The *mec-3* gene of *Caenorhabditis elegans* requires its own product for maintained expression and is expressed in three neuronal cell types

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The homeo-box-containing gene *mec-3* of the nematode *Caenorhabditis elegans*, is expressed in several sensory neurons, as assayed by expression of a *mec-3-lacZ* fusion. These cells are the touch receptors, which mediate the response to gentle touch, and the FLP and PVD neurons. PVD mediates a response to harsh mechanical stimuli, and FLP has an ultrastructure suggestive of a mechanoreceptor, but its function is unknown. *mec-3* is necessary for the differentiation of the touch receptors, because in *mec-3* mutants, the touch receptors do not function and have none of their distinguishing features. *mec-3* is also needed for PVD function: The PVD neurons no longer mediate a response to harsh mechanical stimuli in the mutants. The expression of the *mec-3-lacZ* fusion, and presumably *mec-3* itself, is altered by mutations in several genes originally identified by their effects on touch cell development. *unc-86*, another homeo-box-containing gene, is necessary for all *mec-3-lacZ* expression, but also affects several other lineages and cells in which *mec-3* is not expressed. *mec-3* activity appears to be required for maintained expression of the *mec-3-lacZ* fusion in all cells in which it is expressed. In a *mec-17* mutant, *mec-3-lacZ* expression is not maintained in the touch receptors, but is not affected in the FLP and PVD neurons. These findings suggest that combinatorial mechanisms of gene regulation control both the expression of *mec-3* itself and its action in promoting the terminal differentiation of various cell types.

[Key Words: *Caenorhabditis elegans*; homeo box genes; autoregulation; neuronal differentiation]

Received August 4, 1989; revised version accepted September 25, 1989.

The determination of identity of particular types of cells is a fundamental aspect of development. As a result of cell-type determination, a characteristic set of proteins is synthesized in a given cell. In single cell organisms, considerable information is available on the regulation of cell type, such as the lysis/lysogeny switch for λ phage growth in *Escherichia coli* (Ptashne 1987) or the determination of mating type in the yeast *Saccharomyces cerevisiae* (Herskowitz 1988). In each case, the mechanisms by which the regulatory genes controlling these processes are themselves controlled are reasonably well understood.

In contrast, little is known of how regulatory genes act to induce families of cell type-specific structural genes or how the regulators of cell type are themselves regulated in multicellular animals. In *Drosophila* the determination of the correct cell fate within a region of the developing embryo appears to depend on interactions among a complex pattern of the regulatory genes (for review, see Akam 1987; Ingham 1988), but the molecular mechanisms underlying this combinatorial regulation of development are not well understood.

We have been investigating cell determination by focusing on the *mec-3* gene, which controls the identity of the six touch receptor neurons of the nematode *Caenorhabditis elegans* (Way and Chalfie 1988). The *C. elegans* nervous system is well suited for the study of determination and differentiation of individual cell types, in part because various neuronal cell types mediate easily assayed, nonessential behaviors. For example, the touch cells mediate the animal's response to gentle touch. Loss of these cells results in a specific behavioral defect that is not mimicked by the loss of any other cell type (Chalfie et al. 1985). Thus, mutations affecting the development and function of this cell type can be identified and distinguished from mutations affecting other classes of cells (Chalfie and Sulston 1981; Chalfie and Au 1989).

Mutational analysis has revealed genes that are necessary for the generation, specification, and function of the touch receptors (Chalfie and Au 1989). For example, the homeo-box-containing gene *unc-86* is necessary for cell lineages that generate the touch receptors. Mutations in *unc-86* are pleiotropic: they affect the lineages that gen-

erate the touch receptors, several other neuronal lineages, and the terminal differentiation of the hermaphrodite-specific neurons (Chalfie et al. 1981, Finney et al. 1988). *mec-3* mutants appear to have a defect in the specification of the touch receptors: the pattern of cell division leading to production of these cells is normal, but the cells that are produced have none of the characteristic features of touch receptors. Members of the function class of mutants have recognizable touch receptors that do not mediate a touch response. They appear to encode proteins that are needed for touch receptor activity.

The *mec-3* gene contains a homeo box, suggesting that the *mec-3* protein binds to DNA to regulate transcription of other genes (Way and Chalfie 1988). From the phenotypes and epistatic interactions of different touch-insensitive mutations, it appears that *mec-3* is a regulatory gene that acts upstream of the function genes. A simple hypothesis is that *mec-3* may bind to the DNA of the function genes and induce their transcription. One prediction from this hypothesis is that *mec-3* should be expressed within the touch receptors. To test this hypothesis, we determined the sites of *mec-3* expression within *C. elegans* using a *mec-3-lacZ* fusion. We found that β -galactosidase is localized not only to the six touch receptors, but also to four other neurons. At least two of these neurons are functionally defective in *mec-3* mutants.

To understand how the *mec-3* gene is regulated, we examined the expression of the *mec-3-lacZ* fusion in *C. elegans* mutants that are defective in touch receptor development. For example, *unc-86*, which is necessary for touch receptor generation and shares considerable sequence homology with a number of mammalian tran-

scription factors (Finney et al. 1988), may regulate *mec-3* expression. Our experiments indicate that *mec-3* is expressed in a subset of *unc-86*-dependent lineages, that *mec-3* expression depends on *unc-86*, and that continued expression of *mec-3* requires the *mec-3* product itself as well as the function of the *mec-17* gene.

