Cell, Vol. 44, 817–829, March 28, 1986, Copyright © 1986 by Cell Press

Genetic Control of Programmed Cell Death in the Nematode C. elegans

Hilary M. Ellis,* and H. Robert Horvitz
Department of Biology
Massachusetts Institute of Technology
Cambridge, Massachusetts 02139

Summary

The wild-type functions of the genes *ced-3* and *ced-4* are required for the initiation of programmed cell deaths in the nematode Caenorhabditis elegans. The reduction or loss of *ced-3* or *ced-4* function results in a transformation in the fates of cells that normally die; in *ced-3* or *ced-4* mutants, such cells instead survive and differentiate, adopting fates that in the wild type are associated with other cells. *ced-3* and *ced-4* mutants appear grossly normal in morphology and behavior, indicating that programmed cell death is not an essential aspect of nematode development. The genes *ced-3* and *ced-4* define the first known step of a developmental pathway for programmed cell death, suggesting that these genes may be involved in determining which cells die during C. elegans development.

Introduction

Cell death occurs during the normal development both of vertebrates (Saunders, 1966; Cowan et al., 1984) and invertebrates (Truman, 1984). During the development of the nematode Caenorhabditis elegans, the generation of the 959 somatic nuclei of the hermaphrodite is accompanied by the generation and subsequent deaths of 131 cells (Sulston and Horvitz, 1977; Sulston et al., 1983). As in other organisms, naturally occurring or "programmed" cell death in C. elegans is particularly common during the development of the nervous system; approximately 20% of all presumptive neural cells die (Sulston and Horvitz, 1977; Horvitz et al., 1982a; Sulston et al., 1983).

Morphological and genetic studies have indicated that essentially all programmed cell deaths in C. elegans probably involve the same mechanism. First, nearly all dying cells undergo the same sequence of morphological changes (Sulston and Horvitz, 1977; Sulston et al., 1983). Second, a number of mutations affecting the process of cell death have been isolated. These mutations, which have defined the genes *ced-1* (for cell death abnormal), *ced-2*, and *nuc-1* (nuclease deficient) (Sulston, 1976; Hedgecock et al., 1983), affect all programmed cell deaths. Thus, all cell deaths appear to involve the same genetic (and, hence, molecular) processes. For these reasons we can regard programmed cell death in C. elegans as a specific cell fate and may be able to use cell death as a model system for examining mechanisms that specify cell fates.

* Present address: Department of Biology, University of California, San Diego, La Jolla, California 92093.

The phenomenon of programmed cell death raises a number of questions. Why are cells generated only to die? By what mechanisms do they die? How is it determined during development which cells die? C. elegans is well suited for studies that attempt to answer these questions. This nematode has fewer than 1000 somatic cells, and fixed patterns of cell divisions, migrations, and deaths generate individuals of invariant anatomy (Sulston and Horvitz, 1977; Kimble and Hirsh, 1979; Sulston et al., 1983). Thus, specific developmental events can be examined reproducibly and at the resolution of single cells. In addition, the short generation time (3 days at 20°C) and large brood size of C. elegans facilitate genetic manipulations (Brenner, 1974; Herman and Horvitz, 1980).

We describe here the isolation and characterization of mutations that prevent the initiation of programmed cell death in C. elegans, causing cells that would normally die to survive instead. These mutations define two genes, *ced-3* and *ced-4*, that may be involved in determining which cells express the fate of programmed cell death.

Results

Isolation of Cell Death Mutants by Direct Screening

Programmed cell deaths can easily be identified in living nematodes using Nomarski differential interference contrast microscopy (Sulston and Horvitz, 1977). The first sign of the impending death of a cell is a slight increase in its refractility. The nucleus of the dying cell becomes increasingly refractile until it resembles a flat button; this stage persists for 10–30 min. Subsequently, the nucleus of the dying cell decreases in refractility, begins to appear crumpled, and then gradually disappears. This process is completed in less than 1 hr (Sulston and Horvitz, 1977; Sulston et al., 1983). In *ced-1* and *ced-2* mutants (Hedgecock et al., 1983), dying cells remain in the highly refractile stage for many hours, often persisting in this state through several larval stages. Thus in *ced-1* and *ced-2* animals, deaths that occur over a wide range of developmental times can be observed at once.

To isolate mutations that perturbed the normal pattern of programmed cell deaths, we treated *ced-1* hermaphrodites with the mutagen ethyl methanesulfonate (EMS) (Brenner, 1974) and used Nomarski microscopy to screen their F2 progeny. The progeny of approximately 4000 F1 hermaphrodites were examined. Among the mutants we obtained in this way were two strains in which the characteristic cell deaths of *ced-1* were not seen (Figure 1). From these strains we isolated two recessive mutations, *n717* and *n718*, that define the gene *ced-3* on linkage group IV (Figure 2). (See Experimental Procedures for methods of isolation, mapping, and complementation testing.)

ced-3 Mutations Eliminate Programmed Cell Death

Embryonic and postembryonic cell deaths normally seen in *ced-1* animals are seen only rarely in *ced-1; ced-3* animals (Table 1). The direct observation in *ced-1; ced-3*

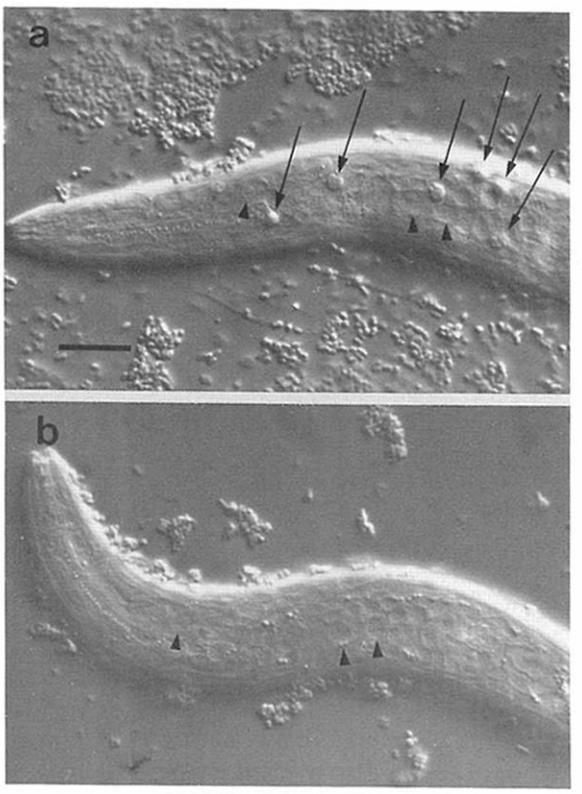

Figure 1. Absence of Cell Deaths in *ced-3* Animals

(a) Nomarski photomicrograph of a newly hatched *ced-1* larva. Arrows indicate dying cells. (b) Nomarski photomicrograph of a newly hatched *ced-1; ced-3* larva. Plane of focus is approximately that shown in (a). Arrowheads indicate several of the nuclei that can be seen in both (a) and (b). No cell deaths are seen in the *ced-1; ced-3* larva. Bar = 10 μ.

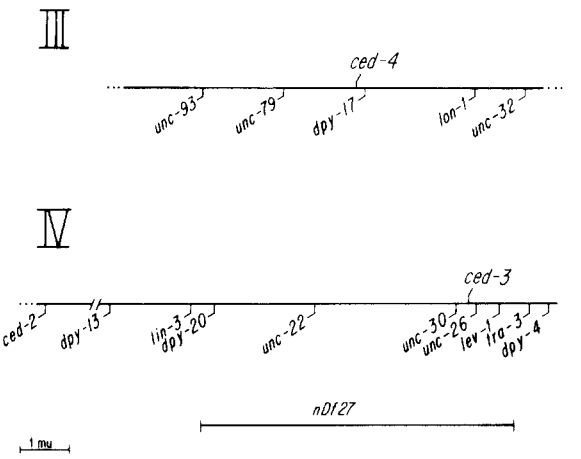

Figure 2. A Partial Genetic Map of Linkage Groups III and IV

or *ced-3* animals of the postembryonic lineages of blast cells that generate the ventral nerve cord (P1-P12), the postdeirid sensilla (V5L.pa and V5R.pa), and neurons located in the lateral hypodermis (QL and QR) indicated that programmed cell deaths do not occur in these lineages. In each case the lineages observed were identical with those of wild-type animals except that cells that normally die in wild-type animals survived in *ced-1; ced-3* or *ced-3* animals (Figure 3).

After determining the postdeirid lineage in several *ced-3* animals, we observed the postdeirid cells through two further larval stages until the animals molted into adults. In no case did we observe the division of a cell death survivor, nor were any delayed deaths observed. Cell death survivors from the Q neuroblast and ventral cord lineages also do not appear to divide; in *ced-3* animals extra postembryonically generated neuronal cells are found in the ventral cord, postdeirids, and lateral hypodermis (Figure 4), and the number of extra cells is consistent with the number of cells that would normally die in the lineages of the Q, V5.pa, and P blast cells. In addition, in our studies of *ced-1; ced-3* or *ced-3* animals we have never seen extra divisions in these lineages. The observation that the lineages of *ced-3* animals are normal, except in the failure of cells to die, suggests that *ced-3* mutations prevent the expression of programmed cell deaths. Presumably the *ced-3* mutation acts prior to *ced-1* to block the initiation of programmed cell death.

