	ssss	9/50
	uuuu	tttt	uuuu	S	ssss	6/50
	S	uuuu	tttt	uuuu	ssss	4/50
	S	uuuu	tttt	S	ssss	4/50
	S	tttt	uuuu	S S	ssss	3/50
	uuuu	tttt	uuuu	S S	ssss	2/50
	S	tttt	uuuu	S ss	ssss	1/50
	uuuu	tttt	uuuu	S ss	ssss	1/50
	uuuu	tttt	ssss	ssss	ssss	1/50
	S	tttt	uuuu	S	ssss	1/50
	S uu	tttt	uuuu	S	ssss	1/50
	ssuu	tttt	uuus	S S	ssss*	1/50
	ssss	tttt	uuuu	S	S	1/50
	ssss	uutt	ttuu	S	ssss	1/50
	S ss	uuuu	tttt	S S	ssss	1/50
	S	uuuu	tttt	uuuu	S S	1/50
cov-4(sy493)	S	tttt	uuuu	S	ssss	3/24
	S	tttt	uuuu	S	S ss	2/24
	S	tttt	uuuu	S	S S	2/24
	S	tttt	S	S	S	2/24
	uuuu	tttt	uuuu	S	S	2/24
	S	S	tttt	S	ssss	2/24
	S	S	S S	S	ssss	1/24

(*continued*)

TABLE 1

(Continued)

Genotype	P4.p	P5.p	P6.p	P7.p	P8.p	*n*
	S	tttt	S	S	S ss	1/24
	uuuu	tttt	S	S	S	1/24
	S	tttt	uuuu	S	S	1/24
	S S	tttt	uuuu	S	S	1/24
	S	tttt	uuuu	S	ss S	1/24
	uuuu	tttt	uuuu	S	ssss	1/24
	S	S	tttt	S	S	1/24
	S	uuut	tttt	S	S	1/24
	S	uuuu	tttt	uuuu	S	1/24
	S	uuuu	tttt	uuuu	S S	1/24
cov-5(mf34)	S	tttt	uuuu	S	S	5/20
	S	tttt	uuuu	S	ssss	2/20
	S	tttt	S	S S	S	1/20
	uuuu	tttt	uuuu	S	S	1/20
	uuuu	tttt	uuuu	ssss	S	1/20
	uuuu	tttt	uuuu	ssss	S S	1/20
	S	uuuu	tttt	uuuu	ssss	2/20
	S	uuuu	tttt	uuuu	S	2/20
	S	S	tttt	S	S	2/20
	S	uuuu	tttt	uuuu	S	1/20
	S	uuuu	tttt	uuuu	ssss	1/20
	S	ssss	uuuu	tttt	uuuu	1/20
Osp1-lin-39	S	S	S	S	S	3
morpholino	S	S	S	ssss	S	1
injection	S	S	tttt	S	S	1
	S	induced	induced	S	S	1
	S	uuuu	tttt	S	ssss	1
	S	S	tttt	uuuu	ssss	1
	S	uuuu	tttt	uuuu	S	1
	S S	uuuu	tttt	uuuu	S	1
	absent	uuuu	tttt	uuuu	S	1
	uuuu	tttt	uuuu	S	S	1
	ssss	uuuu	tttt	uuuu	S	1

The division pattern and fates of P*n*.p cells were scored in individual L4 stage hermaphrodites cultured at 25°. The sublineages of P*n*.p cells, their daughters, or granddaughters are indicated from anterior to posterior, as follows. t, transverse division (left-right) of a P*n*.p granddaughter; u, undivided granddaughter; s, nonvulval ("syncytial," although we do not directly score for fusion in this table). A capital letter indicates the absence of one or two rounds of divisions. S, no division; S S, one division; S ss/ss S, one division followed by the division of one of the daughters; U, vulval fate with only one round of division. The right column indicates the number of animals observed. In *cov-1(mf53)* mutants, some P*n*.p cells cannot be found (especially in the anterior region, apparently because the P*n* mothers do not migrate); the P*n*.p cell below the anchor cell is referred to as P6.p, but could be another P*n*.p cell if P6.p were absent. Some *cov-4(sy493)* animals are also missing some P*n*.p cells and only animals displaying all P*n*.p cells were taken into account in this table. *cov-2(mf79)* mutants sometimes present a small posterior vulval invagination (formed by P6.p or P7.p progeny) in addition to the main invagination. *cov-3* and *cov-4* mutants show bright posterior intestinal granules. For the *lin-39* morpholino injection, only those 13/228 animals displaying an abnormal vulval phenotype are shown; in addition, 35/228 animals showed no (or a single) division of P4.p or P8.p. After injection of the control morpholino, 7/139 animals had an undivided P4.p (a percentage that is similar to untreated CEW1; Dichtel *et al.* 2001), and none showed stronger defects. #, this animal displayed a gonad development problem. *, in this animal, P8.p progeny appeared posterior to P9.p.

the animals are fully vulvaless. *cov-1(mf53)* mutants show a similar phenotype, except that one P*n*.p cell adopts a central vulval fate in about one-half of the animals. *cov-2(mf57)* mutants show an even less penetrant phenotype (Table 1).

In group 2 mutants, the loss of P*n*.p competence is less penetrant and, in addition, the vulval 1° fate is miscentered on P5.p. This relatively large group is composed of seven mutants that define three genes, *cov-3*, *cov-4*, and *cov-5* (Figure 2). The P5.p centering defect is highest in *cov-3* mutants (Table 1 and legend to Table 5), whereas the P4.p (and P8.p to a lesser degree) defect

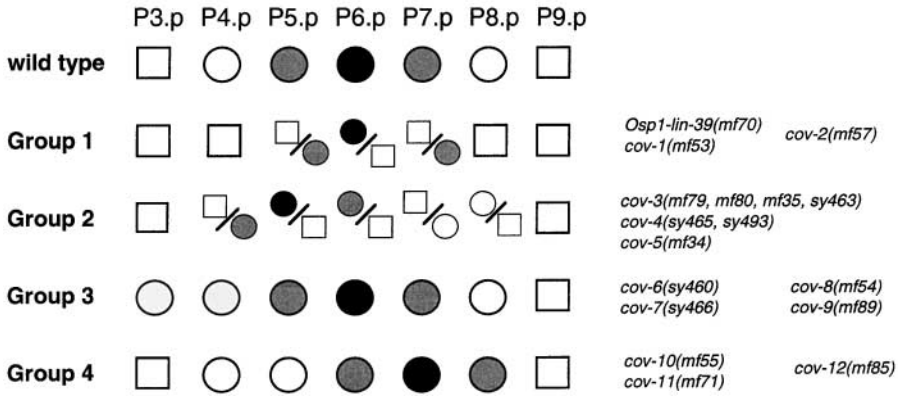

FIGURE 2.—*Oscheius* sp. 1 mutants with an altered competence and/or centering of the vulva. Four phenotypic categories are distinguished among mutations that result in an altered vulval competence and/or centering. P*n*.p cell fates are schematized for each group of mutants. Symbols are as in Figure 1. Squares represent noncompetent cells. The different complementation groups are shown on the right, with different alleles given in parentheses. When two alternative cell fates are observed, the most frequent is indicated above the less frequent.

appears highest in *cov-4(sy493)* and *cov-5(mf34)* mutants and the P7.p defect appears highest in *cov-4(sy493)* mutants.