Results

Construction and transformation of a *mec-3-lacZ* fusion

To identify sites of *mec-3* expression, a *C. elegans* genetic element was generated from a plasmid carrying a *mec-3-lacZ* gene fusion, pTU28 (Fig. 1) and a marker plasmid encoding antisense RNA for the *unc-22* gene, pPD10.41 (A. Fire, pers. comm.). *C. elegans* that are transformed with the second plasmid display the twitcher phenotype of *unc-22* loss-of-function mutants. The entire *mec-3* gene [as defined by rescue of the touch-insensitivity defect of *mec-3(u298)* after transformation] lies within a 3.5-kb DNA fragment: A series of plasmids containing *mec-3* sequences from at least base pairs 1500–5000 all rescue the *mec-3(u298)* mutant on microinjection [Fig. 1; previous experiments had limited the *mec-3* sequence to base pairs 0–5700 (Way and Chalfie 1988)]. The *mec-3* sequences in pTU28 include 3.5 kb at the 5' end and 600 bp at the 3' end in addition to a minimal region required for gene activity. A mixture of pTU28 and pPD10.41 was injected into *C. elegans*. In one transformed animal, the DNAs apparently formed an extrachromosomal element carrying ~40 copies per cell of each plasmid (as estimated by Southern analysis; data not shown). Similar injections of other plasmid mixtures also yield mixed arrays (Stinch-

Figure 1. A plasmid containing a *mec-3-lacZ* fusion and plasmids containing the intact *mec-3* gene. pTU28 contains the *mec-3* sequences from -2 kb to +5.6 kb on the sequence of Way and Chalfie (1988). At the *Xho*I site in the *mec-3* homeo box, a 6-kb fragment of DNA containing most of the *lacZ* gene has been inserted, generating an in-frame translational fusion. The other plasmids were constructed by deletion of pTU23 as described in Methods. All of these plasmids except pTU28 contain a functional *mec-3* gene as defined by transformation rescue of *mec-3(u298)*. The deletion in pTU46 removes the second putative *mec-3* exon. Because of the predicted splicing pattern (Way and Chalfie 1988) this result indicates that the first two suggested exons are nonessential for *mec-3* function. Restriction site code: (V) *Eco*RV; (R) *Eco*RI; (G) *Bgl*II; (C) *Cl*AI; (X) *Xho*I; (Sp) *Spe*I; (Sa) *Sal*I.

comb et al. 1985; A. Fire, pers. comm.). This element, termed *uEx4*, behaves genetically as a free duplication (see Methods). All following experiments use the *uEx4* element.

The *lacZ* fragment in pTU28 disrupts the *mec-3* homeo box, so the *mec-3* gene in *uEx4* should not be active. The *mec-3-lacZ* fusion construction appears not to confer *mec-3* gene activity: *mec-3; uEx4* animals are Mec (the touch-insensitive phenotype conferred by a *mec-3* mutation; Chalfie and Sulston 1981). Moreover, the *uEx4* construction does not interfere with normal *mec-3* activity: Wild-type *uEx4* animals are not Mec (as much as can be determined in spite of the twitching induced by *uEx4*). Furthermore, *mec-4(d); uEx4* animals show the touch-receptor degeneration that results from the dominant *mec-4(e1611)* mutation [*mec-4(d)*] and that requires *mec-3* activity (see below; Chalfie and Sulston 1981; Way and Chalfie 1988).

The mec-3-lacZ fusion is expressed in the touch receptors and the FLP and PVD neurons

C. elegans carrying the fusion construction show β -galactosidase activity, as assayed with the histochemical substrate 5-bromo-4-chloro-3-indolyl- β -galactopyranoside (X-gal). Activity is seen in ten cells at 20°C: the six touch receptors [the ALML/R, AVM, PVM, and PLML/R cells (L/R, left/right)] and the FLPL/R and PVDL/R neurons (Fig. 2, Table 1). The stained cells were identified by their position, time of appearance, and absence in some mutants and apparent mosaics (see Methods and below).

At a low frequency (<5% of animals examined), other cells are stained: usually these cells are neurons, but occasionally intestinal cells show β -galactosidase activity. In adult animals that have begun to lay eggs, one or two stained cells were observed in the region of the vulva (14 of 47 adults with eggs) and anterior to FLP (7 of 52 animals). These cells were not identified. In addition, the frequency of the unusual, faintly staining cells is increased in animals raised at 25°C. In older adults (>20 eggs) the cells that stain occasionally are one to seven neurons in the ventral cord between the vulva and the preanal ganglion, and two cells that may be the AIZ cells, the FLP sisters. We do not know the significance of this staining because it is only seen long after these cells arise, is strongly temperature-dependent, and is generally seen in animals that appear overstained in the touch, FLP, and PVD cells.

Because *C. elegans* neurons are small and have little cytoplasm, it is difficult to determine whether the β -galactosidase activity is localized to the nucleus or to the cell body in staining neurons. However, when the large intestinal cells are stained, the blue X-gal reaction product is clearly localized to the nucleus and not the entire cell. This suggests that the *mec-3* protein contains a nuclear localization sequence between the amino-terminus and the site of the *lacZ* fusion.

In an otherwise wild-type animal carrying the *mec-3-lacZ* fusion, the touch receptors and the FLP and PVD neurons continue to express β -galactosidase activity

throughout the life span of the animal. For the AVM and PVM touch cells and the PVD neurons [all postembryonic cells (Sulston and Horvitz 1977)] staining begins at approximately the time when the cells arise from their respective lineages. We do not know whether the immediate precursors of these cells also stain, because the precursors exist for only about 2 hr before dividing.

Effect of touch-receptor mutations on mec-3-lacZ fusion expression

To determine whether mutations that alter touch receptor development affect the expression of the *mec-3* product, the *uEx4* element was crossed into several mutant strains (see Methods). Expression of β -galactosidase from the *uEx4* element was examined in *unc-86(e1416)*, *mec-3(u298)*, *mec-4(d; e1611)*, and *mec-17(u265)* backgrounds.

