Developing *Caenorhabditis elegans* neurons may contain both cell-death protective and killer activities

Shai Shaham and H. Robert Horvitz

Howard Hughes Medical Institute, Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139 USA

We developed a method for examining the effects of overexpressing cell-death-related genes in specific *Caenorhabditis elegans* neurons that normally live. Using this method, we demonstrated that the cell-death genes *ced-3*, *ced-4*, and *ced-9* all can act cell autonomously to control programmed cell death. Our observations indicate further that not only the protective activity of *ced-9* but also the killer activities of *ced-3* and *ced-4* are likely to be present in cells that normally live. We propose that both in *C. elegans* and in other organisms a competition between antagonistic protective and killer activities determines whether specific cells will live or die. Our results suggest a genetic pathway for programmed cell death in *C. elegans* in which *ced-4* acts upstream of or in parallel to *ced-3* and *ced-9* negatively regulates the activity of *ced-4*.

[Key Words: *C. elegans*; programmed cell death; cysteine protease; *bcl-2* family; ICE family]

Received December 5, 1995; revised version accepted January 23, 1996.

Programmed cell death is a major and apparently universal aspect of metazoan development and tissue homeostasis (Glücksmann 1950; Ellis et al. 1991a). Programmed cell death serves several functions, including the regulation of cell number, the removal of deleterious cells, and the shaping of tissues and organs. Although a diversity of signals can cause different cells to undergo programmed cell death (e.g., Barres et al. 1993; Vaux 1993), the morphology and kinetics of programmed cell deaths are in many cases highly similar (Stanisic et al. 1978; Cohen and Duke 1984; Martin et al. 1988; Arends and Wyllie 1991), suggesting that the mechanisms responsible for the programmed deaths of different cell types and of cells in different organisms could well be the same.

During the development of the nematode *Caenorhabditis elegans*, 131 of the 1090 somatic cells generated undergo programmed cell death (Sulston and Horvitz 1977; Kimble and Hirsh 1979; Sulston et al. 1983). Thirteen genes that affect various aspects of the process of programmed cell death in *C. elegans* have been identified (Hedgecock et al. 1983; Ellis and Horvitz 1986; Ellis and Horvitz 1991; Ellis et al. 1991b; Hengartner et al. 1992; for review, see Horvitz et al. 1994). Three of these genes control the onset of the death process, six act in the phagocytosis of dying cells by their neighbors, and one functions in the digestion of the DNA of cell corpses. Three additional genes affect the fate of programmed cell death of a few specific cells that die during development.

Of the three *C. elegans* genes that control the onset of

programmed cell death, two—*ced-3* and *ced-4* (*ced*, cell death abnormal)—are required for programmed cell death to occur: Loss-of-function mutations in either of these genes cause all 131 cells that normally die instead to survive (Ellis and Horvitz 1986). In contrast, the third gene—*ced-9*—is needed to protect cells that normally survive from undergoing programmed cell death: Loss-of-function mutations in *ced-9* cause cells that normally live instead to undergo programmed cell death (Hengartner et al. 1992). The extra cell deaths that occur in *ced-9* loss-of-function mutants require the activities of *ced-3* and *ced-4*, indicating that *ced-9* acts by preventing *ced-3* and *ced-4* from causing cell death.

Both *ced-9* and *ced-3* have known mammalian counterparts that function in cell death. *ced-9* encodes a protein similar in sequence to the human proto-oncoprotein Bcl-2 (Hengartner and Horvitz 1994a), which like the CED-9 protein can protect cells from programmed cell death (Vaux et al. 1988; Nunez et al. 1990; Hockenbery et al. 1991; Garcia et al. 1992; Sentman et al. 1992; Strasser et al. 1992; for review, see Williams and Smith 1993). Human *bcl-2* expressed in transgenic *C. elegans* can protect nematode cells from undergoing programmed cell death (Vaux et al. 1992; Hengartner and Horvitz 1994a). Human *bcl-2* also can rescue nematode cells that die as a consequence of a *ced-9* loss-of-function mutation, suggesting that *bcl-2* might substitute functionally for *ced-9* (Hengartner and Horvitz 1994a). Thus, *ced-9* and *bcl-2* seem to be structurally similar and at least somewhat functionally interchangeable.

ced-3 encodes a protein similar in sequence to mam-

malian interleukin-1 β (IL-1 β) converting enzyme (ICE) (Yuan et al. 1993), a cysteine protease isolated on the basis of its ability to cleave a 31-kD pro-IL-1 β protein to generate the 17.5-kD mature cytokine (Cerretti et al. 1992; Thornberry et al. 1992). Overexpression of either CED-3 protein or ICE can cause rat fibroblasts to undergo programmed cell death (Miura et al. 1993). Furthermore, the programmed cell death induced when chick dorsal-root ganglion neurons are deprived of nerve growth factor can be inhibited by expression of the cowpox virus protein crmA (Gagliardini et al. 1994), an inhibitor of ICE (Ray et al. 1992), and mice harboring a disruption of the ICE gene are defective in Fas-mediated apoptosis (Kuida et al. 1995). These observations indicate that cysteine proteases of the CED-3/ICE family can cause mammalian cells to undergo programmed cell death and suggest that such proteases act endogenously in the programmed deaths that follow growth factor deprivation and Fas induction.

Both CED-3 and ICE are similar in sequence to the proteins encoded by the mouse *nedd-2* gene and its human homolog *Ich-1*, which can cause cell death when overexpressed in either mouse fibroblasts or neuroblastoma cells (Kumar et al. 1994; Wang et al. 1994), and to the product of the human gene CPP32, which can cause cell death when overexpressed in insect Sf9 cells (Fernandes-Alnemri et al. 1994). In addition, *bcl-2* can inhibit the cell deaths caused by the expression of the CED-3, ICE (Miura et al. 1993), or NEDD-2/ICH-1 proteins (Kumar et al. 1994; Wang et al. 1994) or by the deprivation of nerve growth factor (Gagliardini et al. 1994), suggesting that just as *ced-3* function is inhibited by *ced-9*, the action of ICE-like cysteine proteases can be inhibited by *bcl-2*.

Because mutations in either *ced-3* or *ced-4* block all naturally occurring programmed cell deaths in *C. elegans* (Ellis and Horvitz 1986), both of these genes normally must be functional for the proper execution of programmed cell death. However, this observation does not reveal whether the expression of these genes is restricted to cells that die or whether overexpression of one or both of these genes would suffice to cause a cell that would otherwise survive instead to undergo programmed cell death. To address these issues, we performed the experiments described below.

Results

Overexpression of either *ced-3* or *ced-4* can kill cells that normally live

To test whether expression of *ced-3* or *ced-4* is sufficient to kill cells that normally live, we placed cDNAs for each of these genes under the control of the promoter for the *C. elegans* gene *mec-7*, which is expressed in the six touch neurons (ALML, ALMR, AVM, PVM, PLML, and PLMR) and in a few other cells (Savage et al. 1989; M. Chalfie, pers. comm.). The *P_{mec-7}ced-3* and *P_{mec-7}ced-4* fusion constructs were injected separately into wild-type animals, and independent lines of worms homozygous

for integrated copies of the constructs were established. We obtained three lines (*P_{mec-7}ced-3A*, *P_{mec-7}ced-3B*, and *P_{mec-7}ced-3C*) homozygous for integrated copies of *P_{mec-7}ced-3* and four lines (*P_{mec-7}ced-4A*, *P_{mec-7}ced-4B*, *P_{mec-7}ced-4C*, and *P_{mec-7}ced-4D*) homozygous for integrated copies of *P_{mec-7}ced-4*.

To determine whether cells that normally express *mec-7* were absent in animals carrying the *P_{mec-7}ced-3* or *P_{mec-7}ced-4* transgenes, we scored animals for the presence or absence of the two ALM neurons. We scored the left side of the animal for the presence of the ALML neuron and the right side for the presence of the ALMR neuron. As shown in Table 1, we observed that ALM cells were missing in some of the lines we obtained. For example, ALMs were present on only 9 of the 46 (20%) sides scored in line *P_{mec-7}ced-3A* and on only 4 of the 39 (10%) sides scored in line *P_{mec-7}ced-4A*. We also injected *P_{mec-7}ced-3* and *P_{mec-7}ced-4* constructs together and established two lines containing integrated arrays harboring both constructs (lines *P_{mec-7}ced-3/4A* and *P_{mec-7}ced-3/4B*). Wild-type animals carrying these arrays showed a slight loss of ALM neurons: ALMs were present on 35 of the 37 (95%) and 45 of the 46 (98%) sides scored, respectively. Wild-type animals not carrying these arrays always contained both ALMs ($n = 31$), and animals expressing a *P_{mec-7}lacZ* construct (*jels1*; J. Way, pers. comm.) had ALMs on 40 of the 40 (100%) sides scored, suggesting that the presence of an array or the expression of any protein will not kill these cells (also, see below). These results suggest that overexpression of either *ced-3* or *ced-4* is sufficient to kill the ALMs in wild-type animals and that both *ced-3* and *ced-4* can kill cells in a cell-autonomous fashion. To confirm that the *P_{mec-7}ced-4*

Table 1. Overexpression of *ced-3* or *ced-4* can kill the ALM neurons

	Percent surviving ALMs (no. ALMs/no. sides scored)
	wild-type
—	100 (31/31)
<i>P_{mec-7}lacZ</i>	100 (40/40)
<i>P_{mec-7}ced-3A</i>	20 (9/46)
<i>P_{mec-7}ced-3B</i>	42 (16/38)
<i>P_{mec-7}ced-3C</i>	100 (48/48)
<i>P_{mec-7}ced-4A</i>	10 (4/39)
<i>P_{mec-7}ced-4B</i>	87 (33/38)
<i>P_{mec-7}ced-4C</i>	98 (39/40)
<i>P_{mec-7}ced-4D</i>	98 (40/41)
<i>P_{mec-7}ced-3/4A</i>	95 (35/37)
<i>P_{mec-7}ced-3/4B</i>	98 (45/46)

Each row represents an independently derived line of *C. elegans* homozygous for a given integrated transgene or pair of transgenes. Fractions indicate the number of ALMs per number of animal sides scored. For each line named *P_{mec-7}ced-nX*, n is either 3 or 4 (for *ced-3* or *ced-4*) and X identifies the specific independent line; in rows 10 and 11, n is 3/4, indicating the presence of both *ced-3* and *ced-4* transgenes in each of the two independently derived worm lines.

constructs expressed CED-4 protein, we examined surviving ALMs in $P_{mec-7}ced-4$ -containing lines in a wild-type background for reactivity to anti-CED-4 polyclonal antibodies. ALMs in line $P_{mec-7}ced-4A$ stained more strongly than did ALMs in the other three insertion lines (data not shown), consistent with the observation that ALMs died more frequently in this line. Because no anti-CED-3 antibody was available, we could not directly assess the levels of CED-3 expression in $P_{mec-7}ced-3$ -containing lines.