To study the differences in P*n*.p competence between the wild type and these Cov mutants, we scored the time of fusion of the P*n*.p cells to the epidermal syncytium by staining the worms with the MH27 antibody, which recognizes cell junctions. In wild-type *Oscheius* sp. 1, as in *C. elegans*, P(1,2,9–11).p fuse with an antero-posterior chronology in the late L1 stage to the early L2 stage (Figure 3, A and C). P3.p fuses later, in the late L2 stage (data not shown). In the *cov-(mf70)* mutant, P(3–8).p also fuse in the late L1 stage/early L2 stage, like P(1,2,9–11).p in the wild type (Figure 3, B and D). However, in ~15% (13/98) of the animals, one central cell remains unfused until adulthood and sometimes (3/98 stained animals) appears to divide once (Figure 4C). In the *cov-1(mf53)* mutant, the P(4–8).p cells that do not divide fuse in the late L1 stage, and in half of the animals, the central cell divides and gives rise to central (vulE and vulF) vulval rings ("tttt" lineage), which form a minimal functional vulva (Figures 1 and 4B).

In contrast to previously described mutants such as *dov-4* (in which P4.p and P8.p do not divide, but are competent; DICHTEL *et al.* 2001), group 1 and group 2 mutants have fewer than three induced cells, in a pattern that suggests an altered competence (rather than an altered division or induction): For example, a P*n*.p cell may not divide whereas it is immediately adjacent to that adopting the 1° fate. Moreover, the MH27 stainings show an early fusion of the P*n*.p cells prior to the time of vulval fate induction (Figure 3). Thus, these mutants actually define genes that play a role in P*n*.p vulval competence.

We made use of the incomplete penetrance of some of these mutants to investigate a possible role of the gonad in the competence of P*n*.p cells to later receive the anchor cell inductive signal. In *cov-1(mf53)* mutants, one-half of the animals have one induced cell that is always located below the anchor cell. When this cell is ablated with a laser in the early L2 stage, another cell can adopt a 1° vulval fate in 25% of animals and in this case always moves close to the anchor cell (Table 2). This experiment suggests that the anchor cell or another cell of the gonad could influence the competence of the P*n*.p cells. To test this hypothesis, gonad ablations were performed at different times of development. In wild-type animals, gonad ablation in the L1 stage leads to the adoption of the 3° sublineage (ssss) by P(4–8).p whereas, in *cov-1(mf53)* and *cov-2(mf57)* mutants, all the cells adopt an S fate (Table 2). After ablation of the anchor cell (or of the two anchor cell precursors) in the early L2 or L3 stages, one-fourth of the animals have at least one cell that adopts a ssss sublineage. In contrast, gonad ablation in *cov-3(mf79)* mutants has little effect on cell competence, except possibly for P4.p (Table 2).

These results suggest that the competence of P*n*.p cells is regulated by two mechanisms: an autonomous cell specification mechanism and a gonadal cell signal that can be uncovered in mutant contexts such as *cov-1* and *cov-2*.

To test whether the group 2 mutants had the same effect as gonad ablations in *cov-1(mf53)*, we constructed double mutants between *cov-1* and group 2 mutants. The *cov-1(mf53); cov-3(mf79)* and the *cov-1(mf53); cov-4(sy493)* double mutants both show a strong Vulvaless phenotype: 40/41 and 23/23 animals show no division/induction of any P*n*.p cell (with no enhancement of the P*n*.p generation defect of *cov-1*). Therefore, the number of competent P*n*.p cells is greatly reduced compared to that of either single mutant. Thus, loci defined by group 2 mutants may act in the maintenance of P*n*.p competence by a gonadal signal, whereas those defined by group 1 mutants may act in an automonous cell specification mechanism.

***cov-(mf70)* displays a deletion in the *Osp1-lin-39* gene:** In *C. elegans*, expression of the HOM-C gene *lin-39* in P(3–8).p is necessary to prevent the fusion of P(3–8).p to the hyp7 syncytium in the L1 stage (CLARK *et al.* 1993; WANG *et al.* 1993) and for P(3–8).p to adopt a vulval fate downstream of Ras activation in the L3 stage (CLANDININ *et al.* 1997; MALOOF and KENYON 1998). LIN-39 has recently been shown to downregulate *eff-1*, a gene that is essential for cell fusion (MOHLER *et al.* 2002),

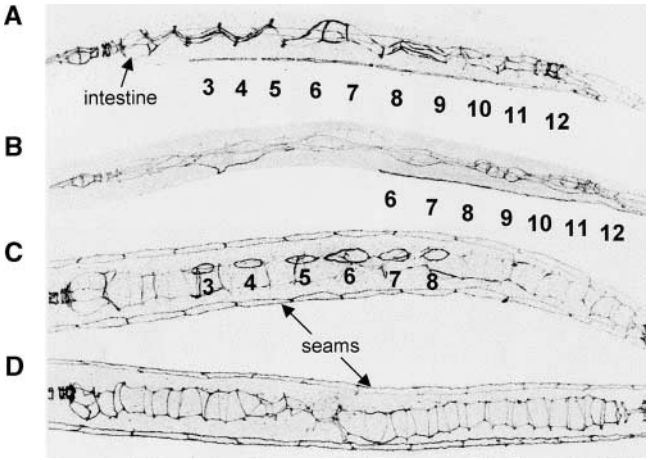

FIGURE 3.—P*n*.p cell fusion to the epidermal syncytium in wild-type and *Osp1-lin-39(mf70)* mutants. Cell fusion is scored by the disappearance of cell junctions that are revealed by immunofluorescence with the MH27 antibody (in black). Anterior is to the left, posterior to the right. (A) Wild-type CEW1 in late L1; lateral view. P1.p and P2.p have fused with the epidermal syncytium. At this stage, P9.p or P10.p or both have sometimes already fused. (B) *Osp1-lin-39(mf70)* mutant in late L1; lateral view. P(1–5).p have fused (typical pattern at this stage). (C) Wild type in mid L2; ventral view. P(3–8).p cells remain unfused. (D) *Osp1-lin-39(mf70)* mutant in mid L2; ventral view. No P*n*.p cell contours can be seen.