In the *unc-86(e1416)* mutant, no staining is seen in the normal positions of the touch receptors and the FLP and PVD cells. *unc-86* mutants show alterations in the lineages that produce these cells [Chalfie et al. 1981; Chalfie and Sulston 1981; J. Sulston, unpubl. (as cited in Finney 1987). Occasionally, a novel cell will stain (not one of the usual set; 2 of 25 animals examined), but most animals show no staining at all.

In the *mec-3(u298)* mutant, the fusion is initially expressed in the correct cells, but β -galactosidase activity in these cells is not seen at later stages of development (Table 1). For example, the ALM, PLM, and FLP cells arise 6 hr before hatching in animals grown at 20°C (Sulston et al. 1983). Staining of these cells can be seen (at a reduced frequency) within 6 hr after hatching. Twenty hours later, in the middle of larval development, these cells no longer stain. Because β -galactosidase activity is seen in wild-type animals throughout development, it appears that an intact *mec-3* gene is necessary for the continuous expression of the *mec-3-lacZ* fusion. (These observations also suggest that the RNA and protein products of the *mec-3-lacZ* gene fusion have a lifetime of less than about 32 hr. It is formally possible that *mec-3* acts to stabilize the fusion protein or mRNA, but this seems unlikely.)

In animals carrying the *mec-4(d)* mutation, the touch receptors degenerate. *mec-4(d)* appears to encode a toxic form of the *mec-4* product that causes cells in which it is expressed to die (Chalfie and Sulston 1981; Herman 1987). No staining corresponding to the touch receptors is seen in adult *mec-4(d); uEx4* animals. This observation uniquely identifies the touch receptors as staining cells because no other cell type reproducibly degenerates in *mec-4(d)* mutants (Chalfie and Sulston 1981; Way and Chalfie 1988). [Occasionally, in an L4 animal, a stained AVM or PVM cell can be seen (Table 1). These cells do not always degenerate by this stage (Chalfie and Sulston 1981).] β -Galactosidase activity continues to be observed in the PVD and FLP cells, which apparently do not degenerate in the *mec-4(d)* background.

Expression of *mec-3-lacZ* was also tested in

Figure 2. Cells expressing the *mec-3-lacZ* fusion in an adult *C. elegans* hermaphrodite. (A) Schematic diagram of the five pairs of cells that express β -galactosidase activity in adults carrying the *uEx4* element. The FLP, ALM, PVD, and PLM neurons are found as bilaterally symmetric pairs; the AVM and PVM neurons arise from virtually identical lineages but are asymmetrically positioned as a result of different migration patterns of their precursors (Sulston and Horvitz 1977). (B) β -Galactosidase staining in a young adult (the AVM, PVM, and PVD neurons are stained too faintly to be seen clearly at this magnification). (C, E, and G) Bright-field exposures of the FLPL, PVDR, and PLML neurons stained with X-gal. (D, F, and H) Same cells and neighboring nuclei stained with the nuclear fluorescent dye DAPI.

mec-17(u265) mutant animals. In *mec-17*, the touch receptors function normally in newly hatched animals, but touch sensitivity is lost as the animals mature (Chalfie and Au 1989). In *mec-17* mutants carrying the *mec-3-lacZ* fusion, the frequency of staining of the touch receptors is reduced by about threefold, whereas the PVD and FLP neurons stain normally. Of the embryonically derived *mec-3-lacZ*-expressing cells, only 37% (84 of 228) of the touch cells (ALML/R and PLML/R)

were stained, whereas 91% (102 of 112) of the FLP cells were stained in L4 larvae and adults (Table 1). The reduction in β -galactosidase activity in the touch receptors occurs in the middle of larval development, at about the same time as the reduction in touch-sensitivity of *mec-17* animals (Table 1; Chalfie and Au 1989). [The AVM/PVM staining appears normal in *mec-17* adults (Table 1). We do not know whether or not the presence of staining is a reflection of the relative age of

Table 1. Expression of the *mec-3*—*lacZ* fusion in wild-type and mutant strains

Strain	Stage	Number of animals	Cells stained (%)				
			ALML/R	AVM + PVM	PLML/R	FLPL/R	PVDL/R
Wild type	L1	24	58	2	73	44	NA
	L2/L3	12	58	8	38	38	46
	L4	15	83	43	70	70	63
	adult	36	75	38	68	81	78
at 25°C	L1	18	83	39	89	67	NA
	adult	25	88	68	84	96	92
<i>unc-86</i>	L1	18	0	0	0	0	NA
	L4/adult	7	0	0	0	0	0
<i>mec-3</i>	L1	21	5	2	26	29	NA
	L2/L3	13	0	8	0	0	8
	adults ^a	12	0	0	0	0	0
<i>mec-4(d)</i>	L4/adults	27	0	17	0	78	35
<i>mec-17</i>	L1	10	85	0	85	75	NA
	at 25°C adults	22	11	68	43	100	91
<i>mec-17 unc-24</i>	L1/L2	24	90	50	65	44	13
	at 25°C L3/young L4	13	72	45	68	70	89
	L4/young adult	15	37	20	33	80	53
	adult with eggs	20	35	48	38	85	88

Animals were identified as carrying the *uEx4* element by their Twitcher phenotype and stained with X-gal. Each cell column represents a pair of cells in each animal. The stage of development was determined either by synchronizing animals at hatching (for L1, L2, and L3 animals) or by examining the development of the gonad and the presence of fertilized eggs. In embryos, it was not possible to identify staining cells. However, embryos with more than six staining cells (i.e., having more than embryonic FLP, ALM, and PLM cells) were not seen. (NA) Not applicable; PVD cells do not arise until the L2 stage.

^aTen of twelve *mec-3*; *uEx4* adults contained embryos that stained with X-gal.

these postembryonic cells compared to that of the ALM and PLM cells.]