Endogenous ced-9 activity protects against killing by overexpressed ced-3 or ced-4

Although, as described above, overexpression of either *ced-3* or *ced-4* caused the deaths of ALM neurons, many ALMs survived in animals transgenic for these cell-death genes. Because the gene *ced-9* can protect cells against cell death mediated by *ced-3* and *ced-4*, it seemed plausible that eliminating endogenous *ced-9* function would result in enhanced killing by a *ced-3* or *ced-4* transgene. To test this hypothesis, we introduced our *ced-3* and *ced-4* transgene constructs into *ced-9(lf)*; *ced-3* or *ced-4* *ced-9(lf)* animals. [*ced-9(lf)*] single-mutant animals die, making it impossible to overexpress *ced-3* or *ced-4* in such a strain, but *ced-9(lf)*; *ced-3* and *ced-4* *ced-9(lf)* double-mutant animals are viable (Hengartner et al. 1992)].

As shown in Table 2A, lines containing a $P_{mec-7}ced-3$ transgene and the chromosomal mutation *ced-9(n2812)*

had fewer ALMs than did lines containing the corresponding transgene in the presence of the wild-type *ced-9* gene. For example, for the $P_{mec-7}ced-3A$ transgene, ALMs were present on 0 of the 29 (0%) sides scored in a *ced-9*; *ced-3* background yet were present on 16 of the 34 (47%) sides scored in a *ced-3* background. Similarly, lines containing a $P_{mec-7}ced-4$ transgene and the chromosomal mutation *ced-9(n2812)* had fewer ALMs than did lines containing the corresponding transgene in the presence of the wild-type *ced-9* gene (Table 2B). For the $P_{mec-7}ced-4A$ transgene, ALMs were present on 0 of the 30 (0%) sides scored in a *ced-4* *ced-9* background yet were present on 12 of the 28 (43%) sides scored in a *ced-4* background. Lines containing integrated copies of both a *ced-3* and a *ced-4* transgene also showed fewer ALMs surviving in a *ced-9(n2812)* background (Tables 2A,B). In line $P_{mec-7}ced-3/4A$, for example, ALMs were present on only 16 of the 37 (43%) sides scored in a *ced-9*; *ced-3* genetic background but were present on 40 of the 40 (100%) sides scored in a *ced-3* background alone (Table 2A). We suspect that the double transgenes were not expressed at levels as high as the single transgenes (see below), which might explain why killing in these lines was reduced in comparison to killing in some of the lines with single transgenes. These results suggest that killing by overexpression of either *ced-3* or *ced-4* is more efficient in the absence of *ced-9* function and are consistent with the hypothesis that *ced-9* acts to regulate negatively the activities of both *ced-3* and *ced-4* (Hengartner et al. 1992; see Discussion). Furthermore, because *ced-9* acts cell autonomously to inhibit cell death (see below), these results suggest that *ced-9* is normally expressed in the ALM neurons (also, see Discussion).

We also tested the effect of the *ced-9* partial loss-of-function allele *n1950 n2161* on ALM cell death in strains containing the $P_{mec-7}ced-3A$ transgene and again observed enhanced killing: ALMs were present on 1 of the 15 (7%) sides scored in an *unc-69(e587) ced-9(n1950 n2161)* background. These results suggest that it is a reduction of *ced-9* activity that allows more efficient killing rather than a specific action of the *ced-9(n2812)* allele.

The *ced-9* gain-of-function allele *n1950* is a missense mutation that acts oppositely to *ced-9(lf)* alleles and prevents the normal cell deaths that occur during *C. elegans* development (Hengartner et al. 1992; Hengartner and Horvitz 1994b). We tested the ability of *ced-9(n1950)* to prevent the deaths of ALM neurons in animals carrying $P_{mec-7}ced-3$ or $P_{mec-7}ced-4$ transgenes. As shown in Table 3, the *n1950* mutation did not consistently alter the abilities of these transgenes to cause ALM death.

Overexpression of ced-3 or ced-4 causes programmed cell death

The experiments described above indicated that overexpression of either *ced-3* or *ced-4* can lead to an absence of ALM neurons. To determine whether *mec-7*-expressing neurons were missing because they underwent programmed cell death, we observed directly the cell divi-

Table 2. Effects of *ced-9* on killing by *ced-3* or *ced-4* overexpression

	Percent surviving ALMs (no. ALMs/no. sides scored)	
	<i>ced-9</i> ; <i>ced-3</i>	<i>ced-3</i>
A. ALM killing by <i>ced-3</i> overexpression is better in a <i>ced-9(lf)</i> background^a		
$P_{mec-7}ced-3A$	0 (0/29)	47 (16/34)
$P_{mec-7}ced-3B$	0 (0/37)	30 (8/27)
$P_{mec-7}ced-3C$	21 (9/43)	100 (34/34)
$P_{mec-7}ced-3/4A$	43 (16/37)	100 (40/40)
$P_{mec-7}ced-3/4B$	67 (18/27)	100 (46/46)
	<i>ced-4</i> ; <i>ced-9</i>	
	<i>ced-4</i>	
B. ALM killing by <i>ced-4</i> overexpression is better in a <i>ced-9(lf)</i> background^b		
$P_{mec-7}ced-4A$	0 (0/30)	43 (12/28)
$P_{mec-7}ced-4B$	53 (18/34)	94 (32/34)
$P_{mec-7}ced-4C$	42 (15/36)	97 (36/37)
$P_{mec-7}ced-4D$	15 (4/27)	100 (36/36)
$P_{mec-7}ced-3/4A$	70 (35/50)	100 (41/41)
$P_{mec-7}ced-3/4B$	74 (37/50)	100 (30/30)

^aThe column headings identify the chromosomal genotypes of the strains examined. The alleles used were *ced-9(n2812)* and *ced-3(n717)*, except for the experiment involving $P_{mec-7}ced-3A$, in which *ced-3(n3002)* was used.

^bThe column headings identify the chromosomal genotypes of the strains examined. The alleles used were *ced-9(n2812)* and *ced-4(n1162)*.

Table 3. *ced-9(n1950)* does not decrease ALM killing by overexpression of *ced-3* or *ced-4*

	Percent surviving ALMs (no. ALMs/no. sides scored)	
	wild type	<i>ced-9(n1950)</i>
$P_{mec-7}ced-3A$	20 (9/46)	58 (18/31)
$P_{mec-7}ced-3B$	42 (16/38)	27 (10/37)
$P_{mec-7}ced-3C$	100 (48/48)	100 (42/42)
$P_{mec-7}ced-4A$	10 (4/39)	4 (2/47)
$P_{mec-7}ced-4B$	87 (33/38)	95 (39/41)
$P_{mec-7}ced-4C$	98 (39/40)	100 (41/41)
$P_{mec-7}ced-4D$	98 (40/41)	100 (42/42)
$P_{med-7}ced-3/4A$	97 (36/37)	100 (46/46)
$P_{mec-7}ced-3/4B$	98 (45/46)	97 (34/35)

The column headings identify the chromosomal genotypes of the strains examined.

sions leading to the formation of the *mec-7*-expressing neuron PVM (Chalfie 1993) in animals carrying trans-

genes $P_{mec-7}ced-3A$, $P_{mec-7}ced-3C$, or $P_{mec-7}ced-3/4A$ in a *ced-9(n2812); ced-3(n717)* background or transgene $P_{mec-7}ced-4A$ in a *ced-4(n1162) ced-9(n2812)* background. These divisions occur postembryonically and are thus easier to follow than the divisions leading to the formation of the ALMs. As illustrated in Figure 1A, presumptive PVM neurons in these lines showed the characteristic refractile appearance of programmed cell deaths viewed using Nomarski optics (17 animals and 12 deaths observed). These deaths were morphologically indistinguishable from normal programmed cell deaths.

We also analyzed the ultrastructure of a cell corpse resulting from a PVM cell death. Figure 1C shows a PVM cell that died and was engulfed by a neighboring cell. The darkly staining cytoplasm and nucleus, the small cytoplasmic volume, and the darkly staining matter within the nucleus are all characteristic features of *C. elegans* cells that normally die by programmed cell death (Robertson and Thomson 1982; Ellis et al. 1991b), suggesting that the deaths we observed were ultrastructurally similar to normal programmed cell deaths.

Figure 1. Cell death induced by overexpression of *ced-3* and *ced-4* resembles normal programmed cell death. (A) Nomarski photomicrograph of a dying PVM cell (arrow) in a *ced-9(n2812); ced-3(n717)* animal carrying the $P_{mec-7}ced-3/4A$ transgene (see text for details). Anterior is to the left; dorsal is on top. (B) Nomarski photomicrograph of dying VD5 (small arrow) and VD6 (large arrow) neurons in a *ced-9(n2812); ced-3(n717)* animal carrying the $P_{unc-30}ced-3/4$ transgene. Anterior is to the left; dorsal is on top. (C) Electron micrograph showing a dying PVM cell (arrow) located dorsolaterally posterior to the primordial gonad in an L1 animal. The cell was engulfed by a neighboring hypodermal cell (see text for details).

Figure 2. Possible genetic pathways for programmed cell death in *C. elegans*. Pointed arrows indicate positive regulation; blunt arrows indicate negative regulation. (A) *ced-4* either activates *ced-3* (top) or acts together with *ced-3* (bottom) to induce programmed cell death. (B) *ced-9* prevents cell killing by *ced-3* by acting at least in part via *ced-4*. We have no evidence for or against the hypothesis that *ced-9* also acts independently of *ced-4* to inhibit *ced-3*-mediated cell killing. This potential interaction is depicted with a broken line. See text for details.