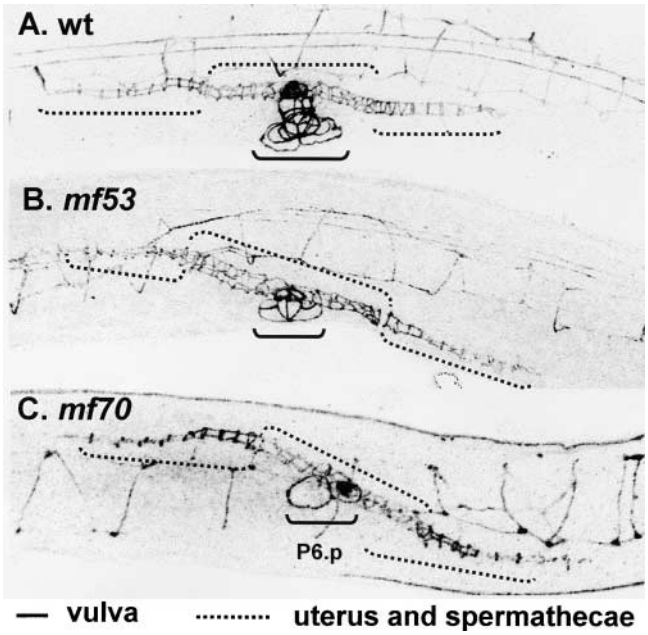

FIGURE 4.—Vulval structures in wild type, *Osp1-lin-39(mf70)*, and *cov-1(mf53)* mutants. Staining is as in Figure 3, in the late L4 stage. Anterior is to left, posterior to the right. (A) Wild-type CEW1; lateral view. The different vulval rings are visible at the end of organogenesis. (B) *cov-1(mf53)* mutant; lateral view. The reduced vulva is composed of the two inner rings vulE and vulF that are connected to the uterus. (C) *Osp1-lin-39(mf70)* mutant; ventral view. This is a rather unusual animal in which two cells (which are likely the progeny of P6.p) are still unfused in the L4 stage.

in vulval cells that fail to fuse to hyp7 (SHEMER and PODBILEWICZ 2002). Because a loss-of-function mutation in the *Cel-lin-39* gene results in a phenotype resembling that of *cov-(mf70)* in *O.* sp. 1, we wondered whether the *lin-39* homolog could be mutated in *cov-(mf70)* (and possibly other mutants). We isolated the *lin-39* homolog of *Oscheius* sp. 1 by RT-PCR of the homeodomain followed by screening of a genomic library and named it *Osp1-lin-39* according to previous nomenclature (EIZINGER and SOMMER 1997). We confirmed the intron-exon structure by sequencing RT-PCR products (Figure 5) and found two splice variants at the beginning and the end of the homeodomain (see MATERIALS AND METHODS). The alignment of the putative protein with the homologs from *C. elegans* and from the other nematode *P. pacificus* is shown in Figure 5B. The homeodomain is highly conserved, as well as the hexapeptide upstream of the homeodomain. The N-terminal is the most divergent part of the protein. The putative C-terminal mitogen-activated protein (MAP) kinase docking site (FXFP) that is present in the *C. elegans* LIN-39 and absent in *P. pacificus* (GRANDIEN and SOMMER 2001) is also present in *O.* sp. 1 (FGFP). However, the MAP kinase phosphorylation site (S/TP) is absent (no proline), rendering it unlikely to be phosphorylated by MAP kinase in *O.* sp. 1.

The *Osp1-lin-39* homeodomain could not be amplified by PCR in *cov-(mf70)* mutant worms, suggesting that it could be deleted. The putative deletion was then further mapped using various primers along the *Osp1-lin-39* genomic sequence. Finally, using a primer at the beginning of intron 2 and a primer 1.5 kb downstream of the stop codon, a band of ~600 bp could be amplified in *mf70* worms and sequenced. The breakpoints of the *mf70* deletion correspond to an AGA triplet found 483 bp after the start of intron 2 and ~1.2 kb downstream of the stop codon. This 3.5-kb deletion thus removes exons 3–6 and therefore the whole homeodomain (Figure 5A). Injection of a morpholino directed against the 5′ end of the *Osp1-lin-39* coding sequence (see MATERIALS AND METHODS) caused a weak *mf70*-like phenotype in the progeny of injected mothers (Table 1), thus confirming that the molecular deletion in *Osp1-lin-39* is the cause of the vulval competence phenotype of *mf70*. Although we cannot rule out that a second mutation plays a role in the loss of P*n*.p competence in the *cov-(mf70)* mutant since it was never outcrossed, on the basis of the strongest phenotypes observed with the morpholino and the molecular deletion of the homeodomain in *mf70*, we conclude that the *cov-(mf70)* mutant is likely to be a null allele of *Osp1-lin-39*.

Since we could not perform complementation experiments with *cov-(mf70)*, we tested whether *cov-2(mf53)* or *cov-3(mf57)* could be alleles of *Osp1-lin-39*. We made use of the *mfP1* polymorphism found in the second intron of *Osp1-lin-39* in the wild strain JU149 *vs.* our reference strain CEW1 (see MATERIALS AND METHODS and Figure

TABLE 2

Cell ablations in mutants with reduced vulval competence

Genotype	Ablation	P4.p	P5.p	P6.p	P7.p	P8.p	*n*
Wild type	Gonad in L1	ssss	ssss	ssss	ssss	ssss	5/5[a]
cov-1(mf53)	Gonad in L1			All P*n*.p: S			17/18
				1 P*n*.p ssss			1/18
cov-1(mf53)	Z1.ppx + Z4.aax			All P*n*.p: S			12/16
	In early L2			1 P*n*.p ssss			4/16
cov-1(mf53)	AC			All P*n*.p: S			18/25
	In early L3			1 P*n*.p ssss			4/25
				2 P*n*.p ssss			3/25
cov-1(mf53)	"P6.p"			All P*n*.p: S			22/30
	In early L2			1 P*n*.p: tttt			8/30
cov-2(mf57)	Gonad in L1	S	S	S	S	S	7/8
		S	S ss	S	S	S	1/8
cov-3(mf79)	Gonad in L1	S	ssss	ssss	ssss	ssss	4/14
		S	S	ssss	S	S	3/14
		S	S ss	ssss	S	ssss	2/14
		S	S	ssss	ssss	ssss	1/14
		S	S	S ss	S S	ssss	1/14
		S	ssss	S S	ssss	S S	1/14
		S	S	S S	ssss	ssss	1/14
		ssss	ssss	ssss	ssss	ssss	1/14

The animals were scored during the L4 stage to determine the cell division pattern and fate. We do not directly score for cell fusion in this table. Abbreviations are as in Table 1. Z1.ppx and Z4.aax are ventral uterine cells, one of which will become the anchor cell (Kimble and Hirsh 1979). In *cov-1(mf53)* mutants, several P*n*.p cells are missing, so the identification of each P*n*.p cell is not possible (the ablated "P6.p" is the cell located below the gonad). P*n*.p cells move posteriorly after gonad ablation in *cov-3(mf79)* animals. AC, anchor cell.

[a] Data from Dichtel *et al.* (2001).

5A). Neither *cov-2(mf53)* nor *cov-3(mf57)* was found to be genetically linked to *Osp1-lin-39*. These two mutations thus define two other genes affecting P*n*.p competence.