The PVD neurons mediate a response to a strong mechanical stimulus

Two observations argue that the chromosomal *mec-3* gene is normally expressed in the FLP and PVD neurons: (1) the *mec-3*—*lacZ* fusion is expressed in these cells, and (2) the wild-type chromosomal gene is needed to maintain expression of the fusion in these cells. Therefore, we sought to identify a behavior mediated by the PVD and FLP neurons and to determine whether this function was altered in *mec-3* mutants. Ward et al. (1975) and E. Hedgecock (pers. comm.), respectively, suggested that the FLP and PVD neurons may be mechanosensory receptors on the basis of their ultrastructure. Each FLP cell has an anterior process with a ciliated ending that is characteristic of many mechanosensory cells. The PVD cells have long anterior and posterior processes that make essentially no synapses, and which therefore may be sensory processes. Both the PVD and FLP cells have ventral processes that synapse onto the interneurons that control forward and backward movement (White et al. 1986).

To test this possibility, animals with or without PVD neurons (and lacking the touch receptors) were tested for their response to a strong mechanical stimulus: a prodding with a platinum wire to the midsection of the body. Several mutant and laser-ablated animals were tested in blind experiments (Table 2). *unc-86* and *lin-32(u282)*

mutants, which lack PVD cells and all or some of the touch receptors, failed to respond to this stimulus. *mec-4(d)* animals, in which the PVD cells appear to be normal but the touch receptors degenerate, responded to being prodded with a platinum wire, usually by moving backwards.

When the PVD neurons are eliminated from *mec-4(d)*, either by laser ablation of PVD or its precursor V5, the response to prodding is lost (Table 2). Ablation of the neighboring cells (V4 or neurons adjacent to PVD) had no effect on this behavior. These results suggest that the response to strong mechanical stimulus is mediated by the PVD cells, and that this behavior can be used to assay PVD function.

mec-3 is required for PVD function

Most *mec-3* mutants do not respond when similarly prodded with a platinum wire, which suggests that *mec-3* is needed for proper function of the PVD neurons as well as for that of the touch receptors (Table 2). [*mec-3(u312)* mutants do respond in this assay, but this mutation appears to be hypomorphic because it only partially fails to complement other *mec-3* mutations for touch insensitivity (M. Chalfie, unpubl.).] In blind experiments in which *mec3*, *mec-4(d)*, and *unc-86* animals are mixed and tested for sensitivity to the platinum wire, *mec-3* and *unc-86* animals are equally insensitive and easily distinguished from the sensitive *mec-4(d)* animals (Table 2).

Because of the apparent loss of PVD function, we

Table 2. Sensitivity of *C. elegans* strains to a strong mechanical stimulus

Genotype or cells ablated	Response to harsh touch ^a	Number responding out of total ^b	Relevant touch receptor or PVD phenotype
<i>mec-4(d; e1611)</i>	+	15/16	touch receptors degenerate
<i>unc-86(e1416)</i>	—	0/8	touch receptors and PVD absent because of lineage alterations ^c
<i>lin-32(u282)</i>	—		AVM, PVM, and PLM touch receptors absent, PVD absent because of lineage alterations ^d
<i>mec-4(d; e1611)</i>			touch receptors degenerate
V5 ablated	—	2/9	PVD absent
V4 ablated	+	6/6	PVD present
PVD ablated	—	1/6	PVD absent
unlasered	+	7/8	PVD present
<i>mec-3(e1338)</i>	—		touch receptors fail to express
(<i>e1498</i>)	—	0/6	any specific features; PVD nonfunctional (as indicated here)
(<i>e1612</i>)	—		
(<i>u6</i>)	—		
(<i>u81</i>)	—	0/8	
(<i>u99</i>)	—	2/5	
(<i>u184</i>)	—		
(<i>u298</i>)	—	2/16	
(<i>u312</i>)	+		
<i>mec-1(e1336)</i>	+		touch receptors differentiate correctly,
<i>mec-2(u447)</i>	+	8/8	but do not function;
<i>mec-4(u441)</i>	+	8/8	PVD functional (as indicated here)
<i>mec-5(e1340)</i>	+		
<i>mec-6(u450)</i>	+		
<i>mec-7(u433)</i>	+		
<i>mec-8(e398)</i>	+	8/8	
<i>mec-9(u258)</i>	+		
<i>mec-10(e1715)</i>	+	7/7	
<i>mec-12(e1605)</i>	+		
<i>mec-14(u82)</i>	+	6/8	
<i>mec-1(e1336); mec-7(e1505)</i>	+		

^aAll strains were tested for sensitivity to a strong mechanical stimulus by examining animals of known genotype.

^bSeveral strains were tested in blind experiments in which individual animals were picked to separate plates and scored in ignorance of the animal's genotype. All laser-ablated animals were scored in blind tests along with unablated animals or control ablations (see Methods).

^cChalfie et al. 1981.

^dChalfie and Au 1989.

wished to determine whether the PVD neurons were morphologically altered in a *mec-3* mutant. *mec-3* mutations cause two of the touch receptors, the ALM cells, to resemble their lineage sister neurons (Way and Chalfie 1988). We sought to test whether, in a *mec-3* mutant, the PVD neurons also resemble their sister cells, which undergo programmed cell death. The PVD neurons were examined in *ced-1; mec-3(u298)* and *ced-1; mec-3(+)* strains. [Normal cell deaths, when viewed with Nomarski optics, are refractile for about 30 min (Sulston and Horvitz 1977); *ced-1* causes cells undergoing programmed cell deaths to remain refractile for several hours (Hedgecock et al. 1983).] Eleven of 12 *mec-3; ced-1* animals examined in the early L3 stage showed refractility of the PVD sister cell, whereas PVD appeared intact. In one animal, the PVD neuron could not be distinguished from other cells in the post-deirid cluster of neurons, and one neuron was missing from this group. Ten *ced-1; mec-3(+)* animals observed at the same stage had the normal pattern of neurons and cell deaths (Ellis and Horvitz 1986). Thus, loss of *mec-3* activity does not cause the PVD cells to assume a fate like

their sisters. In addition, the characteristic large size and position of the cell bodies of the PVD cells do not appear to be altered in *mec-3(u298)* mutants observed with Nomarski optics.