To establish further that the deaths caused by overexpression of *ced-3* or *ced-4* were similar to programmed cell deaths, we examined first larval stage (L1) *ced-1(e1735); ced-4(n1162) ced-9(n2812)* animals containing the $P_{mec-7}ced-4A$ transgene for the presence of unengulfed ALM corpses. Animals carrying the *ced-1(e1735)* mutation fail to engulf many of the corpses that result from programmed cell death, causing these corpses to persist for hours or even days (Hedgecock et al. 1983). We observed corpses on 11 out of 40 sides near the BDU cell (the sister cell of the ALM), and we observed corpses in the normal location of the ALM on 2 out of 40 sides, suggesting that the ALMs often, but not always, died before migrating posteriorly. That 27/40 sides scored did not have observable corpses presumably reflects the incomplete penetrance of the *ced-1(e1735)* mutation (Ellis et al. 1991b). We obtained similar results using the $P_{mec-7}ced-3A$ transgene in a *ced-1(e1735); ced-3(n3002)* background (data not shown). These findings suggest that the gene *ced-1*, which is required for the engulfment of corpses generated by normal programmed cell death, is also required for the engulfment of the corpses generated by overexpression of *ced-3* or *ced-4*.

The gene *nuc-1* is required for the degradation of DNA of cells that die by programmed cell death (Sulston 1976; Hevelone and Hartman 1988). We examined the left sides of *ced-3(n3002); nuc-1(e1392)* animals containing the transgene $P_{mec-7}ced-3A$ and stained with the DNA stain 4,6-diamidino-2-phenylindole (DAPI). Near the po-

sition of the BDU cell, 7 of the 10 sides had DAPI-positive compact structures that did not correspond to visible nuclei when viewed using Nomarski optics and that looked like structures generated by programmed cell death in animals carrying the *nuc-1(e1392)* mutation. These findings suggest that the gene *nuc-1*, which is required for the degradation of the DNA of normally dying cells, is also required for the degradation of the DNA of cells dying because of the overexpression of *ced-3* or *ced-4*.

Programmed cell death caused by overexpression of ced-3 or ced-4 requires functional ced-3 and ced-4 products

To determine whether expression of a functional *ced-3* or *ced-4* cDNA was needed to kill cells in the experiments described above, we constructed and analyzed lines containing *ced-3* or *ced-4* transgenes with missense mutations. Because we did not need to score a given array in a number of different genetic backgrounds, we used extrachromosomal arrays [which are easier to generate than integrated arrays (Fire 1986; Way and Chalfie 1988)] for these experiments. As controls, we used wild-type *ced-3* and *ced-4* constructs maintained on extrachromosomal arrays. As shown in Table 4A, 100% of the ALMs survived in *ced-9(n2812); ced-3(n717)* animals carrying $P_{mec-7}ced-3$ fusion constructs containing a cysteine-to-alanine substitution at position 358 of the CED-3 protein. This mutation alters a residue that corresponds to a cysteine essential both for the protease activity of ICE (Cerretti et al. 1992; Thornberry et al. 1992) and for the protease activity of CED-3 (Xue and Horvitz 1995; D. Xue, S. Shaham, and H.R. Horvitz, unpubl.). In contrast, only ~50% of ALMs survived in animals containing the wild-type constructs. This result suggests that active CED-3 is needed for ALM cell death and supports the notion that CED-3 is a cysteine protease.

Similarly, 100% of the ALMs survived in *ced-4(n1162) ced-9(n2812)* animals carrying $P_{mec-7}ced-4$ fusions containing an isoleucine-to-asparagine substitution at position 258 of the CED-4 protein (Table 4B). This mutation introduces a change identical to that found in the mutant *ced-4* allele *n1948* (Yuan and Horvitz 1992). Only ~50% of ALMs survived in animals containing the wild-type *ced-4* constructs. This result suggests that active CED-4 is needed for ALM killing and confirms that the change observed in *n1948* animals is the cause of the *Ced-4* mutant phenotype. Thus, killing by overexpression of *ced-3* or *ced-4* requires transgenes that encode functional CED-3 or CED-4 proteins.

To determine whether the deaths of the ALM neurons were induced specifically by overexpression of the cell death proteins CED-3 and CED-4, we tested constructs that should have overexpressed other proteins (*Escherichia coli* β -galactosidase, murine ICE, and *C. elegans* NCC-1) under the control of the *mec-7* promoter. The presence of these constructs in *ced-9; ced-3* or *ced-4 ced-9* worms did not cause ALM cell death (data not

Table 4. Mutant *CED-3* or *CED-4* proteins fail to kill ALM neurons

	Percent surviving ALMs (no. ALMs/no. sides scored)
<i>ced-9; ced-3</i>	
A. ALM killing by <i>ced-3</i> overexpression requires a functional <i>CED-3</i> protein ^a	
<i>P_{mec-7}ced-3(+)-1</i>	42 (5/12)
<i>P_{mec-7}ced-3(+)-2</i>	31 (4/13)
<i>P_{mec-7}ced-3(+)-3</i>	60 (9/15)
<i>P_{mec-7}ced-3(C358A)-1</i>	100 (18/18)
<i>P_{mec-7}ced-3(C358A)-2</i>	100 (24/24)
<i>P_{mec-7}ced-3(C358A)-3</i>	100 (17/17)
<i>ced-4 ced-9</i>	
B. ALM killing by <i>ced-4</i> overexpression requires functional <i>CED-4</i> protein ^b	
<i>P_{mec-7}ced-4(+)-1</i>	50 (7/14)
<i>P_{mec-7}ced-4(+)-2</i>	56 (9/16)
<i>P_{mec-7}ced-4(+)-3</i>	43 (9/21)
<i>P_{mec-7}ced-4(I258N)-1</i>	100 (17/17)
<i>P_{mec-7}ced-4(I258N)-2</i>	100 (9/9)
<i>P_{mec-7}ced-4(I258N)-3</i>	100 (17/17)

^aEach row indicates an independent transgenic line of *ced-9(n2812); ced-3(n717)* animals carrying an extrachromosomal array of either a wild-type *ced-3* cDNA fusion to the *mec-7* promoter (+) or a *ced-3* cDNA encoding a C358A mutation in the *CED-3* protein.

^bEach row indicates an independent transgenic line of *ced-4(n1162) ced-9(n2812)* animals carrying an extrachromosomal array of either a wild-type *ced-4* cDNA fusion to the *mec-7* promoter (+) or a *ced-4* cDNA encoding an I258N mutation in the *CED-4* protein.

shown), suggesting that it is not simply excess or foreign protein that killed the ALMs in the experiments described above.

Efficient ectopic killing by overexpression of *ced-4* requires *ced-3* activity

To examine the requirement for endogenous *ced-3* in the killing of ALMs by *P_{mec-7}ced-4* transgenes, we introduced these transgenes into *ced-3* mutant strains. Table 5 shows that killing of ALMs in all four *P_{mec-7}ced-4* insertion lines was strongly inhibited by reduction-of-

function mutations in the endogenous *ced-3* gene by comparison with the level of killing in a wild-type *ced-3* background. For example, in line *P_{mec-7}ced-4A*, ALMs survived on 4 of the 39 (10%) sides scored in a wild-type background but survived on 27 of the 38 (71%) sides scored in a *ced-3* background. Similarly, in line *P_{mec-7}ced-4B*, ALMs survived on 0 of the 30 (0%) sides scored in a *ced-4 ced-9* background but survived on 27 of the 38 (71%) sides scored in a *ced-4 ced-9; ced-3* background. Interestingly, even though reducing *ced-9* function enhanced killing of the ALM neurons with respect to a wild-type background in the presence of a wild-type endogenous *ced-3* gene (*ced-4 ced-9* column vs. wild-type column), killing was greatly reduced in a strain containing mutations in both *ced-9* and *ced-3* (*ced-4 ced-9; ced-3* column). These results suggest that killing by *P_{mec-7}ced-4* is greatly facilitated by the presence of endogenous *ced-3* and that the need for *ced-3* function cannot be overcome by eliminating the function of *ced-9* using a mutation that is likely to have little if any *ced-9* function based on both genetic and molecular criteria (Hengartner and Horvitz 1994a; S. Shaham and H.R. Horvitz, unpubl.).

To determine if the protection against ALM killing by *P_{mec-7}ced-4* constructs observed in these experiments was specific to the *ced-3* allele (*n717*) used, we examined the abilities of other *ced-3* alleles to inhibit killing by *ced-4* overexpression. As shown in Table 6, all five other *ced-3* alleles tested also inhibited killing. Furthermore, the ability of a *ced-3* mutation to cause survival of cells that normally die correlated roughly with its ability to prevent killing of ALM neurons by *P_{mec-7}ced-4A*. Animals carrying the *ced-3* allele *n1040*, for example, contained an average of 7.6 extra cells in the anterior pharynx; these cells would normally have died in wild-type animals. ALMs survived on 35 of the 64 (55%) sides scored in line *P_{mec-7}ced-4A* containing the *n1040* mutation. However, animals harboring the *ced-3* allele *n2433* contained an average of 12.4 extra cells in the anterior pharynx, and ALMs survived on 33 of the 41 (80%) sides scored in line *P_{mec-7}ced-4A* containing this mutation. Although *n2433* is the most severe *ced-3* allele known (S. Shaham and H.R. Horvitz, unpubl.; M. Hengartner, pers. comm.), neither *n2433* nor any other *ced-3* allele characterized to date is unequivocally a null allele based upon molecular and genetic criteria (Yuan et al. 1993; S.

Table 5. ALM killing by *ced-4* overexpression is inhibited by a mutation in the endogenous *ced-3* gene

	Percent surviving ALMs (no. ALMs/no. sides scored)			
	wild type	<i>ced-3</i>	<i>ced-4 ced-9</i>	<i>ced-4 ced-9; ced-3</i>
<i>P_{mec-7}ced-4A</i>	10 (4/39)	71 (27/38)	0 (0/30)	71 (27/38)
<i>P_{mec-7}ced-4B</i>	87 (33/38)	100 (20/20)	53 (18/34)	84 (27/32)
<i>P_{mec-7}ced-4C</i>	98 (39/40)	100 (37/37)	42 (15/36)	98 (39/40)
<i>P_{mec-7}ced-4D</i>	98 (40/41)	100 (36/36)	15 (4/27)	100 (40/40)

The column headings identify the chromosomal genotypes of the strains examined. The alleles used were *ced-9(n2812)*, *ced-3(n717)*, and *ced-4(n1162)*.