Mutations in *ced-3* block the programmed cell deaths that occur during embryonic development as well as those that occur during postembryonic development. We have screened *ced-1; ced-3* hermaphrodites of various stages, both embryonic and postembryonic, for the presence of dying cells. Although occasional dying cells were seen, no particular cell died in all or even many hermaphrodites (Table 1). In addition, in newly hatched *ced-3* larvae a number of extra embryonically generated cells can be observed. For example, *ced-3* mutants have several extra cells in the anterior bulb of the pharynx (Figure 5; also see below). Again, the number of these cells is consistent with the survival of cells that would normally die in lineages that contribute to this structure.

ced-3 mutations appear to block all programmed cell deaths in hermaphrodites. Programmed cell deaths are also blocked in *ced-3* males. However, we have noted one exception, the male-specific linker cell. Unlike most programmed cell deaths in C. elegans (Horvitz et al., 1982a), the death of the linker cell requires the presence of another cell (U.lp or U.rp) (Sulston and White, 1980) and thus could be termed a "murder" (Horvitz et al., 1982a). In three out of five *ced-3* males in which the fate of the linker cell was followed, the linker cell had died by the L4 molt. (In 7 out of 7 wild-type males observed, the linker cell had died by the L4 molt.) Thus, although *ced-3* may play some role in the death of the linker cell, normal *ced-3* function is not essential for linker cell death. We have not examined *ced-3* animals for the deaths of the male-specific cells B.a(l/r)apaav and B-gamma.a(l/r)d, both of which appear to be "murdered" (Sulston and White, 1980).

Cell Death "Survivors" Do Not Divide

As described above, postembryonic cell death "survivors" do not divide. In addition, we have directly followed the fate in *ced-3* animals of a cell that dies during wild-type embryogenesis, Ab.alapapaa. This cell is the grand-

Table 1. Elimination of Cell Death by *ced-3* and *ced-4* Mutations

	Average Number of Deaths Observed			
	Embryonic Deaths	Postembryonic Deaths		
Genotype	Head of L1	Ventral Cord*	Postdeirid†	Q‡
ced-1	28.0 n = 23	8.7 n = 28	0.93 n = 29	1.6 n = 28
ced-1; ced-3 (n717)	0.3 n = 21	0.04 n = 50	0 n = 15	0 n = 24
ced-1; ced-3 (n718)	0.5 n = 26	0.03 n = 35	0 n = 24	0 n = 21
ced-1; ced-3 (n1040)	7.0§ n = 21	0 n = 23	0.05 n = 20	0.06 n = 17
ced-1; ced-3 (n1129)	3.0 n = 22	N.D.	0.13 n = 30	N.D.
ced-1; ced-4 (n1162)	0.6 n = 23	0.04 n = 27	0 n = 21	0 n = 21
Total Number of Deaths That Occur in Wild Type‖	#	10	1	2

Animals of the indicated genotypes were observed using Nomarski differential interference contrast microscopy. The numbers of dead cells present in the head of first larval stage (L1) animals, the ventral cord, and right anteriolateral hypodermis (where progeny of the QR blast cell are generated) of L2 animals, and the right postdeirid of L3 animals were determined.

* In wild-type animals, 10 programmed cell deaths are generated in the ventral cord during the L1 stage by the blast cells W and P1.a-P12.a.

† The lineages of V5R.pa and V5L.pa, which generate the postdeirid sensilla, each include one programmed cell death. Only the right postdeirid was observed, since on the left side of the animal, QL generates cell deaths in the region of the postdeirid.

‡ On the left side of the animal, QL generates two programmed cell deaths posterior to the gonad, near the region of the postdeirid. On the right side of the animal, QR generates two programmed cell deaths in the lateral hypodermis anterior to the gonad. Only deaths in the right anterior lateral hypodermis were observed in order to avoid confusion with the death generated by the left postdeirid lineage.

§ *ced-3 (n1040)* appears to affect these embryonically generated cell deaths less severely than postembryonically generated deaths, suggesting that the embryonic deaths require lower amounts of *ced-3* activity.

‖ The values in this row indicate the total number of deaths that occur in particular regions of the wild-type animal prior to the time at which mutant animals were examined. As can be seen by comparing this row with the first row of this table, not all deaths last long enough to have been observed in *ced-1* animals.

We have not determined which of the deaths that occur during wild-type embryonic development can be visualized within the head region we have examined in *ced-1* mutant animals.

N.D. indicates not determined.

daughter of Ab.alapap, which generates a lineage identical with that generated by another cell, AB.alappp, except that the cell homologous to AB.alapapaa (i.e., AB.alapppaa) divides to generate two neurons. Thus it seemed plausible that in *ced-3* animals the cell death "survivor" AB.alapapaa might divide. We followed the lineage that generates this cell in four *ced-3* animals and observed the cell for 1 to 2 hr after its birth. AB.alapapaa neither divided nor died in any of these animals. In one *ced-3* embryo in which AB.alapapaa was followed for a longer period of time (3.5 hr), this cell eventually became detached from the embryo. (One or two such detached cells are often observed in *ced-3* embryos floating between the embryo and the eggshell.)

Thus, those cell death survivors that we have directly followed do not divide. In addition, there appears to be no gross proliferation of cell number in *ced-3* mutants. These observations suggest that cell death in C. elegans does not function to terminate cell lineages by the elimination of stem or blast cells that would otherwise continue to divide.

Cell Death Survivors Differentiate

At least some cell death survivors assume a differentiated fate that we can identify. For example, the histochemical technique of formaldehyde-induced fluorescence (FIF) can be used to identify dopamine- or serotonin-containing neurons (Sulston et al., 1985; Horvitz et al., 1982b). Animals carrying mutations in *ced-3* often have an extra dopaminergic neuron in each of their postdeirids (Figure 6). In wild-type animals, the postdeirid contains one dopaminergic neuron, V5.paaa. The sister of V5.paaa, V5.paap, divides to generate a programmed cell death, V5.paapp, and a nondopaminergic neuron, V5.paapa, which has a characteristic morphology, as viewed with Nomarski microscopy, that is distinguishable from the other cells of the postdeirid (Figure 7). In *ced-3* animals the postdeirid lineage is identical with that of the wild-type except that the final division generates two neuronal cells. Since the anterior daughter of this division appears identical by Nomarski criteria with the nondopaminergic neuron of the wild-type postdeirid (Figure 7), and since the posterior daughter seems identical with the normal dop-

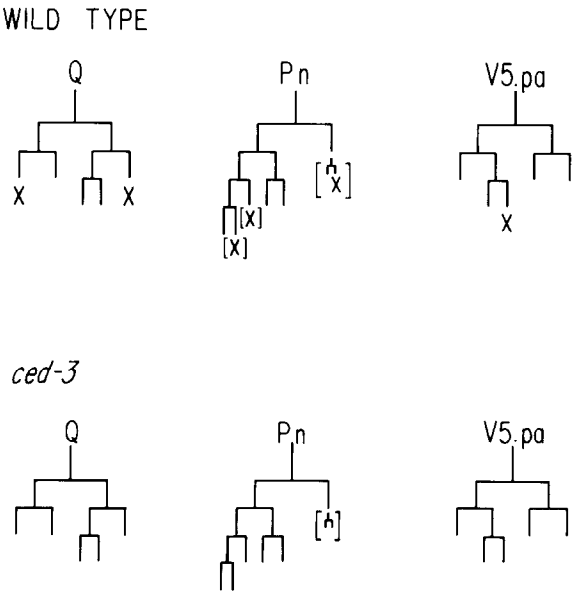

Figure 3. Lineages Generated by the Q, P, and V5.pa Blast Cells in Wild-Type and *ced-3* Animals

Each vertical line represents a cell, and each branch represents a cell division. Cells are named according to their ancestry, for example, V5.pa is the anterior daughter of the posterior daughter of V5. X indicates a programmed cell death. During the first larval stage (L1) of wild-type animals QL and QR (the Q blast cells present on the left and right sides of the animal, respectively), each divide to generate four neural cells and a programmed cell death. In wild-type hermaphrodites, each of the 12 P blast cells (collectively Pn) divides during the L1 to generate a neuroblast, Pn.a and a hypodermal cell, Pn.p. All 12 Pn.a cells undergo the same pattern of divisions, generating five descendants. P1.aap, P2.aap, and P9.aap-P12.aap (but not P3.aap-P8.aap) and P11.aaap and P12.aaap (but not P1.aaap-P10.aaap) undergo programmed cell death. P12.p divides once during the L1 generating a posterior daughter that dies. During the L2 V5L.pa and V5R.pa each divide to generate four neural cells and a programmed cell death. In *ced-3* animals the Q, P, and V5.pa lineages are identical with those of wild-type animals, except that cells that normally die survive instead.

aminergic neuron, it seems very likely that the extra dopaminergic neuron in *ced-3* postdeirids is V5.paapp, the cell that dies in the wild-type postdeirid lineage (Figure 8). While V5.paapp nearly always survives and appears neuronal in *ced-3(n717)* and *ced-3(n718)* animals, only 42 of 79 postdeirids in *ced-3(n717)* animals and 26 of 56 postdeirids in *ced-3(n718)* animals had an extra dopamine-containing neuron. In other words, in *ced-3* animals the choice between death and survival is nearly invariant, but the production of dopamine in the surviving cell is variable. We do not know whether the cell death survivors that fail to make dopamine represent a cell type that differs from the dopaminergic neuron V5.paaa, or whether these survivors simply fail to express one aspect of the differentiated phenotype of the dopaminergic neuron.

ced-3 animals also often have extra dopaminergic neurons in their heads with positions and morphologies similar to the dopaminergic ventral lateral cephalic neurons, CEPVR and CEPVL. Since in wild-type animals CEPVR and CEPVL are generated as "sisters" of deaths (Sulston et al., 1983), it may be that in *ced-3* animals dopaminergic neurons rather than deaths are generated as sisters of CEPVR and CEPVL. The production of dopamine by these cell death survivors also is variable; for example, of 16 *ced-3(n717)* animals observed, six had an extra dopaminergic neuron in the region of the ventral cephalic neuron on one side and five had an extra dopaminergic neuron in this region on both sides.

