Genetic and Molecular Studies of Programmed Cell Death in the Nematode *Caenorhabditis elegans*

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Abstract

This thesis describes a genetic and molecular study of programmed cell death in the nematode *Caenorhabditis elegans*. Programmed cell death is a process common to all metazoans examined, and plays an important role in development and disease. Work on *C. elegans* has demonstrated that in this organism programmed cell death also constitutes a major cell fate. Morphologically, programmed cell deaths in *C. elegans* are similar to programmed cell deaths found in other organisms, including mammals. These similarities can be demonstrated both on the light microscope and the electron microscope levels, and have suggested that the underlying molecular mechanisms of programmed cell death are similar between worm and man.

To characterize the molecular components of cell death in *C. elegans* we have analyzed the cell death gene *ced-3*. Mutations in *ced-3* abolish all programmed cell deaths, suggesting that this gene is necessary for the proper execution of the death program. We have shown that this gene encodes a cysteine protease of the Interleukin-1β converting enzyme (ICE) class and together *ced-3* and ICE define a novel family of cysteine proteases. ICE and subsequently identified family members have been shown to induce cell death in mammals. We have characterized the phenotypes of *ced-3* mutants and determined the mutated sites in these mutants. This study has revealed that sites crucial for *ced-3* function, are conserved with other members of this protease family, and suggest that *ced-3* has cell death activities independent of its proteolytic activity. These experiments are described in chapters 1 and 4.

The cell death gene *ced-4* is necessary for the proper execution of programmed cell death, and the cell death gene *ced-9* is required to negatively regulate the activities of *ced-3* and *ced-4*. Here we present a possible pathway for the action of these three genes in which we propose that *ced-9* acts to negatively regulate *ced-4*, which, in turn, can act to activate *ced-3*. These experiments also suggest that all three genes act in a cell-autonomous fashion and that the activity of these genes is likely to be present in many cells throughout the animal, including cells that do not die. These experiments are described in chapter 2.

Finally, we have demonstrated that the gene *ced-4* can encode two alternative transcripts which have opposite effects. The *ced-4*S transcript can kill cells, and the *ced-4*L transcript can protect cells from death. We present both genetic and molecular evidence that the activity of both transcripts is important *in vivo*. We also suggest that the *ced-9* gene acts to negatively regulate both *ced-4*S and *ced-4*L, so that *ced-9* can have both killing and protecting activities by inhibiting either *ced-4*L or *ced-4*S respectively. These experiments are described in chapter 3 and together with chapter 2 suggest a network of post-translational regulatory interactions which control cell death in *C. elegans*. Because of the molecular similarities between cell death components in mammals and in *C. elegans* we suggest that a similar regulatory network exists in mammals as well.

Thesis Supervisor: Dr. H. Robert Horvitz

עבודה זו מוקדשת לזכרו של אבי היקר פרופ' יעקב שחם

This Work is dedicated to the memory of my dear father Prof. Jacob Shaham

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Chapter 1

Programmed cell death in *Caenorhabditis elegans*

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Abstract

Studies of the nematode *Caenorhabditis elegans* have given us insights into the process of programmed cell death in mammals. Both morphological and molecular similarities exist between programmed cell death in worm and man. Recent work has established a proposed genetic pathway for programmed cell death in worms. In this review we summarize what is known of the functions of the genes *ced-3*, *ced-4* and *ced-9*, which are key regulators of cell death in *C. elegans*. We also address the genetic interactions of these genes and discuss the applicability of these results to the study of mammalian apoptosis. Further analysis of cell death in the nematode is sure to reveal additional evolutionarily conserved functions required for programmed cell death.

Programmed cell death is a fundamental process in the development of metazoan organisms, as well as in disease processes in humans[1,2]. Work on the nematode *Caenorhabditis elegans* has revealed that programmed cell death in this small soil nematode is similar both morphologically and molecularly to the mammalian cell death process termed apoptosis[2,3]. Cell death in *C. elegans* is a common cell fate-- 131 of the 1090 cells born (>12%) in the hermaphrodite undergo programmed cell death after birth[3,4]-- and occurs in a variety of cell types. Work from several laboratories has established a genetic pathway for programmed cell death that correlates with observable changes in the dying cells[3]. A set of genes required for the specification of cell death in a small number of cells has been identified. These genes[6,7] (*ces-1*, *ces-2*, and *egl-1*) are thought to be responsible for determining if the generalized cell death machinery will be turned on in a small subset of cells or not. Three genes[9,10], *ced-3*, *ced-4* and *ced-9*, are key components in the generalized cell death process which is common to all cells in *C. elegans*. Six genes[8], *ced-1*, *ced-2*, *ced-5*, *ced-6*, *ced-7*, and *ced-10*, are required for the proper degradation of the corpses of cells which have died, and a single gene[12,13], *nuc-1*, is required for the degradation of the DNA of dead cells. In this review we focus on the generalized cell death machinery encoded in part by the genes *ced-3*, *ced-4* and *ced-9*. Recent experiments have suggested that these genes can be arranged in a genetic pathway illustrated in figure 1 (S. Shaham and H. R. Horvitz, manuscript in preparation). The evidence leading to this pathway, and the extent to which we can apply information about the molecular nature and genetic interactions of these genes to mammals will be discussed as well.