Table 6. The activity of the endogenous *ced-3* allele determines the extent of ALM killing by *ced-4* overexpression

	Molecular defect	Number of extra cells in anterior of pharynx	Percent surviving ALMs (no. ALMs/no. sides scored)
Wild type	none	0.1 ± 0.4 (40)	10 (4/39)
<i>ced-3(n1040)</i>	L27F	7.6 ± 1.2 (11)	55 (35/64)
<i>ced-3(n1129)</i>	A449V	8.7 ± 1.2 (10)	67 (20/30)
<i>ced-3(n717)</i>	Exon 7 splice acceptor G to A	11.2 ± 2.0 (10)	71 (27/38)
<i>ced-3(n718)</i>	G65R	11.8 ± 1.0 (10)	73 (47/64)
<i>ced-3(n2433)</i>	G360S	12.4 ± 0.9 (10)	80 (33/41)

Rows indicate the genotype of the strain examined. The average number of extra cells in the anterior region of the pharynx in each mutant ± S.D. are shown; the number of animals examined is in parentheses. Fractions indicate the number of ALMs per number of sides scored in a *P_{mec-7}ced-4A* background in a given mutant line. The expected number of cell deaths in the anterior pharynx during normal development is 16.

Shaham and H.R. Horvitz, unpubl.). Thus, it is possible that the complete elimination of *ced-3* function would totally prevent killing by ectopic *ced-4* expression in this line and hence that ALM killing by a *ced-4* transgene absolutely requires *ced-3* function.

Ectopic killing by overexpression of *ced-3* does not require *ced-4* function

Similarly, to examine the requirement for endogenous *ced-4* in the killing of ALMs by *P_{mec-7}ced-3* transgenes, we introduced these transgenes into *ced-4* mutant strains. For these experiments we used the *ced-4* allele *n1162*, which is a nonsense mutation that should result in premature termination of the CED-4 protein at amino acid 79. No detectable *ced-4* RNA or protein is produced by this strain (Yuan and Horvitz 1992; S. Shaham and H.R. Horvitz unpubl.), and, thus, this allele is an excellent candidate for being a molecular null allele. Table 7, shows that ALM survival in *P_{mec-7}ced-3*-containing lines was not consistently greater in a *ced-4(n1162)* mutant background than in a wild-type background. For example, in line *P_{mec-7}ced-3B*, ALMs survived on 16 of the 38 (42%) sides scored in a wild-type background and on 18 of the 61 (30%) sides scored in a *ced-4(n1162)* background. These observations contrast with those presented above, which indicate that *ced-3* mutations reduced the killing efficiency of *P_{mec-7}ced-4* constructs. Together these findings suggest a basic asymmetry between the killing actions of *ced-3* and *ced-4*. Supporting this notion, whereas killing in line *P_{mec-7}ced-4A* was more efficient in a wild-type background than was kill-

ing in line *P_{mec-7}ced-3A*, killing in the former line was much weaker in a *ced-4 ced-9; ced-3* background. These observations suggest that killing by a *ced-4* construct is more dependent on endogenous *ced-3* than is killing by a *ced-3* construct dependent on endogenous *ced-4* and further support the notion that *ced-4* acts upstream of or in parallel to *ced-3*.

When we compared the extent of ALM survival in *P_{mec-7}ced-3*-containing lines in a *ced-9; ced-3* background with survival in a *ced-4 ced-9; ced-3* background, we noticed that the latter background caused reduced killing. As shown in Table 7, fewer ALMs survived in the *ced-9; ced-3* background than in the *ced-4 ced-9; ced-3* background in all *P_{mec-7}ced-3*-containing lines. In line *P_{mec-7}ced-3B*, for example, ALMs survived on 0 of the 37 (0%) sides scored in a *ced-9; ced-3* background but on 12 of the 32 (37%) sides scored in a *ced-4 ced-9; ced-3* background. This result suggests that in the absence of endogenous *ced-9*, *ced-4* promotes killing caused by *ced-3* overexpression (see Discussion).

Protection by *ced-9* against *ced-3*-induced killing is reduced by a mutation that decreases *ced-4* activity

As described above, endogenous *ced-9* function inhibited killing by overexpression of *ced-3* and *ced-4*. Specifically, ALM survival in *P_{mec-7}ced-3* and *P_{mec-7}ced-4* lines was greater in *ced-9(+)* strains than in strains containing a *ced-9* loss-of-function mutation (Table 2A). Does *ced-9* act to inhibit *ced-3* function, *ced-4* function, or both? To address this issue, we examined whether *ced-9* requires *ced-4* function to inhibit killing by *ced-3* overexpression.

Table 7. ALM killing by *ced-3* overexpression can occur in the absence of *ced-4* function

	Percent surviving ALMs (no. ALMs/no. sides scored)			
	wild type	<i>ced-4</i>	<i>ced-9; ced-3</i>	<i>ced-4 ced-9; ced-3</i>
<i>P_{mec-7}ced-3A</i>	20 (9/46)	43 (24/56)	0 (0/29)	27 (8/30)
<i>P_{mec-7}ced-3B</i>	42 (16/38)	30 (18/61)	0 (0/37)	38 (12/32)
<i>P_{mec-7}ced-3C</i>	100 (48/48)	90 (35/39)	21 (9/43)	85 (28/33)

The column headings identify the chromosomal genotypes of the strains examined. The alleles used were *ced-9(n2812)*, *ced-3(n717)*, and *ced-4(n1162)*, except for the line containing the transgene *P_{mec-7}ced-3A*, in which the *ced-3* allele *n3002* was used.

Table 8. Effects of mutations in *ced-3* and *ced-4* on *ced-9* protection against cell death

	Percent surviving ALMs (no. ALMs/no. sides scored)					
	<i>ced-3</i>	<i>ced-9; ced-3</i>	<i>ced-4</i>	<i>ced-4 ced-9</i>	<i>ced-4; ced-3</i>	<i>ced-4 ced-9; ced-3</i>
A. <i>ced-9</i> inhibition of ALM killing by <i>ced-3</i> overexpression is facilitated by <i>ced-4</i> ^a						
<i>P_{mec-7}ced-3A</i>	47 (16/34)	0 (0/29)	43 (34/56)	26 (10/39)	48 (13/27)	27 (8/30)
<i>P_{mec-7}ced-3B</i>	30 (8/27)	0 (0/37)	30 (18/61)	19 (9/47)	17 (5/29)	38 (12/32)
<i>P_{mec-7}ced-3C</i>	100 (34/34)	21 (9/43)	90 (35/39)	94 (73/78)	98 (40/41)	85 (28/33)
	<i>ced-4</i>	<i>ced-4 ced-9</i>	<i>ced-3</i>	<i>ced-9; ced-3</i>	<i>ced-4; ced-3</i>	<i>ced-4 ced-9; ced-3</i>
B. Effects of <i>ced-3</i> on <i>ced-9</i> inhibition of ALM killing by <i>ced-4</i> ^b						
<i>P_{mec-7}ced-4A</i>	43 (12/28)	0 (0/30)	71 (27/38)	69 (69/100)	80 (32/40)	71 (27/38)
<i>P_{mec-7}ced-4B</i>	94 (32/34)	53 (18/34)	100 (20/20)	97 (34/35)	98 (39/40)	84 (27/32)
<i>P_{mec-7}ced-4C</i>	97 (36/37)	42 (15/36)	100 (37/37)	98 (39/40)	97 (37/38)	98 (39/40)
<i>P_{mec-7}ced-4D</i>	100 (36/36)	15 (4/27)	100 (36/36)	100 (42/42)	N.D.	100 (40/40)

^aThe column headings identify the chromosomal genotypes of the strains examined. The alleles used were *ced-9(n2812)*, *ced-3(n717)*, and *ced-4(n1162)*, except for the line containing the transgene *P_{mec-7}ced-3A*, in which the *ced-3* allele *n3002* was used.

^bThe column headings identify the chromosomal genotypes of the strains examined. The alleles used were *ced-9(n2812)*, *ced-3(n717)*, and *ced-4(n1162)*. N.D. Not determined.

Table 8A shows that whereas for *P_{mec-7}ced-3*-containing *ced-4(+)* lines, ALMs survive better if *ced-9(+)* function is present, ALMs in *P_{mec-7}ced-3*-containing *ced-4(-)* lines survive to similar extents in *ced-9(+)* and *ced-9* mutant backgrounds. For example, in line *P_{mec-7}ced-3B*, ALMs survived on 5 of the 29 (17%) sides scored in a *ced-4; ced-3* background and on 12 of the 32 (37%) sides scored in a *ced-4 ced-9; ced-3* background; that is, *ced-9* function did not protect against killing by *ced-3*-overexpression in the absence of *ced-4* function. These results suggest that endogenous *ced-9* inhibits *ced-3* activity in *ced-3*-overexpression strains by acting at least in part via *ced-4*.

A similar analysis using *P_{mec-7}ced-4* transgenes is presented in Table 8B. However, because killing by a *P_{mec-7}ced-4* transgene in a *ced-3(-)* background was inefficient, we cannot assess whether *ced-3* might be required for protection from *ced-4* killing by *ced-9*.

Overexpression of *ced-9* can protect cells killed by overexpression of *ced-4*

Because endogenous *ced-9* could protect against cell death induced by *ced-4* overexpression, we tested whether overexpression of *ced-9* in *mec-7*-expressing cells would prevent the ectopic cell deaths caused by overexpression of *ced-4* in these cells. We examined the effect of a *P_{mec-7}ced-9* fusion (carried as an extrachromosomal array) on survival of ALMs in line *P_{mec-7}ced-4D* in a *ced-4 ced-9* background. We found that in lines transgenic for both *P_{mec-7}ced-4* and *P_{mec-7}ced-9*, more ALMs survived than in the absence of *P_{mec-7}ced-9* or in the presence of a *P_{mec-7}ced-9* construct containing a frame-shift mutation in the *ced-9* gene (see Table 9; Material and methods). These results suggest that overexpression of *ced-9* is sufficient to protect *mec-7*-expressing cells from killing by *ced-4* overexpression and demonstrate

that *ced-9* acts in a cell autonomous fashion to inhibit programmed cell death. We were not able to protect against killing by overexpression of *P_{mec-7}ced-3A* with a *P_{mec-7}ced-9* transgene to an extent greater than the protection conferred by the presence of endogenous *ced-9* (data not shown).