FIF histochemical staining of *ced-3* hermaphrodites has also indicated the presence of two extra serotonergic neurons, one per side, in the anterior bulb of the pharynx (Figure 6). These supernumerary serotonergic neurons are morphologically similar to the normal pharyngeal serotonergic neurons, the NSMs ("neurosecretory motor neurons") (Albertson and Thomson, 1976; Horvitz et al., 1982b). Since in wild-type animals the NSMs are generated as sisters of deaths (Sulston et al., 1983), it may be that in *ced-3* animals serotonergic neurons rather than deaths are generated as sisters of the NSMs. The presence of serotonin in these putative supernumerary NSMs (at least after the uptake of exogenous serotonin; see Figure 6) is less variable than that of dopamine in the supernumerary dopaminergic neurons described above. Specifically, 29 of 34 NSMs in *ced-3(n717)* animals and 36 of 38 NSMs in *ced-3(n718)* animals were adjacent to an extra serotonergic cell.

Sexually dimorphic cell deaths that occur during the embryonic development of C. elegans are also affected by mutations in *ced-3*. In wild-type males the cells that in hermaphrodites become the two hermaphrodite-specific neurons (HSNs) die; in wild-type hermaphrodites the four cells that in males become the cephalic companion neurons (CEMs) die (Sulston et al., 1983). *ced-3* males have an extra cell on each side that by position and morphology (as viewed with Nomarski microscopy) appears to be an HSN (Figure 7). Similarly, *ced-3* hermaphrodites have four extra cells that by position and morphology appear to be the CEMs (Figure 7). In addition, sensory endings with the positions and morphologies of the CEM sensory endings have been observed in serial section electron micrographs of a *ced-3* hermaphrodite (J. G. White, personal communication).

Cell Death Survivors Function

In the absence of programmed cell death, cells that would normally die instead survive and differentiate. Can these cell death survivors function? The following observations suggest that they can. The HSN neurons are required for normal egg-laying (Trent et al., 1983); for this reason *egl-1(n487)* hermaphrodites, which lack HSNs, are egg-laying defective (Trent et al., 1983). Since, as noted above, the HSN homologs normally die during the embryonic development of C. elegans males, it seemed plausible that the phenotype of *egl-1* animals might be caused by the inappropriate death of the HSNs in *egl-1* hermaphrodites. We constructed a *ced-3; egl-1* strain and found that this strain had HSNs and laid eggs normally. The presence of HSNs in *ced-3; egl-1* hermaphrodites indicated that the absence of HSNs in *egl-1* hermaphrodites was caused by cell death, presumably of the HSNs. Furthermore, the egg-laying competent phenotype of the *ced-3; egl-1* strain sug-

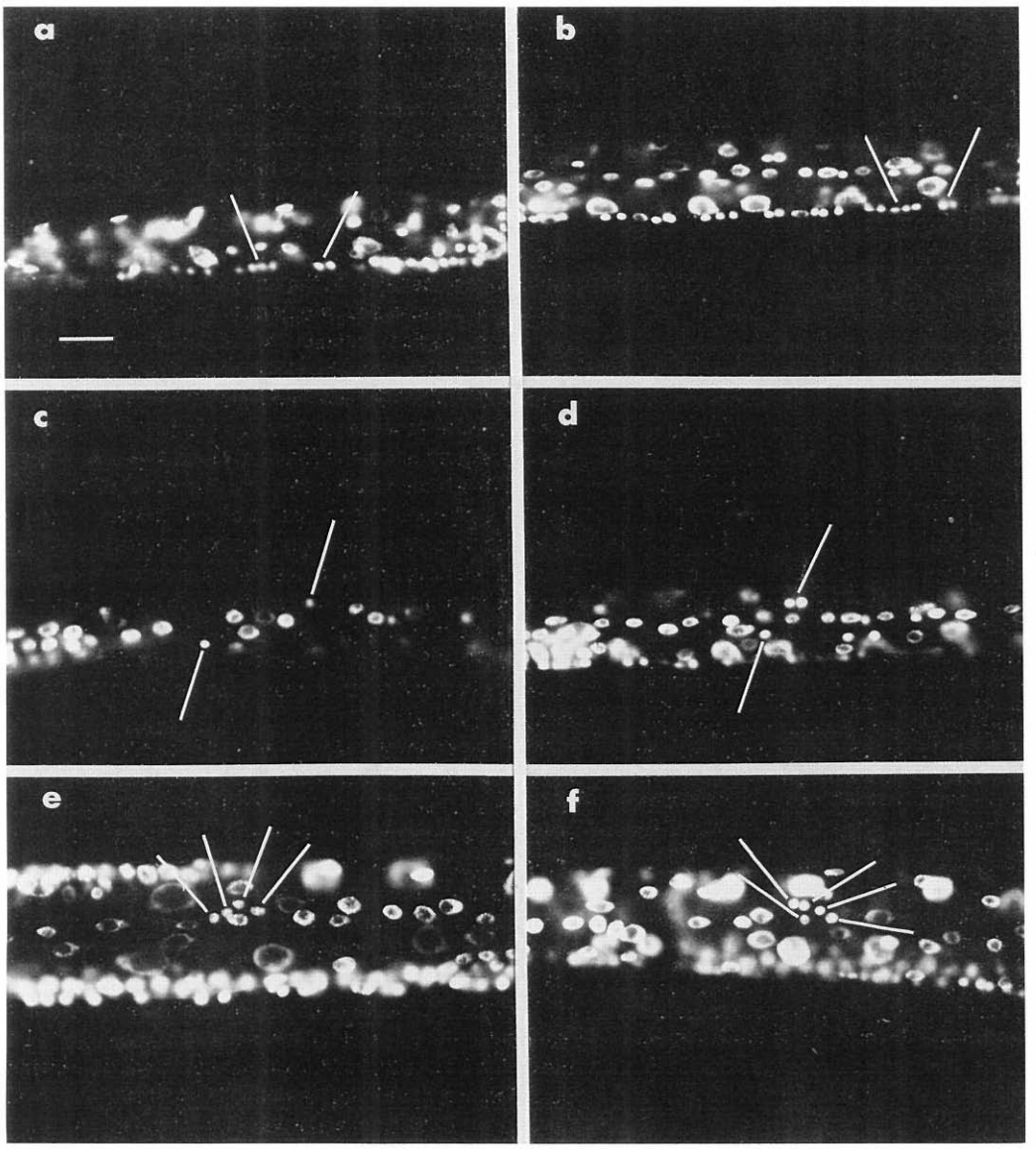

Figure 4. Extra Cells in *ced-3* Animals

Photomicrographs of (a,c,e) wild-type and (b,d,f) *ced-3* animals stained with the fluorescent DNA-binding dye DAPI (diamidinophenolindole). Anterior is to the right and ventral is down. Lines indicate nuclei of (a,b) ventral cord neurons, (c,d) neurons generated by QR, and (e,f) postdeirid neural cells. Bar = 10 μ.

At the anterior end of the ventral cord of (a) wild-type animals, the neuronal nuclei form a characteristic pattern of two nuclei, followed by a space and then four nuclei. In (b) *ced-3* animals, this first pair of nuclei is followed by a set of five nuclei. In (c) wild-type animals QR generates two neurons in the region of the anterior lateral hypodermis, while in (d) *ced-3* animals, QR generates three neurons in the region of the anterior lateral hypodermis. The (e) wild-type postdeirid sensillum consists of four cells; the (f) *ced-3* postdeirid has five cells.

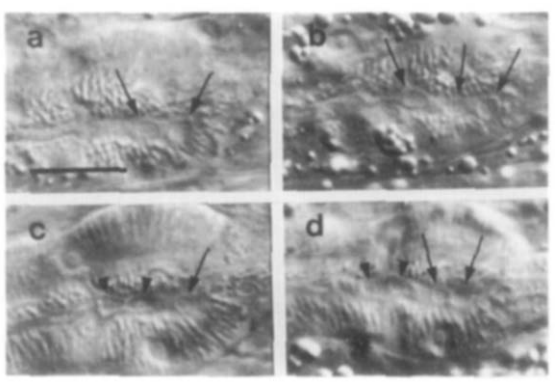

Figure 5. Nomarski Photomicrographs of the Pharynx of Wild-Type and *ced-3* Animals

Anterior is to the left. (a) and (c) show the pharynx of a wild-type animal in two planes of focus. (b) and (d) show the pharynx of a *ced-3* animal in planes of focus equivalent to those shown for wild-type. In (a) arrows indicate the nuclei of the NSM neuron (to the right) and the I2 neuron. In (b) three neuronal nuclei are seen in the equivalent plane of focus in a *ced-3* animal. In (c) the arrow indicates the nucleus of the neuron MCL, and arrowheads indicate two nuclei of the multinucleate muscle cell m1. In (d) an extra neuronal nucleus is seen in the equivalent plane of focus in a *ced-3* animal. Bar = 10 μ.