The fate of the PVD sister cell was unaffected by the *mec-3* mutation: This cell still underwent programmed cell death. Thus, it appears that *mec-3* acts only on a single cell at the end of a lineage (PVD in this case), rather than in a precursor cell.

Several strains with *mec* mutations of the function class (*mec-1*, *mec-2*, *mec-4*, *mec-5*, *mec-6*, *mec-7*, *mec-8*, *mec-9*, *mec-10*, *mec-12*, and *mec-14*, and the double mutant *mec-1; mec-7*) respond normally to prodding with a platinum wire (Table 2). These genes may encode products that are used only in the touch receptors and not in the PVD cells. Alternatively, mutations in these genes may only debilitate partially the touch receptors with the effect of raising the threshold stimulus: In these mutants, the touch receptors could mediate a response to a strong stimulus but not to gentle touch.

It is unclear whether FLP also mediates a response to a mechanical stimulus. *unc-86* animals, which lack FLP,

respond when prodded on the pharynx, in the region of the FLP sensory process. Because a number of additional, putative mechanosensory neurons exist in the head (Ward et al. 1975), any of these could mediate a response in the absence of FLP.

Discussion

mec-3 is expressed in several classes of mechanoreceptors

Originally, *mec-3* was identified by mutations that cause a global disruption of touch-receptor differentiation in *C. elegans* (Chalfie and Sulston 1981). The present results indicate that *mec-3* is expressed in several different cell types (the FLP and PVD neurons as well as the six touch receptor neurons): a *mec-3-lacZ* fusion is expressed in these cells (Fig. 2; Table 1); *mec-3(+)* is necessary for continued expression of the *mec-3-lacZ* fusion in all of these cells (Table 1); and *mec-3(+)* activity is required for function of the touch receptors and PVD, and for the distinctive morphology of the touch receptors (Table 2; Chalfie and Sulston 1981; Way and Chalfie 1988).

All of the *mec-3*-expressing cells appear to be mechanoreceptors. The ALM, AVM, PVM, and PLM cells mediate the animal's response to gentle touch (Chalfie and Sulston 1981). The PVD neurons mediate a response to a harsher mechanical stimulus (Table 2). The FLP cells have a morphology suggestive of a mechanoreceptor (Ward et al. 1975), but their function has not been tested directly.

mec-3 is required for the differentiation of the touch receptor neurons (Chalfie and Sulston 1981; Way and Chalfie 1988). Without this gene, the touch cells fail to express a number of characteristic morphological features. Moreover, unlike the wild-type touch cells, the *mec-3* mutant cells no longer degenerate through the action of dominant *mec-4* mutations.

The FLP and PVD cells do not have characteristic features that can be identified without serial section electron microscopic reconstruction. By Nomarski microscopy these cells appear normal [as do several touch receptor neurons in *mec-3* mutants (Chalfie and Sulston 1981; Way and Chalfie 1988)]. Thus, we do not know how *mec-3* acts in the differentiation and function of the FLP and PVD neurons. We think it likely, however, that *mec-3* serves a similar role in these cells and the touch receptor neurons: the specification of cell fate.

Regulation of *mec-3* expression

The expression of the *mec-3-lacZ* fusion is detectable around the time that the touch receptor neurons and the FLP and PVD cells arise from their respective lineages. Thus, genes that are required for the proper production of these lineages may be good candidates for genes that regulate, directly or indirectly, *mec-3* expression. Two genes, *lin-32* and *unc-86*, are required for the appropriate differentiation of precursors in these lineages (Fig. 3; Chalfie et al. 1981; Chalfie and Au 1989; C. Kenyon and E. Hedgecock, pers. comm.). Mutations in these genes

result in loss of all (*unc-86*) or most (*lin-32*) of the *mec-3*-expressing cells (see Fig. 3 legend). Behaviorally, these mutants lack the function of both the touch receptor neurons and the PVD cells. Moreover, the *unc-86* mutants do not express the *mec-3-lacZ* fusion. Thus, *unc-86* and probably *lin-32* are needed for *mec-3* expression.

How these genes affect *mec-3* expression is unknown. Because they are needed for the differentiation of precursors of the *mec-3*-expressing cells (for *unc-86* some of the affected cells are the direct precursors; see Fig. 3), these genes may act indirectly. Thus, the *unc-86* and *lin-32*-dependent cells may express components necessary for *mec-3* expression, perhaps by regulating *mec-3* transcription or by controlling the segregation of cellular determinants. It may be significant, in this latter context, that the *mec-3*-expressing cells are all anterior daughters of asymmetric cell divisions (Sulston and Horvitz 1977; Sulston et al. 1983). With the rare possible exception of the FLP sister cells (see Methods), the posterior daughters neither express the fusion nor exhibit any mutant phenotype (Fig. 2; Way and Chalfie 1988).

Alternatively, these genes, especially *unc-86*, may directly regulate *mec-3* expression. *unc-86* contains a homeo box and has extended homology to mammalian transcription factors Pit-1, Oct-1, and Oct-2, suggesting that it also regulates transcription (Finney et al. 1988). Using the DNA sequences recognized by the mammalian proteins, Finney et al. (1988) identified possible *unc-86* binding sites within the *mec-3* gene. The functional importance of these sites has not been tested, but

Figure 3. Mutations affecting the lineages giving rise to the AVM and PVM touch receptors and the PVD cells. The two (right and left) Q lineages occur during the first larval stage; the two V5.pa lineages occur during the second. Each lineage gives rise to neurons (designated by their letter names) and cells that undergo programmed cell death (X). In addition, the V5.pa lineage also gives rise to structural cells (PDEsh and PDEso). Genes are positioned on the lineage in association with the cells whose fates appear altered in the respective mutants. This positioning does not necessarily imply the focus of action of these genes. **lin-32* also affects the precursor of the Q cells. This figure is based on data from the following sources: (*lin-14*) Ambros and Horvitz (1984); (*lin-32*) Chalfie and Au (1989) and C. Kenyon and E. Hedgecock (pers. comm.); (*unc-86*) Chalfie et al. (1981), and (*mec-3*) Chalfie and Sulston (1981); Way and Chalfie (1988), and data presented in this paper).

their presence suggests that *unc-86* may directly regulate *mec-3*.