Mutants with a vulval competence group enlarged to P3.p: In *Oscheius* sp. 1 CEW1, P3.p does not divide whereas P4.p divides twice at the end of the L3 stage in most animals (Sommer and Sternberg 1995; Delattre and Félix 2001c). Moreover, P4.p is part of the vulval competence group and is able to replace P(5–7).p (Dichtel *et al.* 2001), whereas P3.p is not part of the vulval competence group: It does not divide and does not acquire a vulval fate after ablation of P(4–8).p in the L1 stage (Table 3, last column).

We found four mutants of *Oscheius* sp. 1 in which P3.p divides twice, with penetrances of 50–100% (group 3 in Figure 2; Table 3). More anterior or posterior P*n*.p cells are not affected [except that additional cells were observed in the P1.p region in 4/50 *cov-9(mf89)* animals]. Each mutation defines a complementation group, which we name *cov-6* to *cov-9*. If P(4–8).p are ablated in the L1 stage in the *cov-8(mf54)* or *cov-9(mf89)* mutants, P3.p is able to adopt a vulval fate (Table 3). Thus, these mutations correspond to an enlargement of the vulval competence group to P3.p.

Mutants with an abnormal centering of the vulva: The vulval pattern is centered on P6.p in species of the family Rhabditidae (to which Caenorhabditis and Oscheius belong) and of the family Diplogastridae (to which Pristionchus belongs; Sommer and Sternberg 1995; Sommer 1997), whereas it is centered between P6.p and P7.p in the more distant families Panagrolaimidae and Cephalobidae (Sternberg and Horvitz 1982; Félix *et al.* 2000a).

We described above a large group of mutants with a reduced competence group and an abnormal centering of the vulva pattern on P5.p (group 2). Additionally, we found three mutants with a normal competence group but a vulval pattern centered on P7.p (group 4 in Figure 2; Table 4) or even sometimes on P8.p, in which case the vulva lacks a posterior 2° fate because P9.p is not competent. These mutants also show a slight increase in vulval induction (strongest for *mf71*), which may be partially explained by the anchor cell being positioned between P6.p and P7.p at the time of the first induction. Indeed, a few animals with a vulval pattern centered between two P*n*.p cells were observed (see legend to Table 4). The three mutations apparently complement and thus define three genes, *cov-10*, *cov-11*, and *cov-12*.

In mutants with an abnormal centering of the vulval

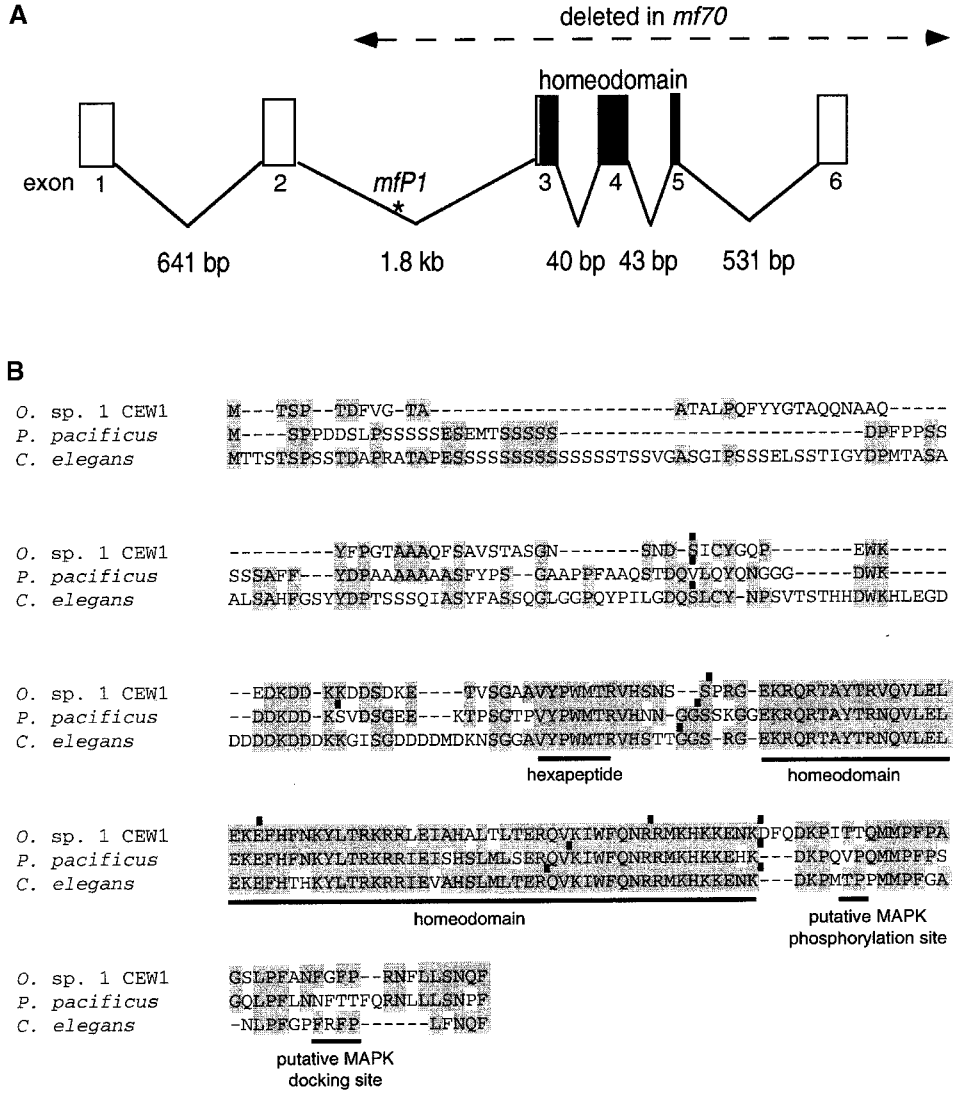

FIGURE 5.—Molecular analysis of the *Oscheius* sp. 1 *lin-39* homolog. (A) *Osp1-lin-39* genomic structure. The homeodomain is shown in black. The size of each intron is indicated below. The drawing is not to scale for intronic sequences and the 3′ endpoint of the *mf70* deletion. (B) Alignment of the full LIN-39 protein sequences of three rhabditid nematodes. Conserved residues between species are indicated by shaded boxes, intron positions by small solid squares. The cDNA sequence of *Osp1-lin-39* is available under accession no. AJ431730.

pattern, either on P5.p (group 2) or on P7.p (group 4), the anchor cell is positioned in the L4 stage immediately dorsal to the cells adopting a central 1° fate. Is the anchor cell mispositioning responsible for a change in vulval centering in these mutants? We first looked at the time of hatching (before anchor cell birth) to see whether the gonad primordium was in its normal position relative to the P*n* cells. In the group 4 *cov-12(mf85)* mutant, the gonad was located more posteriorly than in the wild type, but in the group 2 *cov-3(mf35)* and *cov-3(mf80)* mutants, the gonad position was normal at hatching (Table 5). Following a time course of gonad/anchor cell *vs.* P*n*.p position in these two latter alleles, it appears that P6.p is correctly located below the gonad in the late L1 stage, but by the end of the L2 stage the anchor cell is sometimes displaced above P5.p (or P5.p is displaced below the anchor cell), although not yet at a frequency compatible with the final pattern (Table 5). Thus, the two groups of mutants appear to show an alteration of vulva centering for two distinct reasons. In group 4 (pattern centered on P7.p), the gonad is already displaced posteriorly relative to the P*n* cells at hatching, suggesting an embryonic defect. In group 2 mutants (pattern centered on P5.p and loss of P*n*.p competence), the misalignment of the anchor cell with P5.p occurs later, in the L2 and the L3 stages, close to the time of the first induction. A greater competence of P5.p to adopt a vulval or central vulval fate may be responsible for this change in vulval centering in the group 2 mutants.