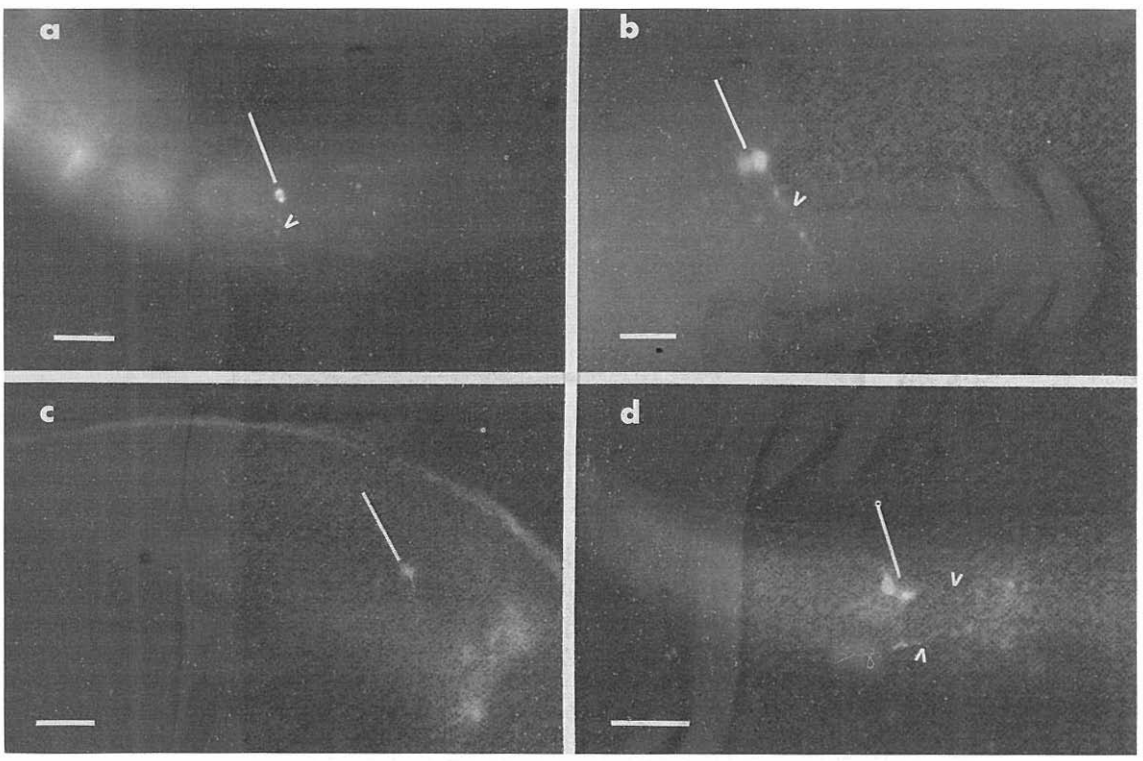

Figure 6. Wild-Type and *ced-3* Animals Stained Using the Technique of Formaldehyde-Induced Fluorescence (FIF)

(a) Wild-type animals have one dopaminergic neuron on each side embedded in the lateral hypodermis, while (b) *ced-3* animals have two dopaminergic neurons per side. Similarly, (c) wild-type animals have one serotonergic neuron on each side of the anterior bulb of the pharynx, while (d) *ced-3* animals have two serotonergic neurons per side. The animals in (c) and (d) were incubated in a solution containing 5 mg/ml serotonin prior to staining by FIF. In both wild-type and *ced-3* animals, the NSM cell bodies can be visualized by FIF only after they have been exposed to exogenous serotonin. The *ced-3* animal in (d) is younger than the other animals shown. Bars = 10 μ.

gests that cell death survivors can function. Direct observations of *egl-1* and *ced-1; egl-1* embryos have also indicated that the HSNs undergo programmed cell death in *egl-1* hermaphrodites (data not shown), thus supporting these conclusions.

ced-3 Mutants Appear Behaviorally Normal

Despite the absence of programmed cell deaths and the presence of many supernumerary cells, *ced-3* mutants appear normal in morphology and behavior as viewed with a dissecting microscope. *ced-3* animals are not uncoordinated in locomotion; they respond as does the wild type by moving forward when gently touched on the tail and backwards when touched on the head (Chalfie et al., 1985), lay eggs at a normal rate, and are stimulated to lay eggs in response to exogenous serotonin and imipramine, which stimulate egg-laying by wild-type hermaphrodites (Trent et al., 1983). We have also shown that animals carrying mutations in *ced-3* move up a gradient of NaCl (Table 2), as does the wild type (Ward, 1973). In addition, male mating ability is not significantly decreased in *ced-3* strains. Specifically, when six wild-type males were crossed with six *dpy-11(e224)* hermaphrodites, 1159 cross progeny were produced. Six *ced-3(n717)* males produced 934 cross progeny, and six *ced-3(n718)* males produced 1181 cross progeny in this assay.

The brood size of *ced-3* hermaphrodites may be somewhat lower than that of wild-type hermaphrodites; the average brood size of eight wild-type hermaphrodites was 308, while the average brood size of eight *ced-3(n717)* hermaphrodites was 256. However, this reduction in brood size may reflect the presence of background mutations in the *ced-3* strain. Consistent with this interpretation, the average brood size of eight *ced-3(n717)/ced-3(n717) dpy-4(e1166)* hermaphrodites was 259.

Additional *ced-3* Alleles

We have identified two additional alleles of *ced-3*. *ced-3(n1129)* was obtained on the basis of its failure to complement *ced-3(n717)* for the suppression of the egg-laying defect caused by *egl-1(n487)* (see Experimental Procedures). *ced-3(n1040)* was isolated in our laboratory by Nancy Tsung (personal communication), who used Nomarski microscopy to screen 6000 F2 progeny of mutagenized hermaphrodites for animals with extra pharyngeal cells near the positions of the NSMs. The general phenotype of both of these mutants is the same as that of *ced-3(n717)* and *ced-3(n718)* mutants. However, *n1040* and *n1129* are of lower expressivity than *n717* or *n718*. For example, while cell deaths are rarely seen in the heads of first larval stage *ced-1; ced-3(n717)* or *ced-1; ced-3(n718)* animals, in *ced-1; ced-3(n1129)* and *ced-1; ced-3(n1040)*

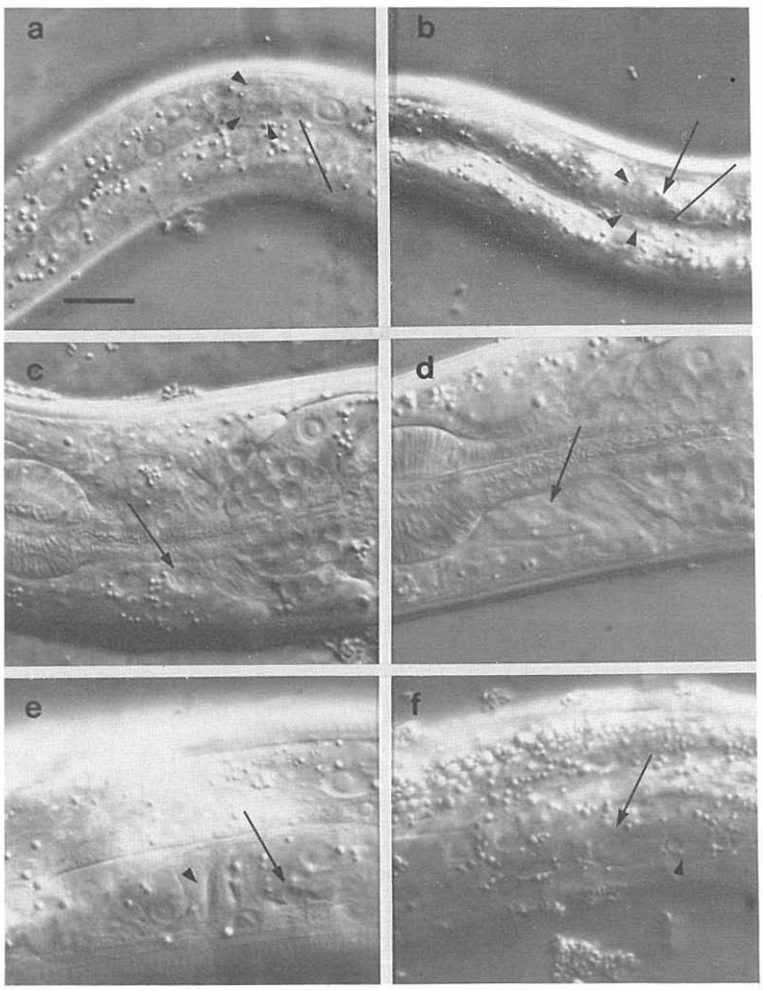

Figure 7. Nomarski Photomicrographs of Postdeirid Cells, CEMs, and HSNs, in Wild-Type and *ced-3* Animals

In (a) and (b) anterior is to the right. In (c), (d), (e), and (f) anterior is to the left. (a) The right postdeirid of a wild-type animal. (b) The right postdeirid of a *ced-3* animal. The postdeirid lineages of the animals shown in (a) and (b) were followed using Nomarski optics; lines indicate the nucleus of the nondopaminergic neuron of the postdeirid, and arrowheads indicate the nuclei of the dopaminergic neuron and two structural cells of the postdeirid sensillum. The arrow in (b) indicates the cell death survivor. (c) The arrow indicates the ventral CEM of a wild-type male. (d) The arrow indicates the ventral CEM in a *ced-3* hermaphrodite. (e) The HSN neuron, indicated by an arrow, in a wild-type hermaphrodite. (f) A cell with the morphology and position (ventral and about midway between anterior and posterior) of an HSN in a *ced-3* male. In (e) the arrowhead indicates the vulva, a hermaphrodite-specific feature. In (f) the arrowhead indicates a coelomocyte. The position of this cell differs between male and hermaphrodites (Sulston and Horvitz, 1977). The appearance of a coelomocyte approximately midway along the length of the animal (next to the HSN) indicates that the animal in (f) is a male. Bar = 10 μ.

first larval stage animals an average of three and seven deaths, respectively, are seen (Table 1).

ced-3 Mutations Result in a Loss or Reduction of Gene Function

Several observations suggest that the phenotype of *ced-3* mutants results from a loss or reduction of wild-type function. First, direct screening by Nomarski microscopy for *ced-3* mutants has produced *ced-3* alleles at a frequency of approximately 2.5×10^{-4} per mutagenized haploid genome (two mutants among the progeny of approximately 4000 F1 animals), which is close to the frequency expected for the generation of null mutations in the average C. elegans gene (Brenner, 1974; Greenwald and Horvitz, 1980). Second, three observations indicate that a deficiency of the *ced-3* locus, *nDf27*, and *ced-3(n717)* behave genetically in the same manner. First, *nDf27/ced-3(n717)* animals are similar in phenotype to *ced-3(n717)/ced-3(n717)* animals, as observed with Nomarski optics. Second, *nDf27/ced-3(n717); egl-1* animals are fully suppressed for the *egl-1* egg-laying defect, just as are *ced-3(n717)/ced-3(n717); egl-1*; animals. Third, *nDf27*, like *ced-3(n717)*, is a semidominant suppressor of *egl-1/ +* heterozygotes (Table 3).