Overexpression of either *ced-3* or *ced-4* can kill DD neurons

To see whether overexpression of *ced-3* or *ced-4* could kill cells other than *mec-7*-expressing cells, we fused a *ced-3* or *ced-4* cDNA to the promoter for the *unc-30* gene, which is expressed in the VD and DD neurons as well as in a few other cells (Jin et al. 1994; Y. Jin, pers. comm.), and obtained lines containing integrated copies of either *P_{unc-30}ced-3* or *P_{unc-30}ced-4*. As Table 10 shows, we observed that *P_{unc-30}ced-3* and *P_{unc-30}ced-4* transgenes could kill DD neurons in *ced-9; ced-3* and *ced-4 ced-9* backgrounds, respectively.

We obtained one line containing integrated copies of both *P_{unc-30}ced-3* and *P_{unc-30}ced-4* (*P_{unc-30}ced-3/4A*). We observed DDs missing in this line as well in both *ced-9; ced-3* and *ced-4 ced-9* backgrounds (data not shown). To confirm that these cells were missing because they were dying by programmed cell death, we observed the pattern of cell divisions leading to the formation of 8 of the 13 VD neurons in the ventral cord of a transgenic animal carrying both *unc-30* fusion constructs. Figure 1B shows that two of these cells underwent a process morphologically similar to normal programmed cell death.

Table 10 also shows that an endogenous *ced-4* mutation inhibited killing by *P_{unc-30}ced-3* transgenes in *ced-9(-)* animals and that an endogenous *ced-3* mutation inhibited killing by *P_{unc-30}ced-4* transgenes in *ced-9(-)* animals. These results parallel our findings with the

Table 9. Overexpression of *ced-9* can protect from killing by overexpression of *ced-4*

	Percent of surviving ALMs (no. ALMs/no. sides scored)
	<i>ced-4 ced-9</i>
$P_{mec-7}ced-4D$	15 (4/27)
$P_{mec-7}ced-4D$; $P_{mec-7}ced-9-1$	70 (14/20)
$P_{mec-7}ced-4D$; $P_{mec-7}ced-9-2$	63 (17/27)
$P_{mec-7}ced-4D$; $P_{mec-7}ced-9-3$	83 (20/24)
$P_{mec-7}ced-4D$; $P_{mec-7}ced-9(lf)-1$	13 (3/24)
$P_{mec-7}ced-4D$; $P_{mec-7}ced-9(lf)-2$	5 (1/20)
$P_{mec-7}ced-4D$; $P_{mec-7}ced-9(lf)-3$	19 (4/21)

Each row indicates an independently derived transgenic line containing the integrated transgene $P_{mec-7}ced-4D$ and (except for row 1) an extrachromosomal transgene containing either a wild-type or a mutant *ced-9* cDNA. Each extrachromosomal array was established in a *ced-4(n1162) ced-9(n2812); nls45; lin-15(n765)* background. $P_{mec-7}ced-9$ arrays contained a wild-type *ced-9* cDNA. $P_{mec-7}ced-9(lf)$ arrays contained a frameshift mutation in an otherwise wild-type *ced-9* cDNA. Each array was coinjected with a plasmid containing the *lin-15* gene (see Materials and methods).

mec-7 promoter fusion constructs in a *ced-9(-)* background.

Unlike the *mec-7* promoter fusion constructs, none of our *unc-30* promoter fusion constructs showed extensive killing of DD neurons in a *ced-9(+)* background, making it impossible to assess if *ced-3* can bypass the requirement for *ced-4* in a *ced-9(+)* animal or if *ced-4* can bypass the requirement for *ced-3* in a *ced-9(+)* animal. That *ced-3* and *ced-4* transgenes failed to kill DD neurons in *ced-9(+)* animals might be a consequence of insufficient expression from the *unc-30* promoter (we never observed complete killing of DD neurons even in a *ced-9* mutant background). Alternatively, this difference might reflect a difference between *unc-30*- and *mec-7*-expressing cells.

Discussion

To study the roles of *ced-3*, *ced-4*, and *ced-9* in programmed cell death in *C. elegans*, we developed a method for assaying the effects of overexpressing these cell-death genes as transgenes in cells that are mutationally defective in specific endogenous cell-death activities. Specifically, we expressed cell-death genes as transgenes under the control of two cell type-specific promoters, the P_{mec-7} promoter, which causes gene expression within a set of mechanosensory neurons, including the ALMs (Savage et al. 1989), and the P_{unc-30} promoter, which causes gene expression in a different set of neurons, including the DDs (Jin et al. 1994). Expression of *ced-3* or *ced-4* killed both ALMs and DDs, suggesting that if overexpressed either gene is sufficient to activate the cell-death program in cells that normally live.

The system we have developed for assessing the effects on cell death and cell survival of specific transgenes

could be used for the analysis of any newly discovered *C. elegans* cell-death genes. Furthermore, because the mechanisms of programmed cell death seem likely to be conserved among organisms as diverse as nematodes, insects, and mammals (e.g., Horvitz et al. 1994), this system also should be useful for the analysis of cell-death genes from other organisms, such as those that encode the human Bcl-2 protein and the baculovirus p35 protein, both of which have been shown to inhibit programmed cell death in *C. elegans* (Vaux et al. 1992; Hengartner and Horvitz 1994a; Sugimoto et al. 1994; Xue and Horvitz 1995).

We suggest that expression of *ced-3* and *ced-4* under the control of other promoters could provide a useful method for specific cell ablation. Such a method would complement that of laser microsurgery (e.g., Sulston and White 1980; Avery and Horvitz 1987; Bargmann et al. 1993), which has been used extensively to define cell functions and reveal cell interactions in *C. elegans*. Whereas laser microsurgery can be used to kill any cell, relatively few cells and animals can be readily analyzed using this approach. The use of *ced-3* or *ced-4* transgenes for cell killing would allow many cells at many times of development to be killed and could generate sufficient numbers of animals lacking specific cells for biochemical studies or mutant hunts. A very strong promoter might allow such killing experiments to be performed using a wild-type genetic background. However, in general, an absence of *ced-9* function most likely would greatly facilitate killing, which leads us to suggest that most such experiments should be attempted using either a *ced-9*; *ced-3* or a *ced-4 ced-9* background.

Killing by overexpression of *ced-3* or *ced-4* is similar to normal programmed cell death

The ectopic cell deaths we observed in lines carrying either P_{mec-7} or P_{unc-30} fusion constructs to *ced-3* or *ced-4* were similar to programmed cell deaths by a number of criteria. First, ectopically dying cells had the same characteristic refractile appearance when viewed with Nomarski optics as normal programmed cell deaths. Second, the characteristic ultrastructural features of programmed cell death—darkly staining cytoplasm, reduced cytoplasmic volume, and darkly staining nuclear matter—were present in the ectopically dying cells. Third, mutations in a gene required for the engulfment of corpses resulting from normal programmed cell deaths prevented the engulfment of corpses from ectopic cell deaths, suggesting that the ectopic cell deaths resembled normal cell deaths. Fourth, a mutation that prevents the degradation of the DNA of cells that normally die prevented also the degradation of the DNA of ALM cells killed ectopically. Fifth, the extent of killing by *ced-3* or *ced-4* overexpression was influenced by endogenous mutations in genes known to affect normal programmed cell deaths (*ced-3*, *ced-4*, and *ced-9*), strongly suggesting that the molecular components responsible for the ectopic cell deaths correspond to those involved in normal programmed cell deaths.

Table 10. Overexpression of *ced-3* or *ced-4* can kill DD neurons

	Percent of surviving DDs (no. DDs scored/no. expected)			
	<i>ced-9; ced-3</i>	<i>ced-3</i>	<i>ced-4 ced-9</i>	<i>ced-4</i>
<i>P_{unc-30}ced-3A</i>	18 (11/60)	95 (38/40)	100 (88/88)	98 (39/40)
<i>P_{unc-30}ced-3B</i>	18 (7/40)	98 (39/40)	100 (40/40)	98 (39/40)
<i>P_{unc-30}ced-4A</i>	100 (40/40)	100 (40/40)	15 (6/40)	100 (40/40)
<i>P_{unc-30}ced-4B</i>	100 (40/40)	100 (40/40)	3 (1/40)	100 (40/40)

Each row indicates an independently derived transgenic line containing a given integrated transgene. Column headings identify the chromosomal genotypes of the strains examined. Fractions indicate the number of DD neurons scored and the number of expected DDs. The alleles used were *ced-9(n2812)*, *ced-3(n717)*, and *ced-4(n1162)*.

ced-3, ced-4, and ced-9 act cell autonomously

All of the ectopic deaths we observed were of cells known to express the promoter we used. No surrounding cells were ever observed to die. These results suggest strongly that killing by overexpression of either *ced-3* or *ced-4* is cell autonomous. Previously, genetic mosaic analyses demonstrated that wild-type copies of *ced-3* and *ced-4* were required in lineages generating cells that normally died to cause the deaths of those cells (Yuan and Horvitz 1990). These experiments, however, did not offer a cellular resolution capable of limiting the requirement for these genes to the dying cell itself. Our results demonstrate that *ced-3* and *ced-4* can act cell autonomously to cause cell death, strongly supporting the hypothesis that *ced-3* and *ced-4* normally do so. In addition, we have found that *ced-9* can act cell autonomously to prevent cell death, because overexpression of *ced-9* in *mec-7*-expressing cells rescued killing by overexpression of *ced-4* in the same cells.

The genetic pathway for programmed cell death in C. elegans

Killing by overexpression of *ced-3* did not require endogenous *ced-4* function, whereas killing by overexpression of *ced-4* was at least in part dependent on endogenous *ced-3* function. These results suggest either that *ced-4* acts upstream of *ced-3* and *ced-4* function can be bypassed by high levels of *ced-3* activity or that *ced-3* and *ced-4* act in parallel, with *ced-3* perhaps having a greater ability to kill (Fig. 2A). Our finding that *ced-4* appears to facilitate the inhibition of *ced-3* by *ced-9* suggests that *ced-9* acts to negatively regulate *ced-4*; our data do not indicate whether *ced-9* also acts to negatively regulate *ced-3* (Fig. 2B). It is conceivable that this proposed pathway applies to cells that die in our overexpression system but not to cells that normally die.