DISCUSSION

We have described 17 mutants of vulval competence and centering in *Oscheius* sp. 1, which define 12 *cov* loci and the *Osp1-lin-39* gene. We grouped together these antero-posterior specification mutants affecting competence and centering of the vulva for two reasons: first, because we (unexpectedly) found a large number of mutations that affect both and, second, because the number of competent cells and the precision of vulval centering are related at the evolutionary level. We now

TABLE 3

Mutants of P3.p fate

Genotype	P3.p ssss	P3.p S S, Sss or ssS	% penetrance	P3.p vulval competence
Wild type	0/100	0/100	0	0/8
cov-6(sy460)	26/50	1/50	54	ND
cov-7(sy466)	74/100	7/100	81	ND
cov-8(mf54)	49/100	2/100	51	10/11
cov-9(mf89)	50/50	0/50	100	10/10

The division pattern of P3.p was scored in the L4 stage on worms cultured at 25°. P3.p competence was tested by ablation of P(4–8).p in the L1 stage (before P*n*.a division) and scored positive if P3.p adopted a vulval fate. Abbreviations are as in Table 1. The *cov-6(sy460)* and *cov-8(mf54)* mutants show a weak egg-laying-defective phenotype. The *cov-7(sy466)* mutant shows a partially penetrant temperature-sensitive sinistral phenotype (Delattre and Félix 2001a) and a gonad morphology defect leading to partial sterility. ND, not determined.

discuss their phenotypes in relation to vulval developmental mechanisms in *Oscheius* sp. 1, compare them with those found in *C. elegans*, and assess their consequence for the phenotypic evolvability of the species.

Vulval developmental mechanisms in *Oscheius* sp. 1 and comparison with *C. elegans*: We previously described vulva mutants of *Oscheius* sp. 1 that affect the P*n*.p division pattern specifically (*dov* mutants); this class is almost completely absent in *C. elegans* (Dichtel *et al.* 2001). Conversely, mutants affecting vulval induction form the most numerous class of vulva development mutants in *C. elegans*, but are very rare in *Oscheius* sp. 1 (M.-L. Dichtel and M.-A. Félix, unpublished data). The other frequent categories of vulva mutants found in *Oscheius* sp. 1 are described here and concern vulval competence and/or centering. The paucity of mutants affecting P*n*.p competence in the first extensive screens for Egl mutants (Horvitz and Sulston 1980; Trent *et al.* 1983) is a bizarre historical feature of *C. elegans* vulva development studies. The *lin-39* locus was found only later, in screens either for an abnormal pattern of cell death (in the P*n*.a lineages) or for suppressors of Multivulva mutations (Clark *et al.* 1993). The *bar-1* and several other similar mutations with a lesser penetrance than that of *lin-39* were found in a screen for animals with a protruding vulva (Eisenmann *et al.* 1998; Eisenmann and Kim 2000), which occurs (as in *Oscheius* sp. 1) when an incomplete vulva is formed from only one or two P*n*.p precursor cells. Strangely, although null mutations in these genes are homozygous viable (except for *apr-1*) and display a high penetrance of egg-laying defects, they were not found in the original Egl screen, even though it was estimated to be close to saturation for such viable mutations (Ferguson and Horvitz 1985). Thus, the comparison of frequency of occurrence of phenotypes in the two different species should be taken with some caution. We will review each group successively.

Within the group 1 mutants (loss of P*n*.p competence), the phenotype of the *Osp1-lin-39(mf70)* mutant closely resembles that of a *C. elegans lin-39* null mutant: All P*n*.p cells (except P12.p) stay undivided. The *lin-39*

TABLE 4

Mutants with posterior vulva centering

Genotype	P7.p centering	P8.p centering	% penetrance of posterior centering	Hyperinduced
Wild type	0/100	0/100	0	0/100
cov-10(mf55)	23/51	0/51	45	3/51
cov-11(mf71)	37/88[a]	1/88	43	21/88
cov-12(mf85)	83/135	9/135	68	1/135

The vulva pattern was scored in the L4 stage.

[a] One animal had a vulval pattern centered between P6.p and P7.p, and one between P7.p and P8.p. Animals scored as hyperinduced show an excess of the first induction, with P5.p or P8.p (depending on vulval centering) abnormally induced (or half-induced). *cov-10(mf55)* is slightly Dumpy and shows an abnormal germ line (no rachis) and some embryonic lethality. *cov-11(mf71)* shows divisions of P3.p in 9/44 animals, a weak egg-laying-defective phenotype, and some embryonic lethality. *cov-12(mf85)* shows a fully penetrant and strong egg-laying-defective phenotype and is slightly Uncoordinated.

TABLE 5

Gonad position in mutants with altered vulval centering

Time of development	Relative positions of vulval precursors and gonad	Genotype			
		Wild type	*cov-3(mf35)*	*cov-3(mf80)*	*cov-12(mf85)*
Early L1	P(5/6)L/R				
	lateral to the gonad	11/15	13/16	13/14	2/13
	at the gonad front edge	4/15	3/16	1/14	6/13
	fully in front of the gonad	—	—	—	5/13
Mid L1	P6 below gonad	11/11	10/10	12/12	1/8
	P7 below gonad	—	—	—	7/8
Late L1	P6.p below gonad	8/8	7/7	8/8	ND
L2 lethargus	AC on P6.p	10/10	4/10	3/10	
	AC between P5.p and P6.p	—	3/10	5/10	ND
	AC on P5.p	—	3/10	2/10	

The position of P*n* or P*n*.p cells relative to the gonad or the anchor cell (AC) was scored at different time points during larval development. "Early L1" is before the rotation of P*n* cells. "Mid L1" is after their anteroposterior alignment and before their division. "Late L1" is after their division. The penetrance of vulval centering on P5.p is high in the two *cov-3* alleles (17/18 *mf35* animals and 15/18 *mf80* animals) and ~68% in the *cov-12(mf85)* mutant (see Table 4).