Amber mutations provide an additional criterion for the identification of the phenotype resulting from the loss of function of a gene. However, we have not yet identified an amber allele of *ced-3*. We have constructed double mutant strains between each *ced-3* allele and the amber suppressor *sup-5* (Wills et al., 1983). Ten or more hermaphrodites of each strain were scored using Nomarski microscopy for the presence of extra cells in the pharynx and postdeirids and for the presence of the normally male-specific CEM neurons. None of the *ced-3* alleles was suppressed.

ced-4 Mutations Also Eliminate Programmed Cell Deaths

ced-4(n1162) was isolated in our laboratory by Chand Desai (personal communication) as a suppressor of the egg-laying defect of the HSN-deficient mutant *egl-1(n1084)*. The phenotype of *ced-4(n1162)* animals (see below) appears identical with that of *ced-3* mutants. However, *ced-4(n1162)* complements *ced-3* mutations and maps to linkage group III (Figure 2; see Experimental Procedures for map data).

In *ced-4* animals, as in *ced-3* animals, cells that would normally die instead survive. The lineages of the V5.pa and Q blast cells in *ced-4* animals were identical with the

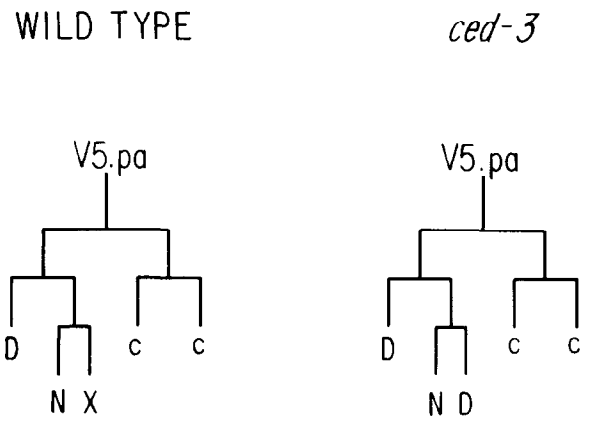

Figure 8. The Lineage of V5.pa in Wild-Type and *ced-3* Animals

In wild-type animals, V5.pa generates four cells of the postdeirid sensillum, a dopaminergic neuron (D), a nondopaminergic neuron (N), two structural cells with compact nuclei (c), and a programmed cell death (X). In *ced-3* animals, this cell death fails to occur. As explained in the text, it seems very likely that the extra domaminergic neuron in *ced-3* animals is the cell that normally dies.

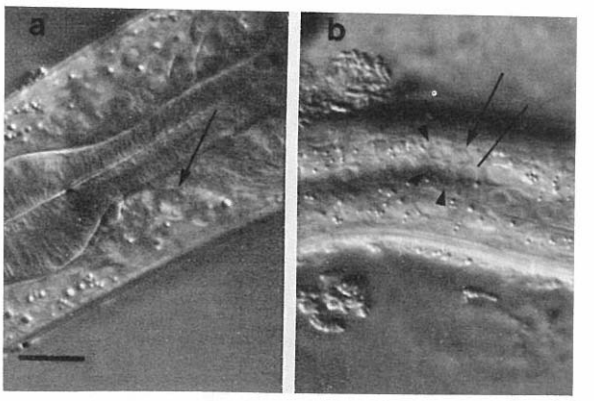

Figure 9. Nomarski Photomicrographs of CEMs and Postdeirid Cells in *ced-4* Animals

(a) Arrow indicates the ventral CEM in a *ced-4* hermaphrodite. The postdeirid lineage of the animal shown in (b) was followed using Nomarski optics. (b) Arrow indicates the cell death survivor, line indicates the nondopaminergic neuron, and arrowheads indicate the dopaminergic neuron and the two structural cells of the postdeirid. Bar = 10 μ.

Table 2. Chemotaxis Assays

Genotype	Average Number of Animals Accumulating	
	With Attractant	Without Attractant
Wild Type	7.0 ± 1.4	0.08 ± 0.08
ced-3(n717)	7.9 ± 1.3	0.50 ± 0.23
che-3(e1124)	0.75 ± 0.35	0.08 ± 0.08

Chemotaxis assays were performed as described in Experimental Procedures. For each genotype, 24 adult hermaphrodites were tested in each of 12 separate experiments. *che-3* is a chemotaxis-defective mutant (Lewis and Hodgkin, 1977). Attractant was placed in the center of the assay plate, and animals to be tested were placed around the edge of the plate. The values indicated are the average number of hermaphrodites that accumulated at the center of the plate after 15 min plus or minus the standard error of the mean (SEM).

Table 3. Suppression of *egl-1(n487)/+* Heterozygotes

Genotype	% Egl⁻	
egl-1(n487)/+	71	n = 300
ced-3(n717)/+; egl-1(n487)/+	30*	n = 212
nDf27/+; egl-1(n487)/+	33	n = 90
ced-4(n1162)/+; egl-1(n487)/+	84	n = 207

The number of animals of the indicated genotype that became bloated with late stage eggs (Egl⁻) was determined.
* The *ced-3* alleles *n718*, *n1040*, and *n1129* are also semidominant suppressors of *egl-1* heterozygotes.

wild-type lineages except that cells that normally die instead survived. *ced-4* animals have extra neuronal cells in the ventral cord, postdeirids (Figure 9) and lateral hypodermis. The number of extra cells is consistent with the number of cells that normally die in the P, V5.pa, and Q lineages. Like *ced-3* mutations, *ced-4(n1162)* blocks essentially all embryonic and postembryonic deaths. In *ced-1; ced-4* hermaphrodites no single cell has been observed to die consistently (Table 1). However, the linker cell was observed to die in two of six *ced-4* males and in one of six *ced-4(n1162); ced-3(n717)* males. Thus, the linker cell death does not require wild-type *ced-3* or *ced-4* function. Extra embryonically generated cells are seen in *ced-4* animals; their number is consistent with the survival of cells that would normally die.

FIF-histochemical staining of *ced-4* animals shows that they often have an extra dopaminergic neuron in each of their postdeirids and in the region of CEPVR and CEPVL. As in *ced-3* mutants, the production of dopamine by the cell death survivor in the postdeirid is variable. Of 60 postdeirids in *ced-4* animals, 27 had an extra dopaminergic neuron. FIF staining of *ced-4* animals also reveals on each side of the anterior bulb of the pharynx the presence of an extra serotonergic neuron that is similar in position and morphology to the NSM.

These observations indicate that the phenotype of *ced-4* mutants is the same as that of *ced-3* mutants. There is, however, one difference between the behavior of *ced-3* mutations and *ced-4(n1162)*. While *ced-3* mutations are semidominant suppressors of the egg-laying defect of *egl-1(n487)* heterozygotes, *ced-4* mutations are recessive suppressors of this defect (Table 3). (*ced-3(n717); egl-1(n487)/+* animals and *ced-4(n1162); egl-1(n487)/+* animals and *ced-4(n1162); egl-1(n487)* animals are egg-laying competent.)

A Developmental Pathway for Programmed Cell Death

Mutations in *ced-1* and *ced-2* prevent the engulfment of dying cells by their neighbors and cause the DNA in dying cells neither to condense nor to be degraded (Hedgecock et al., 1983). The phenotype of *ced-1; ced-2* double mutants has been shown previously to be identical with that of either mutant strain alone (Hedgecock et al., 1983). Thus the order of action of these two genes cannot yet be defined. Mutations in *nuc-1* result in the absence of an endonuclease, slowing the degradation of the DNA of dying cells (Sulston, 1976; Albertson et al., 1978). We have con-

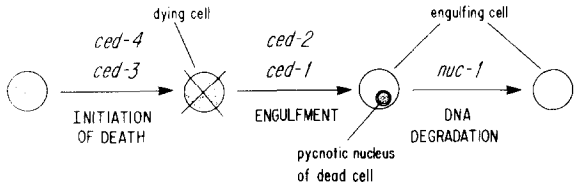

Figure 10. A Proposed Genetic Pathway for Programmed Cell Death in C. elegans

The wild-type functions of *ced-3* and *ced-4* are required for the initiation of programmed cell death. In the absence of *ced-3* or *ced-4* activity, cells that normally die instead survive and may differentiate. In the wild type, dying cells are usually engulfed by neighboring cells. The wild-type *ced-1* and *ced-2* products are required for engulfment or for an earlier step in the death pathway that must precede engulfment. The activities of *ced-1* and *ced-2* are not, however, required for cell death per se (Hedgecock et al., 1983). The wild-type *nuc-1* gene product is required to degrade the DNA of dying cells. Engulfment appears to be a necessary prerequisite for degradation of the DNA of dying cells, suggesting that the *nuc-1* nuclease may be expressed by the engulfing cell (Hedgecock et al., 1983). This pathway appears to be expressed by essentially all cells that undergo programmed cell death during C. elegans development.

structed a *ced-1; nuc-1* strain and have found that *ced-1* is epistatic to *nuc-1*; in *ced-1; nuc-1* animals the DNA of dying cells is neither condensed nor degraded. Similarly, *ced-2* is epistatic to *nuc-1*. Strains that carry mutations in *ced-3* and in one of the three genes *ced-1*, *ced-2*, or *nuc-1*, and strains that carry mutations in *ced-4* and in one of the four genes *ced-1*, *ced-2*, *ced-3*, or *nuc-1*, have also been constructed. Mutations in *ced-3* and *ced-4* are epistatic to mutations in *ced-1*, *ced-2*, and *nuc-1*; no cell deaths are seen in *ced-1; ced-3*, *ced-1; ced-4*, *ced-2 ced-3*, *ced-4; ced-2*, *ced-3; nuc-1*, or *ced-4; nuc-1* animals. Since the phenotype of *ced-4; ced-3* animals is the same as that of either *ced-3* or *ced-4* mutants, we cannot yet define the order of action of these genes. The epistasis of *ced-1* over *nuc-1*, and of *ced-3* and *ced-4* over all three other cell death genes, suggests the genetic pathway for programmed cell death shown in Figure 10.