Interestingly, in the absence but not in the presence of *ced-9* function, ALM killing by overexpression of *ced-3* is potentiated by the presence of a functional *ced-4* gene. Why might *ced-4* function matter only if *ced-9* is inactive? One possible explanation based on the hypothesis that *ced-9* acts upstream of *ced-4* is that the ALMs are cells that normally live and, as such, presumably have active *ced-9* function. This *ced-9* activity might inhibit

any endogenous *ced-4* function, so that the presence or absence of a *ced-4(+)* allele would be irrelevant. However, if *ced-9* were inactivated by mutation, *ced-4* might become functional in the ALMs, thus potentiating killing by a *ced-3* transgene.

The behavior of *ced-4* in our experiments suggests a similarity between the action of the CED-4 protein and the action of Bcl-2-related proteins that induce apoptosis, such as Bax (Oltvai et al. 1993), Bak (Chittenden et al. 1995; Farrow et al. 1995; Kiefer et al. 1995), and Bad (Yang et al. 1995). Overexpression of Bax, for example, results in cell death, just as does overexpression of *ced-4*, and overexpression of *bcl-2* blocks this death (Oltvai et al. 1993) just as overexpression of *ced-9* blocks death caused by overexpression of *ced-4*. Similar interactions exist with Bak (Kiefer et al. 1995) and Bad (Yang et al. 1995). Although CED-4 and the Bcl-2 family do not have obvious sequence similarity, these proteins might similarly mediate signaling between a negative regulator of cell death (e.g., CED-9/Bcl-2) and a cysteine-protease activator of cell death (e.g., CED-3/ICE-like protease). Alternatively, because both *ced-9* and *ced-3* have mammalian counterparts that are involved in programmed cell death, it is possible that a protein similar in both structure and function to CED-4 exists in mammals.

ced-3, ced-4, and ced-9 might all normally be expressed in surviving cells

As discussed above, we found that ALM killing by a *ced-4* transgene was greatly reduced and possibly eliminated in animals that lack *ced-3* function, suggesting that *ced-4*-induced killing requires *ced-3* activity (see Tables 5 and 6). Because *ced-3* acts cell autonomously, this finding suggests that wild-type ALMs have *ced-3* function. Similarly, ALM killing by either a *ced-3* or a *ced-4* transgene was greater in animals that lacked *ced-9* function than in *ced-9(+)* animals (see Tables 2A,B), and *ced-9* can act cell autonomously. These findings suggest that wild-type ALMs have *ced-9* function. Finally, protection by *ced-9* against ALM killing by a *ced-3* transgene might be mediated by *ced-4* function (see Table 8A), and *ced-3*-induced ALM killing was reduced in animals lacking *ced-4* function in the absence of *ced-9* activity (see Table 8A). Because *ced-4* also acts cell auto-

mously, these findings suggest that wild-type ALMs might have *ced-4* function. In short, our observations are consistent with the hypothesis that surviving ALMs contain not only the protective function of *ced-9* but also the killing functions of *ced-3* and *ced-4*. Similar conclusions can be drawn for the DD neurons.

Presumably, the killing functions of *ced-3* and *ced-4* are inhibited in the ALMs and DDs directly or indirectly by the protective function of *ced-9*. Because overexpression of either *ced-3* or *ced-4* in the ALMs can overcome the protective function of *ced-9* (see Table 1), we propose that in these neurons, and perhaps more generally in all *C. elegans* cells, there is an antagonism between functions that activate (e.g., *ced-3* and *ced-4*) and functions that inhibit (e.g., *ced-9*) programmed cell death. Because *ced-3*, *ced-4*, and *ced-9* do not seem to affect each other's transcription (S. Shaham and H.R. Horvitz, unpubl.; M. Hengartner, pers. comm.; Yuan and Horvitz 1992; Yuan et al. 1993), it is likely that this antagonism is not at the transcriptional level but rather involves the activities of the proteins encoded by these genes. Cells might initiate programmed cell death either by reducing a protective activity or by increasing a killing activity. Interestingly, not all of our overexpression lines could overcome the protective effects of *ced-9* (see Table 1). This observation suggests that the dosage of the *ced-3* or the *ced-4* product in these lines was insufficient to overcome *ced-9* protection. Supporting this notion is our observation that lines heterozygous rather than homozygous for the insertions $P_{mec-7}ced-3B$, $P_{mec-7}ced-3A$, and $P_{mec-7}ced-4A$ showed little ALM death (S. Shaham and H.R. Horvitz, unpubl.). For example, ALMs survived on 23 of the 25 (92%) sides scored in animals heterozygous for the $P_{mec-7}ced-4A$ transgene in a wild-type background as opposed to 4 of the 39 (10%) sides in $P_{mec-7}ced-4A$ homozygotes, indicating that gene dosage is important for killing by either *ced-3* or *ced-4*.

Our hypothesis that surviving cells in *C. elegans* might contain antagonistic protective and killing cell-death functions is consistent with a number of findings from studies of programmed cell death in other organisms. For example, in many cases cells can be induced to undergo programmed cell death in the absence of macromolecular synthesis (for review, see Vaux and Weissman 1993), suggesting that the protein components needed for cell death are present in living cells. Similarly, many and perhaps all mammalian cells are protected by exogenous growth factors from dying by programmed cell death, which has led Raff (1992) to propose that all cells contain cell-death killing factors. Our findings provide direct evidence that at least some living cells are indeed poised for death in this way.

Materials and methods

General methods and strains

We cultured *C. elegans* as described by Brenner (1974). All strains were grown at 20°C. The wild-type strain used was *C. elegans* variety Bristol strain N2. Genetic nomenclature follows the standard *C. elegans* system (Horvitz et al. 1979). The mu-

tations used have been described by Ellis and Horvitz (1986), Hedgecock et al. (1983), Hengartner et al. (1992), and Sulston (1976) or were isolated by us. These mutations are as follows: LG I: *ced-1*(e1735); LG III: *ced-4*(n1162), *ced-9*(n2812, n1950, n1950 n2161), *unc-69*(e587); LG IV: *ced-3*(n717, n718, n1040, n1129, n2433, n3002); LG X: *nuc-1*(e1392), *lin-15*(n765); Allele designations for the integrated lines are as follows: $P_{mec-7}ced-3A$ is *nls50*, $P_{mec-7}ced-3B$ is *nls38*, $P_{mec-7}ced-3C$ is *nls33*, $P_{mec-7}ced-4A$ is *nls31*, $P_{mec-7}ced-4B$ is *nls44*, $P_{mec-7}ced-4C$ is *nls47*, $P_{mec-7}ced-4D$ is *nls45*, $P_{mec-7}ced-3/4A$ is *nls32*, $P_{mec-7}ced-3/4B$ is *nls29*, $P_{unc-30}ced-4A$ is *nls46*, and $P_{unc-30}ced-4B$ is *nls48*. The integrated *mec-7-lacZ* fusion construct is designated as allele *jels1* (J. Way, pers. comm.).

Plasmid constructions

$P_{mec-7}ced-3$: The vector pPD52.102 (A. Fire, M. Hamelin, and J. Culotti, pers. comm.) was digested with the restriction enzymes *NheI* and *EcoRV* and was ligated to an *SpeI-SmaI* fragment obtained from plasmid pS126, which contains the full-length *ced-3* cDNA. $P_{mec-7}ced-4$: The vector pPD52.102 was digested with the restriction enzymes *NheI* and *EcoRV* and was ligated to an *SpeI-SmaI* fragment obtained from plasmid pS125, which contains a full-length *ced-4* cDNA. $P_{unc-30}ced-3$: Plasmid pS126 was digested with the enzyme *SpeI*, dephosphorylated using calf intestinal alkaline phosphatase (New England Biolabs, Beverly, MA) and ligated to a 3.5-kb *XbaI* fragment that contains non-coding sequences upstream of the *unc-30* start codon sufficient to express a *lacZ* reporter gene in the VD and DD neurons of *C. elegans* (Jin et al. 1994; Y. Jin, pers. comm.). $P_{unc-30}ced-4$: Plasmid pS125 was digested with the enzyme *SpeI* as above and ligated to the same *XbaI* fragment used to construct $P_{unc-30}ced-3$. $P_{mec-7}ced-9$: The *ced-9* cDNA insert of plasmid B30 (Hengartner and Horvitz 1994b) was amplified using the polymerase chain reaction and cloned into the vector pPD52.102 using its *NheI* and *EcoRV* sites. $P_{mec-7}ced-9(lf)$: Plasmid $P_{mec-7}ced-9$ was digested with *BamHI*, the overhangs were filled with Klenow enzyme and religated to create a frameshift mutation (Hengartner and Horvitz 1994b). Plasmid pS172 was made as follows: We replaced the sequence TG at the codon encoding cysteine 358 of CED-3 with the sequence GC using an oligonucleotide-mediated site-directed mutagenesis kit and following the instructions of the manufacturer (Amersham, Arlington Heights, IL). An oligonucleotide that encodes the peptide QAARG (5'-CGTTTTTGTGCAGGCTGCTCGAGGCGAACGTCGT-3') was used to introduce the mutation, and plasmid pS126 was used as the template. The sequence of the entire mutated plasmid was determined to confirm that only the desired mutation was introduced. The resulting plasmid was then digested with the enzymes *SpeI* and *SmaI* and ligated to the plasmid pPD52.102 as described above to generate plasmid pS172. Plasmid pS178 (which contains a T-to-A substitution at the codon encoding isoleucine 258 of CED-4) was made as above except that an oligonucleotide encoding the peptide TNRWA (5'-GC-TCTGAGCCCAACGATTTGTTTCTTCTTGAAC-3') was used to introduce the mutation, and plasmid pS125 was used as the template.