At least one *unc-86*-dependent lineage does not produce any *mec-3* expressing cells: The T blast cell gives rise to several neurons, none of which express detectable levels of *mec-3-lacZ* fusion product (Sulston and Horvitz 1977; Chalfie et al. 1981; data presented herein). These results suggest that additional, unidentified regulatory genes restrict *mec-3* expression to only a subset of *unc-86*-dependent lineages (Q and V5 but not T) and to only one of the cells affected by *unc-86* in each of these lineages.

Autoregulation of *mec-3*

Autoregulation is a common feature of developmental switch genes. Lambda repressor (Ptashne 1987), *Sex-lethal* (Cline 1984) and *Drosophila* homeo-box-containing genes such as *fushi tarazu* (Hiromi and Gehring 1987), *Ultrabithorax* (Bienz and Tremml 1988), and *Deformed* (Kuziora and McGinnis 1988) all show autoregulation, albeit by different mechanisms. In each case, autoregulation functions to maintain a determined state during cell proliferation after proteins that induce the initial synthesis of the gene in question are degraded, lost by dilution, or both.

In a *mec-3* mutant, expression of the *mec-3-lacZ* fusion occurs in the correct set of cells at early times. By adulthood, however, no expression of the *lacZ* fusion can be detected, implying that an intact *mec-3* gene is necessary for maintenance of *mec-3* expression. Unlike the above examples of autoregulation, the *mec-3*-expressing cells do not divide after *mec-3* is expressed. However, the cells enlarge and greatly increase the number of their cell-specific microtubules throughout development (Chalfie and Thomson 1982). Moreover, many genes of the function class have temperature-sensitive periods that span larval development (Chalfie and Au 1989). Thus, a continuous supply of *mec-3* may be necessary. Such maintenance may be particularly important during periods of arrested development, for example, during starvation.

The effect of *mec-3* on its own expression may be direct or indirect. As a homeo-domain-containing protein, the *mec-3* product may bind to *mec-3* DNA sequences and thus effect further expression. Alternatively, *mec-3*-dependent differentiation of the touch receptor neurons and the FLP and PVD cells may produce cellular environments required for the stability of the *mec-3* message or product.

The *mec-17* product may also be involved in the maintenance of *mec-3* expression, at least in the touch receptor neurons, although any hypothesis about *mec-17* must be regarded as tentative because there is only one mutant allele of the gene. The single *mec-17* mutation causes initially touch-sensitive animals to become touch-insensitive as they develop (Chalfie and Au 1989). The adult touch receptors in this mutant have a reduced number of the cell-specific microtubules, an indication that their development may have been blocked. In this

mutant, expression of the *mec-3-lacZ* fusion is gradually lost from the touch receptors, but not from the FLP or PVD cells. Thus, *mec-17* may be needed for continued *mec-3* expression. Alternatively, the *mec-17* mutation product may somehow poison the touch receptors so that, as an indirect consequence, protein synthesis is inhibited. Further analysis of the function of *mec-17* may require the isolation of null mutants.

Combinatorial determination of cell fate

mec-3 is expressed in more than one cell type, so it alone cannot regulate cell-specific differentiation. Other factors must act with *mec-3* or modify its action so that touch receptors, FLP cells, and PVD cells can be generated. Although the identity of these other factors is unknown, one gene whose product may be required is *lin-14*. Among other abnormalities, loss of *lin-14* gene activity changes the lineages that normally produce the AVM and PVM touch receptors into lineages that produce the PVD cells (Ambros and Horvitz 1984). The *lin-14* protein is normally localized to nuclei of the touch receptor precursors, but not to the those of PVD precursors (Ruvkun and Giusto 1989). This product may contribute to the determination of the fate of these post-embryonic *mec-3*-expressing cells.

A number of genes (e.g., *mec-4* and *mec-7*) appear to be selectively expressed in touch receptor neurons, and we have hypothesized that *mec-3* is required for their expression (Way and Chalfie 1988). We presume that other *mec-3*-dependent genes are expressed in the FLP and PVD cells. Because all the cells affected by *mec-3* mutations appear to be mechanoreceptors (although the function of the FLP cells has not been tested), one may expect that some *mec-3*-dependent genes may be shared by the different cell types. However, none of the genes required for the morphology or function of differentiated touch receptor neurons appears to be required for PVD function (Table 2). Thus, the requirement for *mec-3* in these different cell types may not be for the expression of shared components. Perhaps *mec-3* activity is modified significantly by other transcription factors so that completely different sets of genes are activated in the various cell types. Further analysis of *mec-3*, its regulators, and targets may help elucidate how this gene specifies the differentiation of such morphologically distinct neurons.

Methods

Growth and maintenance of strains

All *C. elegans* strains were propagated as described in Way and Chalfie (1988) and Brenner (1974).

Mutant strains

All of the *mec* mutant strains, and *egl-5(u202)* and *lin-32(u282)* are described in Chalfie and Au (1989) and references therein. *ced-1(e1735)* (Hedgecock et al. 1983) was obtained from the lab of H.R. Horvitz. *unc-24(e138)* *mec-3(u467)* *dpy-20(e1282)* is described in Way and Chalfie (1988).