The phenotypes of these cell death mutants are consistent with the pathway suggested on the basis of genetic interactions. In *ced-3* and *ced-4* mutants no signs of cell death are seen in cells that would normally die; no increase in refractility is seen with Nomarski microscopy and no condensation of the DNA is seen when *ced-3* animals are stained with diamidinophenolindole (DAPI). In contrast, cell death begins normally in *ced-1* and *ced-2* mutants, but the dying cells are not engulfed and remain in a highly refractile state for an abnormally long time. In addition, the nuclei of dying cells in *ced-1* and *ced-2* mutants are only slightly condensed, and the DNA of these cells is not degraded (Hedgecock et al., 1983). Finally, in *nuc-1* mutants dying cells become refractile, are engulfed, and their cytoplasm is removed, leaving the DNA highly condensed; this DNA is visible after staining with DAPI (Sulston, 1976).

Not all cell deaths appear to involve this pathway for programmed cell death. For example, as discussed above, the linker cell death can occur in *ced-3* and *ced-4* mutants. Furthermore, in the dominant mutant *mec-4(e1611)* six mechanosensory neurons, the microtubule neurons, die (Chalfie and Sulston, 1981). Hedgecock et al. (1983) found that these cell deaths were not affected by mutations in *ced-1* or *ced-2*. Similarly, we have observed that these deaths still occur in *ced-3(n717); mec-4(e1611)* animals. In addition, the deaths of the ventral hypodermal (Pn.p) cells in the dominant mutant *lin-24(n432)* (Ferguson and Horvitz, 1985) are not suppressed in *lin-24(n432) ced-3(n717)* animals (H. Ellis, E. Ferguson, and P. Sternberg, unpublished observations). The cell deaths caused by *mec-4(e1611)* and *lin-24(n432)* differ in morphology from cell deaths that occur during the development of the wild type (e.g., Chalfie and Sulston, 1981). These observations suggest that the mutations *mec-4(e1611)* and *lin-24(n432)* result in the cell-specific expression of a cytotoxic product.

Discussion

Recessive mutations in the C. elegans genes *ced-3* or *ced-4* cause cells that would normally die instead to survive and, in at least some cases, to differentiate. Genetic studies indicate that the *ced-3* phenotype is likely to result from the absence of wild-type *ced-3* function. The recessive nature of the phenotype of *ced-4(n1162)* animals suggests that this mutation also results in a reduction of wild-type function. Since these mutations result in a reduction or loss of gene activity, it is unlikely that the differentiated phenotypes of cell death survivors in *ced-3* or *ced-4* animals are produced de novo. Rather, mutations in these genes may be revealing underlying developmental potentials. These underlying potentials may reflect evolutionarily more primitive states of the lineage.

For example, the presence of HSN neurons in *ced-3* and *ced-4* males suggests that the male homologs of the HSNs, which normally die, have an underlying potential to differentiate into HSNs. Thus, sexual dimorphism for the presence of these neurons appears to be generated by programmed cell death. Similarly, the presence of the normally male-specific CEM neurons in *ced-3* and *ced-4* hermaphrodites suggests that sexual dimorphism for the presence of these neurons is also generated by programmed cell death.

Other cell death survivors may also have fates that correspond to those of homologous cells. For example, the cell P2.aap in *ced-3* hermaphrodites has characteristics that suggest that P2.aap, which normally dies in wild-type animals, has an underlying potential that is similar to that of its spatial homologs P3.aap-P8.aap. Specifically, during the first larval stage of the hermaphrodite, 12 neuroblasts (P1.a-P12.a) undergo identical patterns of cell divisions to generate five neural cells each (Sulston, 1976). The fates of these cells are strongly correlated with their lineage histories. For example, all 12 Pn.apa cells become AS motoneurons. However, only P3.aap-P8.aap become VC neurons; the other six Pn.aap cells undergo programmed cell death. Reconstruction from serial section electron micrographs of the anatomy of the anterior region of the ventral cord of a *ced-3* hermaphrodite has indicated that P2.aap in this animal had many of the properties of its nor-

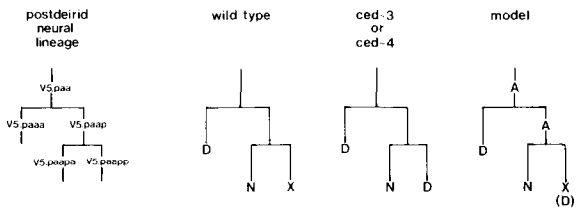

Figure 11. *ced-3* and *ced-4* Mutants Suggest an Underlying Reiterative Nature to the Postdeirid Lineage

In wild-type animals, V5.paa divides to generate a dopaminergic neuron (D) (V5.paaa) and a daughter (V5.paap) that, in turn, divides to generate a cell (V5.paapp) that dies (X) and a cell (V5.paapa) that becomes a nondopaminergic neuron (N). In *ced-3* and *ced-4* animals, V5.paa divides to generate a dopaminergic neuron and a daughter, which divides to generate a dopaminergic neuron rather than a cell death and a nondopaminergic neuron. This observation suggests that in wild-type animals, V5.paapp has a cryptic potential fate, the ability to become a dopaminergic neuron, and that V5.paa and V5.paap both have the potential to generate dopaminergic daughters. Thus, V5.paa and V5.paap can be considered to be of the same cell type (A).

mal spatial homologs, the VC neurons (J. G. White, personal communication). Thus, in the case of P2.aap, cell death may act to modify spatial repeats of a specific lineage pattern.

In other instances cell death survivors may express fates normally expressed by their sisters. For example, the extra serotonergic neurons in *ced-3* and *ced-4* mutants seem likely to be the sisters of the two NSMs. Similarly, the extra dopaminergic neurons often found in *ced-3* and *ced-4* animals in the region of the ventral cephalic neurons are probably the sisters of the ventral cephalic neurons.

In contrast, the cell death survivor in the postdeirid in many instances expresses the fate normally associated with its mother's sister. The dopaminergic phenotype of this cell suggests an underlying stem cell or "reiterative" (Chalfie et al., 1981) nature to this lineage. Such reiterative cell lineages, in which a cell divides to produce one or more descendant cells like itself, occur in many animals, including C. elegans. In *ced-3* or *ced-4* mutants, the postdeirid neuroblast V5.paa divides to generate a daughter (V5.paaa) that becomes a dopaminergic neuron, as it does in the wild type, and a daughter (V5.paap) that also generates a dopaminergic daughter, the cell death survivor V5.paapp. Thus, V5.paa and its daughter V5.paap are alike in that they share the potential to generate dopaminergic progeny cells. This observation suggests that in wild-type animals as well, V5.paa and V5.paap share the potential to produce dopaminergic progeny, a potential normally masked by the death of V5.paapp (Figure 11). The underlying reiterative nature of the postdeirid lineage was previously suggested on the basis of observations of mutants defective in the gene *unc-86* (Chalfie et al., 1981).

The absence of programmed cell death in *ced-3* or *ced-4* animals results in the presence of many extra cells. For example, during the normal development of C. elegans approximately 20% of the neural cells generated subsequently die. Thus, *ced-3* and *ced-4* animals may have up to 20% more neural cells than wild-type animals. It is striking, however, that mutations in *ced-3* and *ced-4* do not have any obvious deleterious effects on behavior. Although *ced-3* or *ced-4* mutations may cause behavioral defects that we have not detected, it seems clear that the absence of cell death is not grossly detrimental to the nematode. Thus, we are left with the question of what selective advantage may be associated with the extensive programmed cell death that occurs during C. elegans neurogenesis. Even small effects on behavior might provide a selective advantage. Alternatively, the elimination of unnecessary cells that would otherwise differentiate may decrease energy consumption and hence increase fitness.

The phenotypes of strains carrying mutations in pairwise combinations of the genes involved in cell death have suggested that these genes act sequentially in a developmental pathway for cell death. *ced-3* and *ced-4* define the first known step in this pathway. Since relatively few mutants affecting cell death have been identified, it seems likely that other genes in this pathway remain to be defined. Furthermore, in addition to genes involved in all cell deaths, there presumably are genes involved in the specification of particular cell deaths. For example, *egl-1* may be one such gene; dominant mutations in *egl-1* appear to result in the expression in hermaphrodites of the normally male-specific fate of HSN cell death (Trent et al., 1983), but do not affect other cell deaths (H. Ellis, unpublished observations).