(mf70) mutation is very likely to be a null allele since it deletes the whole homeodomain. However, unlike in a *C. elegans lin-39* null mutant, at least one P*n*.p cell stays unfused until adulthood in ~15% of the animals (but it usually does not divide and it does not form vulval tissue). Thus, in *Oscheius* sp. 1, LIN-39 may be not be absolutely necessary to prevent fusion, and it is required for vulval competence. We have found that the few cells that evade cell fusion do not usually divide. Thus, LIN-39 in *Oscheius* sp. 1 may be required for cell proliferation as was recently found in *C. elegans* (Shemer and Podbilewicz 2002). The conserved role of *lin-39* in preventing P*n*.p cell fusion and division in *C. elegans* and *Oscheius* sp. 1 contrasts with its role in inhibiting P*n*.p cell death in the more distantly related nematode *P. pacificus* (Eizinger and Sommer 1997; Sommer *et al.* 1998).

Although they do not map to the *Osp1-lin-39* locus, the phenotypes of the other group 1 mutants resemble hypomorphic *Cel-lin-39* mutants (Clandinin *et al.* 1997), except that P4.p is not affected in the latter. The *cov-1* and/or *cov-2* mutations may define regulators of *Osp1-lin-39* expression in the L1 stage.

A specific feature of the control of P*n*.p fusion and competence that is unraveled in *Oscheius* sp. 1 is its dependence on the gonad in *cov-1* and *cov-2* mutants (Table 2). As was previously found in the *dov-1(sy543)* mutant for P*n*.p cell divisions (Dichtel *et al.* 2001), this signaling from the gonad is apparent only in a mutant context and does not show in wild-type animals, probably because of redundant processes ensuring P*n*.p cell competence. In other words, competence (or the maintenance of this competence) to receive the later inductive signal is itself subject to inductive signaling from the gonad. This signal may act by repressing cell fusion, like *lin-39* and the regulators of cell fusion *ref-1* and *ref-2* in *C. elegans* (Alper and Kenyon 2001, 2002). Dependence of cell competence on intercellular signaling has been suggested in *C. elegans* by the fact that a hypomorphic mutation in the LET-23 receptor (which acts in receiving the anchor cell inductive signal) and gonad ablation enhance the effect of the *bar-1* mutation (Eisenmann *et al.* 1998).

The group 2 mutants are the most frequent in *Oscheius* sp. 1 (Figure 2) and display an Egl defect; however, such a phenotype with an altered centering on P5.p, in addition to a P*n*.p competence defect, has not been found in *C. elegans*. The anterior vulva displacement has been found only at low penetrance in Lon (long) mutants (Sternberg and Horvitz 1986), and the *Oscheius* sp. 1 group 2 mutants are not long. In *cov-3* mutants, the gonad appears at its normal position at hatching and the anchor cell aligns only with P5.p late during the L2 or L3 stages (Table 5). P5.p thus appears more competent to adopt a 1° sublineage than P6.p and the vulva pattern most often remains incomplete on the anterior side because P4.p is not competent in this mutant. The higher penetrance of defects in P7.p *vs.* P8.p (Table 1) is also surprising. In our screen, this combination of phenotypes has been found at three distinct loci, with four alleles for one locus (including two obtained after TMP-UV mutagenesis), suggesting that they reflect the loss of function of a pathway. Since group 2 mutations have an enhancing effect on P*n*.p competence loss in *cov-1* mutants that is similar to gonad ablation, it is possible that they define an intercellular signaling pathway from the gonad to the P*n*.p cells that regulates their competence and time of fusion (which

would act redundantly in wild-type animals since gonad ablation has no effect in this context).

In *C. elegans*, some components of the Wnt pathway act in maintaining *lin-39* expression in the L2 stage (although no ligand, receptor, or defined cell interaction has been demonstrated). In mutants of this pathway, the affected cells fuse during the late L2 stage, like P3.p in wild type. The *C. elegans bar-1/Armadillo* mutant is mostly affected for P3.p and P4.p (but less for P8.p; Eisenmann *et al.* 1998). Other loci, such as *mig-14/mom-3*, *egl-18*, and *sem-4*, show a mutant phenotype similar to *bar-1* (Eisenmann and Kim 2000). Tissue-specific inactivation of the *apr-1/APC* gene causes L1 fusion of P(3–8).p, also with a higher penetrance for the distal cells (Hoier *et al.* 2000). It is possible that group 2 mutations define a Wnt pathway.

The phenotype of the group 3 mutants corresponds to an evolutionary change within the family Rhabditidae: In *C. elegans*, P3.p divides in one-half of the animals (in the reference strain N2; Sulston and Horvitz 1977) and can adopt a vulval fate upon removal of P(4–8).p (Sternberg and Horvitz 1986; Delattre and Félix 2001c), whereas in *Oscheius* sp. 1, P3.p fuses in the late L2 stage, does not divide, and cannot replace P(4–8).p. Two mutants, *cov-6(sy460)* and *cov-8(mf54)*, show a 50% occurrence of P3.p division similar to that of the *C. elegans* reference strain N2, whereas the *cov-9(mf89)* mutant shows a complete penetrance of P3.p division (Table 3), as was noticed in *C. elegans* by G. Jongeward in *lin-22/Hairy* mutants (mentioned in Wrischnik and Kenyon 1997). Because of the variability of P3.p division, a phenotype of loss or gain of P3.p division/competence is much more difficult to screen for in *C. elegans* than in *Oscheius* sp. 1. In our mutagenesis, these mutants were first picked because of their low-penetrance Egl phenotype and were easily found in a secondary Nomarski screen.

During development, P3.p and P4.p (or at least their respective mothers) are initially equivalent. The 12 P*n* cells (the P*n*.p mother cells) are found at hatching as six symmetric left-right pairs and subsequently align along the ventral midline, in a random orientation for the P(3/4)L/R cell pair as for most P*n* cell pairs in *C. elegans* (Sulston and Horvitz 1977). In *Oscheius* sp. 1, the left and right cells of the P(3/4)L/R cell pair also appear equivalent before rotation (Sommer and Sternberg 1995). They (or their daughters) must later acquire different fates, since only P4.p is competent and divides. The group 3 mutants clearly demonstrate the existence of a specific mechanism of inhibition of P3.p competence that results in a vulval equivalence group of five rather than six cells.

The phenotype of the group 4 mutants (centering of the vulval pattern on P7.p) has also not been specifically screened for in *C. elegans*, but has been observed at low penetrances in some mutant contexts: at ~25% penetrance in mutants for *mab-5*, the HOM-C gene posterior to *lin-39* that is expressed in P(7–11).p (Clandinin *et al.* 1997), and at ~8% penetrance in mutants for the *C. elegans even-skipped* homolog *vab-7* (50% penetrance in the *P. pacificus vab-7* mutants; Jungblut and Sommer 2001). Here we describe three mutants, which cause up to 68% posterior displacement of the vulval 1° fate (Table 4). As in *P. pacificus vab-7* mutants (Jungblut and Sommer 2001), this posterior vulva shift appears to result from aberrant gonad positioning at hatching (Table 5). None of the three *Oscheius* sp. 1 mutants shows the bobbed tail phenotype that is characteristic of *C. elegans* and *P. pacificus vab-7* mutants (Ahringer 1997; Jungblut and Sommer 2001). Interestingly, the two-step mechanism of vulval induction in *Oscheius* sp. 1 could allow the 1° sublineage to be shared between daughters of two different P*n*.ps (as happens for P6.pp and P7.pa in *Panagrolaimus* sp.; Félix and Sternberg 1997). However, we find only a few such animals (Table 4), suggesting the existence of a mechanism of centering on one P*n*.p, for example, by alignment of the anchor cell with a P*n*.p cell before its division.