Germ-line transformation and integration of extrachromosomal arrays

Our procedure for microinjection and germ-line transformation followed that of Fire (1986) and Mello et al. (1991). DNA for injections was purified using a Qiagen system and following the instructions of the manufacturer (Qiagen Inc., Chatsworth, CA). The concentrations of all plasmids used for injections were

between 50 and 100 $\mu\text{g/ml}$. All constructs were coinjected with the pRF4 plasmid, which contains the *rol-6(su1006)* allele as a dominant marker. Animals carrying the pRF4 plasmid exhibit a roller (Rol) phenotype. We transformed strains of genotype *ced-9(n2812); ced-3(n717)*, *ced-4(n1162) ced-9(n2812)*, or wild type. Approximately 30 animals were injected in each experiment, and 50–100 F1 Rol animals were picked onto separate plates. F1 animals segregating Rol progeny were established as lines containing extrachromosomal arrays (Way and Chalfie 1988). To assay the activity of $P_{mec-7}ced-9$ and $P_{mec-7}ced-9(lf)$, we injected animals of genotype *ced-4(n1162) ced-9(n2812); nIs45; lin-15(n765)* with each plasmid and with a plasmid containing the wild-type *lin-15* gene (Clark et al. 1994; X. Lu, pers. comm.). Approximately 40 F1 non-Lin-15 animals were obtained in each experiment, and lines transmitting the non-Lin-15 phenotype were established.

To obtain lines containing integrated copies of the $P_{mec-7}ced-3$, $P_{mec-7}ced-4$, $P_{unc-30}ced-3$, or $P_{unc-30}ced-4$ constructs, we exposed a plate of worms containing a given construct as an extrachromosomal array to γ rays or X rays at a dose of 4500 rads. Thirty to 50 fourth larval stage animals (L4s) were picked from the plate onto a separate plate and allowed to generate self progeny. F1 Rol progeny of the mutagenized animals were picked onto individual plates and allowed to generate self progeny. Six to eight Rol F2 animals were picked from each F1 plate and allowed to generate self progeny. F2 plates containing 100% Rol animals were maintained as integrated lines. The integration event was confirmed by a cross with wild-type animals. Putative heterozygote animals from these crosses were allowed to generate self progeny and were shown to segregate homozygous Rol animals at a frequency of approximately one in three animals picked. These results also showed that all our integrated lines had a dominant Rol phenotype. All integrated strains we obtained were backcrossed at least twice either to N2 or to another strain when appropriate. Overall, we screened $\sim 30,000$ F2 animals to obtain three independent $P_{mec-7}ced-3$ integrants, 30,000 F2 animals to obtain four independent $P_{mec-7}ced-4$ integrants, 10,000 F2 animals to obtain two independent $P_{unc-30}ced-3$ integrants, 10,000 F2 animals to obtain two independent $P_{unc-30}ced-4$ integrants, 5000 F2 animals to obtain two independent integrants containing both the $P_{mec-7}ced-3$ and $P_{mec-7}ced-4$ constructs, and 1000 F2 animals to obtain one integrant containing both $P_{unc-30}ced-3$ and $P_{unc-30}ced-4$ constructs. None of the integrated lines used in our experiments exhibited any obvious abnormality besides the Rol, Mec, or Unc-30 phenotypes.

Assays for ALM and DD cell death

ALM cell death was assayed by scoring transgenic animals for the presence of ALM nuclei as follows: approximately 40 early L1 animals were mounted onto a drop of 50 mM NaN_3 in M9 buffer (Sulston and Hodgkin 1988) on a slide containing a pad of 5% agar in water and were covered with a coverslip. Animals were then observed using Nomarski optics (Sulston and Horvitz 1977). The Rol phenotype conferred by the pRF4 plasmid is not expressed in L1 larvae, which thus are easier to score for the presence or absence of the ALMs. The left side of animals was scored for the presence of an ALML nucleus, and the right side of the animals was scored for the presence of the ALMR nucleus. After scoring L1s for the presence or absence of ALMs, we allowed them to mature; only Rol animals or animals segregating Rol progeny were included in our data.

DD cell death was assayed as follows; Young L1 animals (at a stage prior to the migration of the P-cell nuclei) from an integrated line were scored using Nomarski optics for the presence

of 15 neuronal nuclei located between the retrovesicular ganglion and the pre-anal ganglion. Four of these 15 nuclei are DD nuclei (Sulston and Horvitz 1977), some of which were missing in strains containing $P_{unc-30}ced-3$ or $P_{unc-30}ced-4$ constructs.

We also directly observed the deaths of PVM neurons in *ced-9(n2812); ced-3(n717) P_{mec-7}ced-3/4A* and in *ced-4(n1162) ced-9(n2812); P_{mec-7}ced-4A* animals by following the QL cell lineage (Sulston and Horvitz 1977) in living larvae ($n = 12$). Occasionally, we also saw the sister of the PVM neuron, SDQL, undergo programmed cell death. The *mec-7* promoter is known to be expressed weakly in this cell (M. Chalfie, pers. comm.), supporting our hypothesis that the level of overexpression of *ced-3* or *ced-4* is important for the penetrance of cell killing. We also observed directly the deaths of the VD neurons in one *ced-9(n2812); ced-3(n717); P_{unc-30}ced-3/4A* animal by following the P5–P12 cell lineages in that animal.

Acknowledgments

S.S. would like to dedicate this paper to the memory of his father, Prof. Jacob Shaham. We thank Ewa Davison, Michael Koelle, Mark Metzstein, Beth Sawin, and Gillian Stanfield for helpful comments about the manuscript, Erika Hartwig for help with the electron microscopy, Yishi Jin for sharing unpublished results and Jeff Way for the allele *jeIs1*. S.S. was supported by a William Keck Foundation fellowship and a fellowship from the Glaxo Research Institute. H.R.H. is an Investigator of the Howard Hughes Medical Institute.

The publication costs of this article were defrayed in part by payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 USC section 1734 solely to indicate this fact.

References

- Arends, M.J. and A.H. Wyllie. 1991. Apoptosis: Mechanisms and roles in pathology. *Int. Rev. Exp. Path.* **32**: 223–254.
- Avery, L. and H.R. Horvitz. 1989. A cell that dies during wild-type *C. elegans* development can function as a neuron in a *ced-3* mutant. *Cell* **51**: 1071–1078.
- Bargmann, C.I., E. Hartwig, and H.R. Horvitz. 1993. Odorant-selective genes and neurons mediate olfaction in *C. elegans*. *Cell* **74**: 515–527.
- Barres, B.A., R. Schmid, M. Sendtner, and M.C. Raff. 1993. Multiple extracellular signals are required for long-term oligodendrocyte survival. *Development* **118**: 283–295.
- Brenner, S. 1974. The genetics of *Caenorhabditis elegans*. *Genetics* **77**: 71–94.
- Cerretti, D.P., C.J. Kozlosky, B. Mosley, N. Nelson, K.V. Ness, T.A. Greenstreet, C.J. March, S.R. Kronheim, T. Druck, L.A. Cannizzaro, K. Huebner, and R.A. Black. 1992. Molecular cloning of the interleukin-1 β converting enzyme. *Science* **256**: 97–100.
- Chalfie, M. 1993. Touch receptor development and function in *Caenorhabditis elegans*. *J. Neurobiol.* **24**: 1433–1441.
- Chittenden, T., E.A. Harrington, R. O'Connor, C. Flemington, R.J. Lutz, G.I. Evan, and B.C. Guild. 1995. Induction of apoptosis by the Bcl-2 homologue Bak. *Nature* **374**: 733–736.
- Clark, S.G., X. Lu, and H.R. Horvitz. 1994. The *Caenorhabditis elegans* locus *lin-15*, a negative regulator of a tyrosine kinase signaling pathway, encodes two different proteins. *Genetics* **137**: 987–997.
- Cohen, J.J. and R.C. Duke. 1984. Glucocorticoid activation of a calcium-dependent endonuclease in thymocyte nuclei leads to cell death. *J. Immunol.* **32**: 38–42.