The identification of a genetic pathway used for most C. elegans programmed cell deaths helps us to distinguish three distinct classes of cell deaths. First, almost all of the cell deaths that occur during wild-type development involve all four *ced* genes; deaths of this class appear to be suicides, that is, they are cell autonomous and can be induced by mutations (e.g., *egl-1*) that lead to the ectopic expression of the complete cell death pathway. Second, at least one death that occurs during normal development (that of the male linker cell) involves *ced-1* and *ced-2*, but can take place without normal *ced-3* and *ced-4* function; this death (as well as those of a few others that we have not tested for *ced-3* or *ced-4* involvement but that seem likely to be of the same class) appears to be a murder, that is, it occurs only in the presence of another cell. Third, certain mutation-induced deaths do not require the functions of any of the four *ced* genes; these deaths (such as those caused by the dominant mutations *mec-4(e1611)* and *lin-24(n432)* are morphologically abnormal and apparently result from the cell-specific synthesis of cytotoxic products.

Although *ced-3* and *ced-4* appear to act at the same step in the developmental pathway for cell death, genetic evidence suggests that these genes may differ functionally. The suppression of *egl-1/ +* heterozygotes by *ced3/ +* heterozygotes but not by *ced-4/ +* heterozygotes indicates that *ced-3* function is relatively sensitive to the dosage of the wild-type gene product (a 2-fold reduction of wild-type product can lead to a mutant phenotype), while *ced-4* function may be relatively less sensitive to dosage.

A number of observations have suggested that most

cell deaths, like most other cell fates in C. elegans, are specified in a cell autonomous manner (see Horvitz et al., 1982a, for discussion). Although the specification of which cells die is likely to be cell autonomous, cell extrinsic factors could still be involved in cell death. In particular, the experiments described in this paper do not indicate whether *ced-3* and *ced-4* act within dying cells (or their precursors) or act extrinsically to the dying cells. For example, at least one of these genes could act to control a hormonal signal that triggers cell death; such hormonal influences on neuronal and muscle cell deaths have been identified in the moth Manduca sexta (Truman and Schwartz, 1982a, 1982b).

The reduction or loss of *ced-3* or *ced-4* function results in a transformation of cell fates; in *ced-3* or *ced-4* mutants, cells that would normally die instead adopt fates normally associated with other cells. Other "homeotic" mutations, which cause specific transformations in developmental fates, have been identified in Drosophila (for a review see Lawrence and Morata, 1983) and in C. elegans (Greenwald et al., 1983; Horvitz et al., 1983). Such mutations appear to be good candidates for defining genes involved in the specification of cell fates. It remains possible, though, that the functions of *ced-3* and *ced-4* are simply necessary for the "differentiation" of the fate of cell death. For example, the products of *ced-3* and *ced-4* may directly "kill" cells rather than control the activities of other genes that act to kill cells. Nonetheless, the homeotic nature of *ced-3* and *ced-4* suggests that these genes may be involved in the determination of a specific cell fate, programmed cell death.

Experimental Procedures

General Methods and Strains

The techniques used for the culturing and ethyl methanesulfonate (EMS) mutagenesis of C. elegans were as described by Brenner (1974). All strains were grown at 20°C unless otherwise indicated. The wild-type parent of all strains described here was C. elegans variety Bristol, strain N2 (Brenner, 1974). The genetic markers used are as follows: LG I *dpy-5(e61)*, *ced-1(e1735)*, *che-3(e1124)*; LG II *dpy-10(e128)*, *bli-2(e768)*; LG III *unc-93(e1500)*, *unc-79(e1068)*, *dpy-17(e164)*, *unc-32(e189)*; LG IV *ced-2(e1752)*, *dpy-13(e184)*, *lin-3(e1417)*, *dpy-20(e1362)*, *unc-22(e66)*, *unc-30(e191)*, *unc-26(e205)*, *lev-1(x22)*, *tra-3(e1767)*, *dpy-4(e1166)*; LG V *dpy-11(e224)*, *egl-1(n487, n1084)*; LG X *dpy-3(e27)*, *nuc-1(e1392)*, *lon-2(e678)*. These markers have been described by Brenner (1974) or by Swanson et al. (1984). This paper conforms to the standard system of C. elegans genetic nomenclature (Horvitz et al., 1979).

Cell Lineage

The use of Nomarski differential interference contrast microscopy for the determination of cell lineages has been described by Sulston and Horvitz (1977). All lineages discussed in this manuscript were observed in at least three animals. The nomenclature used to designate individual cells reflects their lineage history (Sulston and Horvitz, 1977). Blast cells are designated by upper case letters, which may be followed by numbers to indicate a set of analogous blast cells, for example, V1 through V6. L (left) or R (right) are added to blast cell names to indicate identical pairs of postembryonic cells found on the left and right sides of the animal, for example, V5L and V5R. The progeny of a blast cell are represented by adding to the blast cell name a lower case letter indicating the relative positions of the daughters immediately following division; for example, V5.a is the anterior daughter of V5.

Histochemistry

Formaldehyde-induced fluorescence (FIF) was used to identify serotonin- or dopamine-containing cells (Sulston et al., 1975; Horvitz et al., 1982b). Diamidinophenolindole (DAPI) (Sigma) was used to stain nuclei. Animals were fixed in 6:3:1 ethanol:chloroform:acetic acid (Carnoy's solution) and stained with 1.0 μg/ml DAPI and 1.0 μg/ml phenoxypropanol in M9 buffer (W. Fixsen, Ph.D. thesis, M.I.T., Cambridge, Massachusetts, 1985).

Behavioral Assays

Chemotaxis

The chemotaxis assays used were modified from those described by Ward (1973). A 100 mm tissue culture dish was spread evenly with 5 ml of a thick slurry of Sephadex G-200-40 gel beads (Sigma) swelled overnight in 0.1 M HEPES and 2.5% sucrose (pH 7.2). Five microliters of 2 M NaCl was placed in the center of the dish and 4½ to 5 hr later, 24 adult hermaphrodites were placed around the edge of the dish. After 15 min, the number of worms that had accumulated within a radius of 1.6 cm from the center of the dish was determined.

Male Mating

The efficiency of male mating was tested as described by Hodgkin et al. (1979). Six L4 males and six L4 *dpy-11(e224)* hermaphrodites were placed on a petri dish seeded with a small (~1 cm) circle of Escherichia coli, and 24 hr later the males were removed. The hermaphrodites were transferred to fresh plates each day and total cross progeny (non-Dpy animals) were counted.

Pharmacological Tests of Egg Laying

Exogenously added serotonin or imipramine stimulate gravid wild-type adults to lay eggs (Horvitz et al., 1982b; Trent et al., 1983). The response of adult *ced-3* hermaphrodites to serotonin and imipramine was tested as described by Trent et al. (1983). Gravid adults were placed in microtiter wells containing serotonin (Sigma) (5 mg/ml) or imipramine (Sigma) (0.75 mg/ml), and 60 min (serotonin) or 90 min (imipramine) later the number of eggs laid by each hermaphrodite was counted.

All behavioral assays were performed at 20°C.

Genetic Protocols

Mapping

ced-3 was tested for linkage to markers on all linkage groups and weak linkage to *dpy-13(e184) IV* was observed (data not shown). *ced-3* was more precisely mapped to a position between *unc-30* and *unc-26* on linkage group IV (Figure 2). The results of a three-factor cross confirmed the assignment of *ced-3* to linkage group IV: 21/21 Dpy non-Unc and 0/26 Unc non-Dpy recombinant progeny of *unc-26 dpy-4/ ced-3* heterozygotes segregated *ced-3* animals. A second three-factor cross established that *ced-3* mapped between *unc-30* and *dpy-4*; 5/6 Dpy non-Unc and 1/10 Unc non-Dpy recombinant progeny of *unc-30 dpy-4/ ced-3* heterozygotes segregated *ced-3* animals. In these crosses, the Ced-3 phenotype was scored using Nomarski optics to detect the presence of extra cells. A four-factor cross ordered *ced-3* with respect to *unc-26*. Heterozygotes of genotype *ced-3 unc-26/unc-30 dpy-4; egl-1* were constructed and from their progeny Ced-3 non-Unc-26 and Unc-26 non-Ced-3 recombinants were picked and scored for the segregation of *unc-30* or *dpy-4* animals. (*ced-3* mutants are indistinguishable from the wild-type as viewed with a dissecting microscope. However, the ability of mutations in *ced-3* to suppress the egg-laying defect of *egl-1* animals allowed us to score *ced-3* in an *egl-1* background using a dissecting microscope.) Three out of three Ced-3 non-Unc-26 recombinants segregated *dpy-4* but not *unc-30* animals, while 3/3 Unc-26 non-Ced-3 recombinants segregated *unc-30* but not *dpy-4* animals.

ced-4 was tested for linkage to markers on all linkage groups and was found to be linked only to linkage group III; 2/15 Ced-4 non-Unc animals segregating from *ced-4/unc-32* heterozygotes gave Unc progeny (frequency of recombination [p] approximately equal to 0.07, since the frequency of animals segregating Unc-32 progeny should be 2p/(1+p), that is, 2/3 if *ced-4* and *unc-32* were unlinked and 2p if they were closely linked). A three-factor cross suggested that *ced-4* mapped to the left of *dpy-17*; 3/3 Lon non-Dpy recombinant progeny of *dpy-17 lon-1/ced-4* heterozygotes segregated *ced-4* animals. A second three-factor cross indicated that *ced-4* mapped between *unc-93* and *dpy-17*; 1/8 Dpy non-Unc and 8/8 Unc non-Dpy recombinant progeny of *unc-93 dpy-17/ced-4* heterozygotes segregated *ced-4* progeny. An additional

three-factor cross indicated that *ced-4* mapped between *unc-79* and *dpy-17* (Figure 2); 5/5 Unc non-Dpy and 1/7 Dpy non-Unc recombinant progeny of *unc-79 dpy-17/ced-4* heterozygotes segregated *ced-4* animals. In these crosses, the Ced-4 phenotype was scored using Nomarski optics to detect the presence of extra cells.