In summary, the group 1 mutants resemble the *C. elegans* competence mutants, such as *lin-39*, and demonstrate the existence of a gonadal signal that maintains vulval competence in *Oscheius* sp. 1; the many group 2 mutants do not resemble any *C. elegans* (nor *P. pacificus*) mutants and suggest that P*n*.p competence and centering of the 1° fate are coregulated by the same pathway; the group 3 mutants resemble the *C. elegans* wild-type phenotype and demonstrate the existence of a specific developmental mechanism repressing P3.p competence in *Oscheius* sp. 1; the group 4 mutants have not been found at such high penetrance in *C. elegans* and suggest the existence of a mechanism for centering of the vulva on a single P*n*.p cell in *Oscheius* sp. 1.

Comparison with phenotypes found in wild populations: The high number of mutations affecting vulval divisions described in Dichtel *et al.* (2001) demonstrates the high mutability of *Oscheius* sp. 1 CEW1 to these phenotypes. Correspondingly, we found many similar variations in vulval divisions in wild populations of *Oscheius* sp. 1 and in closely related species, whereas such mutants and wild variations do not exist within *C. elegans* (Delattre and Félix 2001b,c). Here we review the occurrence of phenotypes in wild strains corresponding to that of the Cov mutants.

Animals with a loss of competence such as found in group 1 and group 2 mutants are not found in wild isolates, which is likely due to a negative selection pressure acting on egg-laying-defective animals.

By contrast, occasional centering of the vulva pattern on P5.p or P7.p occurs in wild-type animals of both species (Delattre and Félix 2001c). Such noise in centering of the vulval pattern constitutes a selection pressure for a five-cell competence group (one more competent cell on each side of the vulva). It is not clear whether there are selection pressures in *C. elegans* and

Oscheius sp. 1 acting to include or exclude the sixth cell P3.p from the competence group. This character evolves within each genus. A few animals with divided P3.p can be found in some *Oscheius* sp. 1 strains and in closely related species in about one-fourth of the animals (Delattre and Félix 2001c). We show here that a mutable mechanism specifies a distinct noncompetent fate in P3.p compared to P4.p (whereas we did not find any mutants abolishing specifically P4.p or P8.p competence). The existence of such a mechanism suggests that some evolutionary pressure acts (perhaps indirectly) to reduce the competence group to five rather than six cells in *Oscheius* sp. 1. It will be interesting to study the polarity of change in vulval competence group size within the genus Oscheius and the family Rhabditidae. Some nematodes such as *Panagrolaimus* spp. (family Panagrolaimidae) have a vulval competence domain that is not larger than the cells actually adopting a vulval fate and may rely on a greater precision of centering (Sommer and Sternberg 1996; Félix *et al.* 2000a). Thus, the combination of the number of competent cells and of the precision of vulval centering is important for the reproducibility of vulva formation.

We are very grateful to Marie-Laure Dichtel for great help with the screens, to Marie Delattre for finding the *mf89* mutant, and to both of them for comments on the manuscript. We thank Evelyne Pierre for help with genomic library construction and freezing many strains. We thank Paul Sternberg in whose lab the screen was started and Christophe Antoniewski, Jean-Louis Bessereau, and Ralf Sommer for advice and discussions. This work was supported by a Human Frontier Science Program collaborative grant to M.-A. F. and B.P., the Centre National de la Recherche Scientifique (France), and the Association pour la Recherche sur le Cancer.

LITERATURE CITED

Ahringer, J., 1997 Maternal control of a zygotic patterning gene in *Caenorhabditis elegans*. Development **124:** 3865–3869.

Alper, S., and C. Kenyon, 2001 REF-1, a protein with two bHLH domains, alters the pattern of cell fusion in *C. elegans* by regulating Hox protein activity. Development **128:** 1793–1804.

Alper, S., and C. Kenyon, 2002 The zinc finger protein REF-2 functions with the Hox genes to inhibit cell fusion in the ventral epidermis of *C. elegans*. Development **129:** 3335–3348.

Clandinin, T. R., W. S. Katz and P. W. Sternberg, 1997 *Caenorhabditis elegans* HOM-C genes regulate the response of vulval precursor cells to inductive signal. Dev. Biol. **182:** 150–161.

Clark, S. G., A. D. Chisholm and H. R. Horvitz, 1993 Control of cell fates in the central body region of *C. elegans* by the homeobox gene *lin-39*. Cell **74:** 43–55.

Delattre, M., 2001 Variants de développement et évolution chez les nématodes: études sur la chiralité de P11/12 et sur le lignage des cellules précurseurs de la vulve. Ph.D. Thesis, Université de Paris 6, Paris.

Delattre, M., and M.-A. Félix, 2001a Evolution and development of a variable left-right asymmetry in nematodes: the handedness of P11/P12 migration. Dev. Biol. **232:** 362–371.

Delattre, M., and M.-A. Félix, 2001b Microevolutionary studies in nematodes: a beginning. Bioessays **23:** 807–819.

Delattre, M., and M.-A. Félix, 2001c Polymorphism and evolution of vulval precursor cell lineages within two nematode genera, *Caenorhabditis* and *Oscheius*. Curr. Biol. **11:** 631–643.

Dichtel, M.-L., S. Louvet-Vallée, M. E. Viney, M.-A. Félix and P. W. Sternberg, 2001 Control of vulval cell division number in the nematode *Oscheius/Dolichorhabditis* sp. CEW1. Genetics **157:** 183–197.

Eisenmann, D. M., and S. K. Kim, 2000 Protruding vulva mutants identify novel loci and Wnt signaling factors that function during *Caenorhabditis elegans* development. Genetics **156:** 1097–1116.

Eisenmann, D. M., J. N. Maloof, J. S. Simske, C. Kenyon and S. K. Kim, 1998 The β-catenin homolog BAR-1 and LET-60 Ras coordinately regulate the Hox gene *lin-39* during *Caenorhabditis elegans* vulval development. Development **125:** 3667–3680.

Eizinger, A., and R. J. Sommer, 1997 The homeotic gene *lin-39* and the evolution of nematode epidermal cell fates. Science **278:** 452–455.

Epstein, H. F., and D. C. Shakes (Editors), 1995 *Caenorhabditis elegans: Modern Biological Analysis of an Organism*. Academic Press, San Diego.