DNA constructions

Plasmids pTU41–pTU51 (Fig. 1) were constructed by BAL-31 exonuclease digestion followed by religation in the presence of *NotI* linkers of pTU28 from the following unique restriction sites: *BglII* (pTU41, pTU42); *Clal* (pTU46); *Sall* (pTU48, pTU49, pTU51). All these plasmids received *NotI* linkers except pTU48. Extents of deletion were determined by restriction digestion with *NotI* and other appropriate enzymes, except for pTU48, which was analyzed with *Sau3A*. The sizes of the resulting restriction fragments were estimated within an accuracy of 50 bp. pTU37 was constructed by ligation of the *mec-3*-containing *ScaI*–*SacI* fragment from pTU23 [base pairs 910–5660 on the sequence of Way and Chalfie 1988] into *EcoRV*, *SacI* cut pIB130 (International Biotechnologies Incorporated).

Construction of *uEx4*

To construct a *mec-3-lacZ* fusion, a *lacZ*-containing DNA fragment was inserted into pTU48, which carries the entire *mec-3* gene. pTU48 has an intact *mec-3* gene, as defined by rescue of touch sensitivity following transformation, and contains base pairs –2,000 to +5660 of the *mec-3* sequence (Way and Chalfie 1988). Furthermore, it is possible to delete up to base pair +1495, from the 5' end of *mec-3*, back to base pair 5030, from the 3' end, or from base pair 2335 to 2580 internally, and still retain *mec-3* gene function (as assayed by rescue of touch sensitivity following transformation). Thus, it seemed reasonable that pTU48 would contain sequences sufficient for accurate *mec-3* gene expression. pTU28 was constructed by insertion of a *XhoI*–*Sall* *lacZ* fragment from PS118 (Silver et al. 1988) into the unique *XhoI* site in pTU48, generating an in-frame translational fusion between the homeo box of *mec-3* and *lacZ*.

Equal amounts of pTU28 and pPD10.41, a plasmid that expresses an *unc-22* antisense RNA and that causes transformed *C. elegans* to twitch (A. Fire, pers. comm.), were coinjected at a concentration of 100 ng/μl into wild-type *C. elegans* according to Fire (1986). One transformed animal was identified that contained a mixed array of pTU28 and pPD10.41, with ~40 copies per cell of each plasmid, as determined by Southern blotting (data not shown). This array, termed *uEx4*, behaves genetically as a free duplication: It has an inheritance rate of about 50% per generation, and animals carrying it often appear to be genetic mosaics. This element has been propagated for over 50 generations and crossed into several mutant backgrounds, always followed by direct screening for the twitching phenotype and subsequent checking for β-galactosidase activity. No line has ever been identified that completely fails to express β-galactosidase activity. *uEx4* has been crossed from a wild-type background into *unc-24 mec-17* and back into wild type without a change in the wild-type staining pattern.

Identification of cells with β-galactosidase activity

C. elegans strains carrying *uEx4* were stained with the β-galactosidase substrate X-gal and the fluorescent nuclear stain 4,6-diamidino-2-phenylindole dihydrochloride (DAPI), according to a procedure developed by A. Fire (pers. comm.). Stained animals were observed with a Zeiss Universal microscope using bright-field and fluorescence illumination. Cells were scored as staining if any observable blue precipitate was present.

Stained cells were identified by their overall positions within the animal, their positions relative to other neuronal nuclei,

their time of appearance, and their absence in certain mutants (Table 1). Cells were identified according to White et al. (1986). Each FLP neuron was found in a characteristic position in a row of nuclei lateral to the rear bulb of the pharynx. Each PVD cell was the most anterior neuron in the post-deirid cluster (Sulston and Horvitz 1977; White et al. 1986; H.R. Horvitz, pers. comm.). Each PLM was located in a large cluster of neurons posterior to the anus. The identification of this staining cell was also based on its absence in the *mec4(d)* mutant. Each ALM was found dorso-lateral and anterior to the vulva, and did not have a fixed geometry relative to its neighbors because it underwent a long posterior migration. The identification of this cell was based on its absence in *mec-4(d)* and the fact that it was the only neuron evident in this general region of the animal. AVM was found to be ventro-lateral, about halfway between the vulva and the rear bulb of the pharynx. This cell, which does not have a fixed position relative to its neighbors due to migration of its precursors, is the only neuron in this vicinity, and is absent in *mec-4(d)*. PVM was intercalated into the postdeirid cluster in a variable position, because it arose at a different time from other post-deirid neurons. This cell was absent in *mec-4(d)*. AVM and PVM were also identified by the fact that they were not bilaterally symmetric (AVM was only on the right side, PVM on the left), and because staining of these neurons was first seen in the late L1 stage.

Individuals containing *uEx4* were identified as twitchers, either by visual inspection, the nicotine assay of Moerman and Baillie (1979), or by the *Mec* phenotype of twitchers (Chalfie and Sulston 1981), then picked into staining solution and stained overnight at 37°C. Because the *uEx4* element is a free duplication, its copy number could vary and lead to variations in β-galactosidase expression. To select for animals that would have the highest copy of *uEx4*, we generally stained animals with the most severe Twitcher phenotype, that is, animals that could be seen to twitch without nicotine. Such animals can be recognized during adulthood and the L4 larval stage, but not at earlier stages. Twitching appears to habituate the touch receptor cells (Chalfie and Sulston 1981). We do not feel, however, that the *unc-22* antisense construct significantly affects the expression of the *mec-3-lacZ* fusion because the touch receptors in otherwise wild-type animals are functional (a touch response can sometimes be elicited) and because the cells degenerate in the *mec-4(d)* background.