Complementation Tests

Putative *ced-3* alleles (*ced-3**) were tested for failure to complement *ced-3(n717)*. Heterozygotes of genotype *ced-3(n717)/ced-3** were constructed and scored using Nomarski microscopy for the presence of cell death survivors.

ced-4(n1162) was tested for complementation of *ced-3(n717). ced-4(n1162); egl-1(n1084)* males were crossed with *ced-3(n717) dpy-4(e1166); egl-1(n487)* hermaphrodites. The resulting cross progeny (non-Dpy) hermaphrodites were egg-laying defective (non-Ced-3), and 12/12 cross progeny scored using Nomarski microscopy did not have a Ced-3 phenotype.

Complementation Screens

ced-3(n1129) was isolated on the basis of its failure to complement *ced-3(n717)*. L4 *egl-1(n487)* males were mutagenized with EMS and crossed with *ced-3 dpy-4; egl-1; dpy-3* hermaphrodites. The majority of the hermaphrodite cross progeny were non-Dpy and egg-laying defective (genotype *ced-3 dpy-4/+ +; egl-1; dpy-3/+)* rare animals were phenotypically wild-type and candidates for harboring a new *ced-3* allele (genotype *ced-3 dpy-4/ced-3* +; egl-1; dpy-3/+)*. Putative new *ced-3* alleles were isolated from these heterozygotes on the basis of their failure to segregate *dpy-4* progeny and were phenotypically and genetically characterized. In this experiment approximately 12,000 F1 cross progeny were examined, and one new *ced-3* allele, *n1129*, was generated.

***Generation of Deficiencies for* ced-3**

To generate the deficiency *nDf27 IV*, L4 males on petri dishes were irradiated for 2.5 min at a dose rate of approximately 3000 rad/min in a Gamma Cell 220 Co source (Atomic Energy of Canada, Ltd.) (Greenwald and Horvitz, 1980) and then crossed with L4 *lev-1(x22) dpy-4(e1166)* hermaphrodites. The recessive mutation *lev-1(x22)* results in resistance to the antihelmenthic drug levamisole (Lewis et al., 1980). Rare levamisole-resistant non-Dpy F1 animals (four of 5000 F1 animals examined) were selected. These animals were candidates for carrying deficiencies of the *lev-1* locus. Two strains segregated approximately one-quarter inviable zygotes and were tested to determine whether they contained a lethal levamisole-resistant allele that failed to complement other loci in the region (i.e., to ascertain whether the lethal levamisole-resistant allele was a deficiency). One of these strains contained a lethal *lev-1* allele (*nDf27*) that failed to complement *unc-26, ced-3, unc-30, unc-22* (H. Ellis, unpublished observations), and *dpy-20* (E. Ferguson, personal communication) and complemented *lin-3* (E. Ferguson, personal communication), *tra-3* and *dpy-4* (Figure 2). The second strain, which was subsequently lost, also contained a deficiency of the *lev-1* locus.

Acknowledgments

We thank Ed Hedgecock for sharing with us his strains, thoughts, and unpublished data. We thank John Sulston for encouragement and advice, and Nancy Tsung and Chand Desai for isolating some of the mutants we have studied. We also thank Leon Avery, Marty Chalfie, Chip Ferguson, Bill Fixsen, Ed Hedgecock, Jonathan Hodgkin, and Carol Trent for discussions and comments on the manuscript. This research was supported by U.S. Public Health Service grants GM24663 and GM24943 and Research Career Development Award HD00369 to H. R. Horvitz.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received September 30, 1985; revised December 23, 1985.

References

Albertson, D., and Thomson, N. (1976). The pharynx of *Caenorhabditis elegans*. Phil. Trans. Roy. Soc. (Lond.) B *275*, 299–325.

Albertson, D., Sulston, J. E., and White, J. G. (1978). Cell cycling and DNA replication in a mutant blocked in cell division in the nematode *Caenorhabditis elegans*. Dev. Biol. *63*, 165–178.

Brenner, S. (1974). The genetics of *Caenorhabditis elegans*. Genetics *77*, 71–94.

Chalfie, M., and Sulston, J. E. (1981). Developmental genetics of the mechanosensory neurons of *Caenorhabditis elegans*. Dev. Biol. *82*, 358–370.

Chalfie, M.,Horvitz, H. R., and Sulston, J. E. (1981). Mutations that lead to reiterations in the cell lineages of C. elegans. Cell *24*, 59–81.

Chalfie, M., Sulston, J. E., White, J. G., Southgate, E., and Thomson, J. N. (1985). The neural circuit for touch sensitivity in *Caenorhabditis elegans*. J. Neurosci. *5*, 956–964.

Cowan, W. M., Fawcett, J., O'Leary, D., and Stanfield, B. (1984). Regressive events in neurogenesis. Science *225*, 1258–1265.

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

Greenwald, I. S., and Horvitz, H. R. (1980). *unc-93(e1500)*: a behavioral mutant of *Caenorhabditis elegans* that defines a gene with a wild-type null phenotype. Genetics *96*, 147–164.

Greenwald, I. S., Sternberg, P. W., and Horvitz, H. R. (1983). The *lin-12* locus specifies cell fates in Caenorhabditis elegans. Cell *34*, 435–444.

Hedgecock, E., Sulston, J. E., and Thomson, N. (1983). Mutations affecting programmed cell deaths in the nematode *Caenorhabditis elegans*. Science *220*, 1277–1280.

Herman, R., and Horvitz, H. R. (1980). Genetic analysis of *Caenorhabditis elegans*. In Nematodes as Biological Models, B. Zuckerman, ed. (New York: Academic Press), pp. 227–264.

Hodgkin, J., Horvitz, H. R., and Brenner, S. (1979). Nondisjunction mutants of the nematode *Caenorhabditis elegans*. Genetics *91*, 67–94.

Horvitz, H. R., Brenner, S., Hodgkin, J., and Herman, R. (1979). A uniform genetic nomenclature for the nematode *Caenorhabditis elegans*. Mol. Gen. Genet. *175*, 129–133.

Horvitz, H. R., Ellis, H. M., and Sternberg, P. W. (1982a). Programmed cell death in nematode development. Neurosci. Comment. *1*, 56–65.

Horvitz, H. R., Chalfie, M., Trent, C., Sulston, J., and Evans, P. (1982b). Serotonin and octopamine in the nematode *C. elegans*. Science *216*, 1012–1014.

Horvitz, H. R., Sternberg, P. W., Greenwald, I. S., Fixsen, W., and Ellis, H. M. (1983). Mutations that affect neural cell lineages and cell fates during the development of the nematode *Caenorhabditis elegans*. Cold Spring Harbor Symp. Quant. Biol. *48*, 453–463.

Kimble, J. E., and Hirsh, D. (1979). The postembryonic cell lineages of the hermaphrodite and male gonads in *Caenorhabditis elegans*. Dev. Biol. *70*, 396–417.

Lawrence, P. A., and Morata, G. (1983). The elements of the bithorax complex. Cell *35*, 595–601.

Lewis, J., and Hodgkin, J. (1977). Specific neuroanatomical changes in chemosensory mutants of the nematode *Caenorhabditis elegans*. J. Comp. Neurol. *172*, 489–510.

Lewis, J., Wu, C. H., Berg, H., and Levine, J. (1980). Genetics of levamisole resistance in the nematode *Caenorhabditis elegans*. Genetics *95*, 905–928.

Saunders, J. (1966). Death in embryonic systems. Science *154*, 604–612.

Sulston, J. E. (1976). Post-embryonic development in the ventral cord of *Caenorhabditis elegans*. Phil. Trans. Roy. Soc. (Lond.) B *275*, 287–297.

Sulston, J. E., and Horvitz, H. R. (1977). Post-embryonic cell lineages of the nematode *Caenorhabditis elegans*. Dev. Biol. *82*, 110–156.

Sulston, J. E., and White, J. G. (1980). Regulation and cell autonomy during postembryonic development of *Caenorhabditis elegans*. Dev. Biol. *78*, 577–597.

Sulston, J. E., Dew, M., and Brenner, S. (1975). Dopaminergic neurons in the nematode *Caenorhabditis elegans*. J. Comp. Neurol. *163*, 215–226.

Sulston, J. E., Schierenberg, E., White, J. G., and Thomson, N. (1983).

The embryonic cell lineage of the nematode *Caenorhabditis elegans*. Dev. Biol. *100*, 64–119.

Swanson, M. M., Edgeley, M. L., and Riddle, D. L. (1984). *Caenorhabditis elegans*. In Genetic Maps, S. O'Brien, ed. (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory), pp. 244–258.

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

Truman, J. W. (1984). Cell death in invertebrate nervous systems. Ann. Rev. Neurosci. *7*, 171–188.

Truman, J. W., and Schwartz, L. M. (1982a). Insect systems for the study of programmed neuronal death. Neurosci. Comment. *1*, 66–72.

Truman, J. W., and Schwartz, L. M. (1982b). Programmed cell death in the nervous system of a moth. Trends Neurosci. *5*, 270–273.

Ward, S. (1973). Chemotaxis by the nematode *Caenorhabditis elegans*: identification of attractants and analysis of response by use of mutants. Proc. Natl. Acad. Sci. USA *70*, 817–821.

Wills, N., Gesteland, R. F., Karn, J., Barnett, L., Bolten, S., and Waterston, R. H. (1983). The genes *sup-7 X* and *sup-5 III* of C. elegans suppress amber nonsense mutations via altered transfer RNA. Cell *33*, 575–583.