Félix, M.-A., and P. W. Sternberg, 1997 Two nested gonadal inductions of the vulva in nematodes. Development **124:** 253–259.

Félix, M.-A., P. De Ley, R. J. Sommer, L. Frisse, S. A. Nadler *et al.*, 2000a Evolution of vulva development in the Cephalobina (Nematoda). Dev. Biol. **221:** 68–86.

Félix, M.-A., M. Delattre and M.-L. Dichtel, 2000b Comparative developmental studies using *Oscheius/Dolichorhabditis* sp. CEW1 (Rhabditidae). Nematology **2:** 89–98.

Félix, M.-A., A. Vierstraete and J. Vanfleteren, 2001 Three biological species related to *Rhabditis (Oscheius) pseudodolichura* Körner in Osche, 1952. J. Nematol. **33:** 104–109.

Ferguson, E., and H. R. Horvitz, 1985 Identification and characterization of 22 genes that affect the vulval cell lineages of *Caenorhabditis elegans*. Genetics **110:** 17–72.

Ferguson, E. L., P. W. Sternberg and H. R. Horvitz, 1987 A genetic pathway for the specification of the vulval cell lineages of *Caenorhabditis elegan*s. Nature **326:** 259–267.

Finney, M., and G. Ruvkun, 1990 The *unc-86* gene product couples cell lineage and cell identity in *Caenorhabditis elegans*. Cell **63:** 895–905.

Francis, R., and R. H. Waterston, 1991 Muscle cell attachment in *Caenorhabditis elegans*. J. Cell Biol. **114:** 465–479.

Grandien, K., and R. J. Sommer, 2001 Functional comparison of the nematode Hox gene *lin-39* in *C. elegans* and *P. pacificus* reveals evolutionary conservation of protein function despite divergence of primary sequence. Genes Dev. **15:** 2161–2172.

Hoier, E. F., W. A. Mohler, S. K. Kim and A. Hajnal, 2000 The *Caenorhabditis elegans* APC-related gene *apr-1* is required for epithelial cell migration and *Hox* gene expression. Genes Dev. **14:** 874–886.

Horvitz, H. R., and P. W. Sternberg, 1991 Multiple intercellular signalling systems control the development of the *C. elegans* vulva. Nature **351:** 535–541.

Horvitz, H. R., and J. E. Sulston, 1980 Isolation and genetic characterization of cell-lineage mutants of the nematode *Caenorhabditis elegans*. Genetics **96:** 435–454.

Jungblut, B., and R. J. Sommer, 2001 The nematode *even-skipped* homolog *vab-7* regulates gonad and vulva position in *Pristionchus pacificus*. Development **128:** 253–261.

Kimble, J., and D. Hirsh, 1979 Post-embryonic cell lineages of the hermaphrodite and male gonads in *Caenorhabditis elegans*. Dev. Biol. **70:** 396–417.

Maloof, J. N., and C. Kenyon, 1998 The Hox gene *lin-39* is required during *C. elegans* vulval induction to select the outcome of Ras signaling. Development **125:** 181–190.

Maniatis, T., E. F. Fritsch and J. Sambrook, 1989 *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Mohler, W. A., G. Shemer, J. J. del Campo, C. Valansi, E. Opoku-Serebuoh *et al.*, 2002 The type I membrane protein EFF-1 is essential for developmental cell fusion. Dev. Cell **2:** 355–362.

Podbilewicz, B., 1996 ADM-1, a protein with metalloprotease- and disintegrin-like domains, is expressed in syncytial organs, sperm, and sheath cells of sensory organs in *Caenorhabditis elegans*. Mol. Biol. Cell **7:** 1877–1893.

Podbilewicz, B., and J. G. White, 1994 Cell fusions in the developing epithelia of *C. elegans*. Dev. Biol. **161:** 408–424.

Sharma-Kishore, R., J. G. White, E. Southgate and B. Podbilewicz, 1999 Formation of the vulva in *Caenorhabditis elegans*: a paradigm for organogenesis. Development **126:** 691–699.

Shemer, G., and B. Podbilewicz, 2000 Fusomorphogenesis: cell fusion in organ formation. Dev. Dyn. **218:** 30–51.

Shemer, G., and B. Podbilewicz, 2002 LIN-39/Hox triggers cell division and represses EFF-1/fusogen-dependent vulval cell fusion. Genes Dev. **15:** 3136–3141.

Shemer, G., R. Kishore and B. Podbilewicz, 2000 Ring formation drives invagination of the vulva in *C. elegans*: Ras, cell fusion and cell migration determine structural fates. Dev. Biol. **221:** 233–248.

Sommer, R. J., 1997 Evolutionary changes of developmental mechanisms in the absence of cell lineage alterations during vulva formation in the Diplogastridae (Nematoda). Development **124:** 243–251.

Sommer, R. J., and P. W. Sternberg, 1995 Evolution of cell lineage and pattern formation in the vulval equivalence group of rhabditid nematodes. Dev. Biol. **167:** 61–74.

Sommer, R. J., and P. W. Sternberg, 1996 Apoptosis and change of competence limit the size of the vulva equivalence group in *Pristionchus pacificus*: a genetic analysis. Curr. Biol. **6:** 52–59.

Sommer, R., A. Eizinger, K.-Z. Lee, B. Jungblut, A. Bubeck *et al.*, 1998 The *Pristionchus* HOX gene *Ppa-lin-39* inhibits programmed cell death to specify the vulva equivalence group and is not required during vulval induction. Development **125:** 3865–3873.

Sternberg, P. W., and H. R. Horvitz, 1982 Postembryonic nongonadal cell lineages of the nematode *Panagrellus redivivus*: description and comparison with those of *Caenorhabditis elegans*. Dev. Biol. **93:** 181–205.

Sternberg, P. W., and H. R. Horvitz, 1986 Pattern formation during vulval development in *Caenorhabditis elegans*. Cell **44:** 761–772.

Sulston, J., and H. R. Horvitz, 1977 Postembryonic cell lineages of the nematode *Caenorhabditis elegans*. Dev. Biol. **56:** 110–156.

Trent, C., N. Tsung and H. R. Horvitz, 1983 Egg-laying defective mutants of the nematode *Caenorhabditis elegans*. Genetics **104:** 619–647.

Wang, B. B., M. M. Muller-Immergluck, J. Austin, N. T. Robinson, A. Chisholm *et al.*, 1993 A homeotic gene cluster patterns the anteroposterior body axis of *C. elegans*. Cell **74:** 29–42.

Wang, M., and P. W. Sternberg, 2001 Pattern formation during *C. elegans* vulval induction. Curr. Top. Dev. Biol. **51:** 189–220.

Wood, W. B., 1988 *The Nematode Caenorhabditis elegans*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Wrischnik, L. A., and C. J. Kenyon, 1997 The role of *lin-22*, a hairy/Enhancer of split homolog, in patterning the peripheral nervous system of *C. elegans*. Development **124:** 2875–2888.

Communicating editor: P. Anderson