- Ellis, H.M. and H.R. Horvitz. 1986. Genetic control of programmed cell death in the nematode *C. elegans*. *Cell* **44**: 817–829.
- Ellis, R.E. and H.R. Horvitz. 1991. Two *C. elegans* genes control the programmed cell deaths of specific cells in the pharynx. *Development* **112**: 591–603.
- Ellis, R.E., J. Yuan, and H.R. Horvitz. 1991a. Mechanisms and functions of cell death. *Annu. Rev. Cell Biol.* **7**: 663–698.
- Ellis, R.E., D. Jacobson, and H.R. Horvitz. 1991b. Genes required for the engulfment of cell corpses during programmed cell death in *Caenorhabditis elegans*. *Genetics* **129**: 79–94.
- Farrow, S.N., J.H. White, I. Martinou, T. Raven, K.T. Pun, C.J. Grinham, J.C. Martinou, and R. Brown. 1995. Cloning of a *bcl-2* homologue by interaction with adenovirus E1B 19K. *Nature* **374**: 731–733.
- Fernandes-Alnemri, T., G. Litwack, and E.S. Alnemri. 1994. CPP32, a novel human apoptotic protein with homology to *Caenorhabditis elegans* cell death protein Ced-3 and mammalian interleukin-1 beta-converting enzyme. *J. Biol. Chem.* **269**: 30761–30764.
- Fire, A. 1986. Integrative transformation of *Caenorhabditis elegans*. *EMBO J.* **5**: 2673–2680.
- Gagliardini, V., P. Fernandez, R.K.K. Lee, H.C.A. Drexler, R.J. Rotello, M.C. Fishman, and J. Yuan. 1994. Prevention of vertebrate neuronal death by the *crmA* gene. *Science* **263**: 826–828.
- Garcia, I., I. Martinou, Y. Tsujimoto, and J.C. Martinou. 1992. Prevention of programmed cell death of sympathetic neurons by the *bcl-2* proto-oncogene. *Science* **258**: 302–304.
- Glücksman, A. 1950. Cell deaths in normal vertebrate ontogeny. *Biol. Rev. Cambridge Philos. Soc.* **26**: 59–86.
- Hedgecock, E., J.E. Sulston, and N. Thomson. 1983. Mutations affecting programmed cell deaths in the nematode *Caenorhabditis elegans*. *Science* **220**: 1277–1280.
- Hengartner, M.O., R.E. Ellis, and H.R. Horvitz. 1992. *Caenorhabditis elegans* gene *ced-9* protects cells from programmed cell death. *Nature* **356**: 494–499.
- Hengartner, M.O. and H.R. Horvitz. 1994a. *C. elegans* cell survival gene *ced-9* encodes a functional homolog of the mammalian proto-oncogene *bcl-2*. *Cell* **76**: 665–676.
- . 1994b. Activation of *C. elegans* cell death protein CED-9 by an amino-acid substitution in a domain conserved in *Bcl-2*. *Nature* **369**: 318–320.
- Hevelone, J. and P.S. Hartman. 1988. An endonuclease from *Caenorhabditis elegans*: Partial purification and characterization. *Biochem. Genet.* **26**: 447–461.
- Hockenbery, D.M., M. Zutter, W. Hickey, M. Nahm, and S.J. Korsmeyer. 1991. BCL2 protein is topographically restricted in tissues characterized by apoptotic cell death. *Proc. Natl. Acad. Sci.* **88**: 6961–6965.
- Horvitz, H.R., S. Brenner, J. Hodgkin, and R.K. Herman. 1979. A uniform genetic nomenclature for the nematode *Caenorhabditis elegans*. *Mol. & Gen. Genet.* **175**: 129–133.
- Horvitz, H.R., S. Shaham, and M.O. Hengartner. 1994. The genetics of programmed cell death in the nematode *Caenorhabditis elegans*. *Cold Spring Harbor Symp. Quant. Biol.* **59**: 377–385.
- Jin, Y., R. Hoskins, and H.R. Horvitz. 1994. Control of type-D GABAergic neuron differentiation by *C. elegans* UNC-30 homeodomain protein. *Nature* **372**: 780–783.
- Kiefer, M.C., M.J. Brauer, V.C. Powers, J.J. Wu, S.R. Umansky, L.D. Tomei, and P.J. Barr. 1995. Modulation of apoptosis by the widely distributed *Bcl-2* homologue *Bak*. *Nature* **374**: 736–739.
- Kimble, J. and D. Hirsh. 1979. The postembryonic cell lineages of the hermaphrodite and male gonads in *Caenorhabditis elegans*. *Dev. Biol.* **70**: 396–417.
- Kuida, K., J.A. Lippke, G. Ku, M.W. Harding, D.J. Livingston, M.S. Su, and R.A. Flavell. 1995. Altered cytokine export and apoptosis in mice deficient in interleukin-1 beta converting enzyme. *Science* **267**: 2000–2003.
- Kumar, S., M. Kinoshita, M. Noda, N.G. Copeland, and N.A. Jenkins. 1994. Induction of apoptosis by the mouse *Nedd-2* gene, which encodes a protein similar to the product of the *Caenorhabditis elegans* cell death gene *ced-3* and the mammalian IL-1 β -converting enzyme. *Genes & Dev.* **8**: 1613–1626.
- Martin, D.P., R.E. Schmidt, P.S. Distefano, O.H. Lowry, J.G. Carter, and E.M. Johnson Jr. 1988. Inhibitors of protein synthesis and RNA synthesis prevent neuronal death caused by nerve growth factor deprivation. *J. Cell Biol.* **106**: 829–844.
- Mello, C.C., J.M. Kramer, D. Stinchcomb, and V. Ambros. 1991. Efficient gene transfer in *C. elegans*: Extrachromosomal maintenance and integration of transforming sequences. *EMBO J.* **10**: 3959–3970.
- Miura, M., H. Zhu, R. Rotello, E.A. Hartwig, and J. Yuan. 1993. Induction of apoptosis in fibroblasts by IL-1 beta-converting enzyme, a mammalian homolog of the *C. elegans* cell death gene *ced-3*. *Cell* **75**: 653–660.
- Nunez, G., L. London, D. Hockenbery, M. Alexander, J.P. McKeen, and S.J. Korsmeyer. 1990. Deregulated *Bcl-2* gene expression selectively prolongs survival of growth factor-deprived hematopoietic cells lines. *J. Immun.* **144**: 3602–3610.
- Oltvai, Z.N., C.L. Millman, and S.J. Korsmeyer. 1993. *Bcl-2* heterodimerizes in vivo with a conserved homolog, *Bax*, that accelerates programmed cell death. *Cell* **74**: 609–619.
- Raff, M.C. 1992. Social controls on cell survival and death: An extreme view. *Nature* **356**: 397–400.
- Ray, C.A., R.A. Black, S.R. Kronheim, T.A. Greenstreet, P.R. Sleath, G.S. Salvesen, and D.J. Pickup. 1992. Viral inhibition of inflammation: Cowpox virus encodes an inhibitor of the interleukin-1 β converting enzyme. *Cell* **69**: 597–604.
- Robertson, A.M.G. and J.N. Thomson. 1982. Morphology of programmed cell death in the ventral nerve cord of *Caenorhabditis elegans* larvae. *J. Embryol. Exp. Morph.* **67**: 89–100.
- Savage, C., M. Hamelin, J.G. Cullotti, A. Coulson, D.G. Albertson, and M. Chalfie. 1989. *mec-7* is a beta tubulin gene required for the production of 15 protofilament microtubules in *Caenorhabditis elegans*. *Genes & Dev.* **3**: 870–881.
- Sentman, C.L., J.R. Shutter, D. Hockenbery, O. Kanagawa, and S.J. Korsmeyer. 1992. *Bcl-2* inhibits multiple forms of apoptosis but not negative selection in thymocytes. *Cell* **67**: 879–888.
- Stanisic, T., R. Sadlowski, C. Lee, and J.T. Grayhack. 1978. Partial inhibition of castration induced ventral prostate regression with actinomycin D and cyclohexamide. *Invest. Urol.* **16**: 19–22.
- Strasser, A., A.W. Harris, and S. Cory. 1992. *bcl-2* transgene inhibits T cell death and perturbs thymic self-censorship. *Cell* **67**: 889–899.
- Sugimoto, A., P.D. Friesen, and J.H. Rothman. 1994. Baculovirus p35 prevents developmentally programmed cell death and rescues a *ced-9* mutant in the nematode *Caenorhabditis elegans*. *EMBO J.* **13**: 2023–2028.
- Sulston, J.E. 1976. The ventral cord of *Caenorhabditis elegans*. *Phil. Trans. R. Soc. Lond. B* **275**: 287.
- Sulston, J.E. and H.R. Horvitz. 1977. Post-embryonic cell lineages of the nematode *Caenorhabditis elegans*. *Dev. Biol.* **82**: 110–156.
- Sulston, J.E. and J.G. White. 1980. Regulation and cell auton-

- omy during postembryonic development of *Caenorhabditis elegans*. *Dev. Biol.* **78**: 577–597.
- Sulston, J. and J. Hodgkin. 1988. Methods. In *The nematode Caenorhabditis elegans* (ed. W.B. Wood and the community of *C. elegans* researchers), pp. 491–584. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
- Sulston, J.E., E. Schierenberg, J.G. White, and N. Thomson. 1983. The embryonic cell lineage of the nematode *Caenorhabditis elegans*. *Dev. Biol.* **100**: 64–119.
- Thornberry, N.A., H.G. Bull, J.R. Calaycay, K.T. Chapman, A.D. Howard, M.J. Kostura, D.K. Miller, S.M. Molineaux, J.R. Weidner, J. Aunins, K.O. Elliston, J.M. Ayala, F.J. Casano, J. Chin, J.F. Ding, L.A. Egger, E.P. Gaffney, G. Limjoco, O.C. Palyha, S.M. Raju, A.M. Rolando, J.P. Salley, T. Yamin, T.D. Lee, J.E. Shively, M.M. MacCross, R.A. Mumford, J.A. Schmidt, and M.J. Tocci. 1992. A novel heterodimeric cysteine protease is required for interleukin-1 β processing in monocytes. *Nature* **356**: 768–774.
- Vaux, D.L. 1993. Toward an understanding of the molecular mechanisms of physiological cell death. *Proc. Natl. Acad. Sci.* **90**: 786–789.
- Vaux, D.L. and I.L. Weissman. 1993. Neither macromolecular synthesis nor myc is required for cell death via the mechanism that can be controlled by Bcl-2. *Mol. Cell. Biol.* **13**: 7000–7005.
- Vaux, D.L., S. Cory, and J.M. Adams. 1988. Bcl-2 gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. *Nature* **335**: 440–442.
- Vaux, D.L., I.L. Weissman, and S.K. Kim. 1992. Prevention of programmed cell death in *Caenorhabditis elegans* by human bcl-2. *Science* **258**: 1955–1957.
- Wang, L., M. Miura, L. Bergeron, H. Zhu, and J. Yuan. 1994. *Ich-1*, an *Ice/ced-3*-related gene, encodes both positive and negative regulators of programmed cell death. *Cell* **78**: 739–750.
- Way, J.C. and M. Chalfie. 1988. *mec-3*, a homeobox-containing gene that specifies differentiation of the touch receptor neurons in *C. elegans*. *Cell* **54**: 5–16.
- Williams, G.T. and C.A. Smith. 1993. Molecular regulation of apoptosis: Genetic controls on cell death. *Cell* **74**: 777–779.
- Xue, D. and H.R. Horvitz. 1995. Inhibition of the *Caenorhabditis elegans* cell-death protease CED-3 by a CED-3 cleavage site in baculovirus p35 protein. *Nature* **377**: 248–251.
- Yang, E., J. Zha, J. Jockel, L.H. Boise, C.B. Thompson, and S.J. Korsmeyer. 1995. Bad, a heterodimeric partner for Bcl-XL and Bcl-2, displaces Bax and promotes cell death. *Cell* **80**: 285–291.
- Yuan, J. and H. R. Horvitz. 1990. Genetic mosaic analyses of *ced-3* and *ced-4*, two genes that control programmed cell death in the nematode *C. elegans*. *Dev. Biol.* **138**: 33–41.
- . 1992. The *Caenorhabditis elegans* cell death gene *ced-4* encodes a novel protein and is expressed during the period of extensive programmed cell death. *Development* **116**: 309–320.
- Yuan, J., S. Shaham, S. Ledoux, H.M. Ellis, and H.R. Horvitz. 1993. The *C. elegans* cell death gene *ced-3* encodes a protein similar to mammalian interleukin-1 β -converting enzyme. *Cell* **75**: 641–652.

Developing *Caenorhabditis elegans* neurons may contain both cell-death protective and killer activities.

S Shaham and H R Horvitz

Genes Dev. 1996, **10**:

Access the most recent version at doi:[10.1101/gad.10.5.578](https://doi.org/10.1101/gad.10.5.578)

References

This article cites 63 articles, 21 of which can be accessed free at:
<http://genesdev.cshlp.org/content/10/5/578.full.html#ref-list-1>

License

Email Alerting Service

Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article or [click here](#).