In some experiments, no effort was made to select extreme Twitchers, and the staining was less complete. This variation may account for the apparent overall reduction in staining in the wild-type L2/L3 animals in Table 1. In addition, animals stained for less than 15 hr often had a reduced frequency of staining cells (data not shown). The low frequency of staining of the AVM and PVM cells probably resulted from insufficient levels of *mec-3-lacZ* expression (staining of these cells was often weak), and not from somatic loss of *uEx4*, because such loss should occur just as often for PVD as for AVM and PVM. When animals were raised at 25°C, either the entire set of cells were stained, or the animals were clearly mosaic (Table 1). Growth at this temperature may stabilize the β-galactosidase activity of the *mec-3-lacZ* fusion or may reduce the frequency of somatic loss of the *uEx4* element.

Many animals appear to be genetic mosaics in that an incomplete set of cells stained. Among 25 *uEx4* animals raised at 25°C (Table 1), 5 appeared to have lost *uEx4* during somatic cell divisions. One lost the element between ABpr and ABprapa (no FLPR, PVDR, AVM, or PLMR staining), one lost it between ABpl and ABplapa (no FLPL, PVDL, PVM, or PLML staining), one between ABa and ABarpp (no ALM staining), and two appeared to have lost *uEx4* in the QL/V5L precursor (no PVDL or

Way and Chalfie

PVM staining). Among animals raised at 20°C, the pattern of unstained cells could not be rationalized by single loss events. We do not know if these animals sustained multiple losses of the free duplication, or whether some cells expressed β -galactosidase activity below the threshold of detection.

Crossing the *uEx4* element into mutant backgrounds

The *uEx4* element was crossed into *mec-3(u298)*, *unc-86(e1416)*, *mec-4(e1611)*, and *egl-5(u202)* mutant backgrounds by a two-step procedure. These constructions were complicated by the fact that the twitching of *uEx4*-containing animals reduces male mating (Hodgkin 1983) and results in a *Mec* phenotype (Chalfie and Sulston 1981). Wild-type males were mated with each mutant and heterozygous male progeny were mated into *uEx4* animals to generate a *+/m*; *uEx4* cross-progeny. Because these animals could not be distinguished from other types of cross- or self-progeny, 50 to 75 twitching (*uEx4*-containing) hermaphrodites were picked to separate plates and screened for segregation of one-quarter of *m/m* animals among the nontwitching progeny. *m/m*; *uEx4* animals were identified as twitching animals whose nontwitching progeny were all *m/m*. (In addition to the strains discussed in Results, *egl-5*; *uEx4* animals were also examined and showed the wild-type staining pattern.)

Because the *mec-17(u265)* phenotype is difficult to score, a *mec-17*; *uEx4* strain was constructed with the aid of the linked marker *unc-24(e138)*. *mec-17* maps about 0.1 cM to the right of *unc-24*: 1 of 10 *Unc* recombinants and all three *Dpy* recombinants from *+ mec-17 ++ / unc-24 + mec-3 dpy-20(e1282)* contained the *mec-17* mutation on the recombinant chromosome, as determined by progeny testing and test mating with *mec-17*; *him-5(e1490)* males.

An *unc-24 mec-17*; *uEx4* strain was constructed and tested in the same way as the *mec*; *uEx4* strains described above. *mec-17*; *him-5* males were mated into this strain, and non-*Unc-24*, twitching progeny were identified (*+ mec-17/ unc-24 mec-17*; *uEx4*), from which *mec-17*; *uEx4* animals were obtained by segregation.

A *ced-1*; *mec-3* strain was constructed by mating spontaneous *ced-1(e1735)* males with *mec-3(u298)* hermaphrodites. From non-*Mec* *+/ mec-3*; *+/ ced-1* hermaphrodite cross-progeny, *Mec* L1 self-progeny were identified and screened for the presence of persistent cell deaths (the *Ced-1* phenotype) using Nomarski optics. One *mec-3*; *ced-1* animal was thus identified and used to test for the presence of cell deaths in the post-deirid region (see results).

Behavioral assay for sensitivity to strong mechanical stimuli

To test for sensitivity to harsh touch, non-moving gravid adults were prodded with a platinum wire at or just posterior to the vulva. Animals with a functional PVD neuron moved, usually backing up, whereas animals lacking this neuron did not respond. Only *Mec* mutants (mutants that lack touch receptor function and do not respond to gentle touch with an eyebrow hair; Chalfie and Sulston 1981; Chalfie and Au 1989) have been tested in this way: Functional touch receptors would otherwise mediate a vigorous response to this stimulus. *Mec* animals lacking PVD still respond when prodded at the tail or in the region of the pharynx. Animals tested were grown from hatching in the presence of food at 20°C. Animals recovering from the dauer state or a starved state often fail to respond regardless of the presence of the PVD neurons.

In several experiments (Table 2), approximately equal numbers of sensitive and insensitive animals were scored blind.

Laser ablation

Cells were ablated essentially as described in Sulston and White (1980), except that a Laser Science VS337 laser was used and 50–100 pulses were used to kill each cell. The V4 and V5 cells were killed in newly hatched animals (<5 hr old) by killing the cell on the upper side of the animal, then focusing through the animals and killing the cell on the other side. The PVD and other post-deirid cells were killed in L3 animals. After ablation of PVD on one side of the animal, the animals were allowed to recover, were remounted, and the cell on the other side was ablated.

Acknowledgments

We wish to thank A. Fire for plasmids, procedures, and advice about expression of *lacZ* in *C. elegans*; Geraldine Seydoux and Iva Greenwald for help with the laser ablations, and Mark Osborne and Iva Greenwald for reading the manuscript. This research was supported by National Institutes of Health (NIH) grant GM30997 to M.C., NIH Post-doctoral Fellowship, Merck Research Fellowship, and NIH First Award HD25557 to J.C.W.

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The *mec-3* gene of *Caenorhabditis elegans* requires its own product for maintained expression and is expressed in three neuronal cell types.

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Genes Dev. 1989, **3**:

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