ced-3 is similar to the mammalian enzyme Interleukin-1β converting enzyme

Mutations in the gene *ced-3* prevent programmed cell deaths in *C. elegans*, suggesting that this gene is necessary for the proper execution of cell death[9]. Over 50 mutations have been currently analyzed both genetically and molecularly (S. Shaham, B. Davies, and H. R. Horvitz, unpublished data). Most are partial loss-of-function, and none are good candidates for complete loss-of-function mutations, so that we do not know the phenotype of animals carrying a *ced-3* null mutation. Mosaic analysis of the *ced-3* gene demonstrated that the activity of this gene within a lineage leading to a cell death is required for that cell death to occur[14], suggesting that *ced-3* might act within dying cells. Recent data (S. Shaham and H. R. Horvitz, manuscript in preparation) has shown that overexpression of *ced-3* in cells that normally live using cell-specific promoters for the genes *mec-7* (expressed in mechanosensory neurons), and *unc-30* (expressed in a group of motorneurons) fused to a *ced-3* cDNA can cause these cells to die, supporting the notion that *ced-3* acts in a cell-autonomous fashion to regulate cell

death. The product encoded by the *ced-3* gene is similar to the family of mammalian proteins represented by the cysteine protease Interleukin-1β converting enzyme (ICE)[11]. ICE is a cysteine protease needed for the processing of the cytokine IL-1β from its pro-form to a mature form[15,16]. Recent experiments have shown that overexpression of ICE[17] and other family members (ICH-1[18]/NEDD-2[19], CPP32[20], TX[21]) in culture can cause cell death, and that a knockout of ICE in mice results in defects in cell death mediated by the Fas receptor[22], suggesting that ICE family members have a role in mammalian cell death similar to that of *ced-3* in *C. elegans*.

***ced-4* encodes death-preventing and death-promoting transcripts**

The *ced-4* gene encodes two novel proteins, CED-4L (for CED-4Long) and CED-4S (for CED-4Short) which are produced by alternative splicing (ref. 42; S. Shaham and H. R. Horvitz, manuscript in preparation). The *ced-4*L transcript encodes a protein containing a 24 amino-acid insertion relative to CED-4S. Overexpression of CED-4S in cells that normally live using the cell-specific promoters for the genes *mec-7* and *unc-30* (see above) can kill these cells, suggesting that CED-4S acts in a cell-autonomous fashion to induce programmed cell death. Mosaic analysis of *ced-4* also suggests that the killing activity of this gene is required within the lineages of cells that die[14]. Interestingly, overexpression of the CED-4L transcript using a heatshock promoter which is ubiquitously expressed will prevent normally-occuring cell deaths (S. Shaham and H. R. Horvitz, manuscript in preparation), suggesting that this protein normally acts to prevent cell death.

Loss-of-function mutations in the *ced-4* gene which appear to be null by both genetic[9] and molecular[42] criteria eliminate both *ced-4*S and *ced-4*L transcripts and result in the absence of programmed cell death, suggesting that *ced-4* is crucial for the proper execution of the death program.

A partial loss-of-function mutation, *n2273*, in the *ced-4* gene acts genetically as if a death-protecting aspect of *ced-4* activity has been disrupted (S. Shaham and H. R. Horvitz, manuscript in preparation). Animals with this mutation also have a weak defect in *ced-4*'s killing activity. The mutation has been shown to reside in the splice site leading to the selective production of the *ced-4*S product[42] and results in the production of mutant *ced-4*S and *ced-4*L transcripts (S. Shaham and H. R. Horvitz, manuscript in preparation). These observations suggest the tempting conclusion that the genetically-defined protecting and killing aspects of *ced-4* correlate with the alternate transcripts produced by the gene, however, this assertion has not yet been rigorously demonstrated.

CED-9 is a member of the BCL2 family of proteins

Loss-of-function (lf) mutations in the *ced-9* gene which appear to represent null alleles by both genetic and molecular criteria result in a maternal effect lethality[10,23]. Animals heterozygous for a *ced-9*(lf) allele give rise to live homozygous *ced-9*(lf) animals which in turn produce only dead embryos. These embryos usually arrest early during development for an unknown reason. However, a few embryos that develop longer, as well as embryos which have been derived from animals carrying a weaker loss-of-function mutation show massive programmed cell death, suggesting that *ced-9* normally acts to prevent programmed cell deaths in cells that normally survive. Why embryos carrying strong *ced-9*(lf) alleles arrest early with no apparent cell deaths is not yet understood.

Overexpression of *ced-9* can prevent programmed cell death. This result has been demonstrated using gene fusions to a ubiquitously expressed heatshock promoter and showing that cells that normally die can survive[23,24], and using gene fusions to the *mec-7* promoter to rescue the ectopic deaths induced by overexpression of the CED-4S protein (S. Shaham and H. R. Horvitz, manuscript in preparation). Thus, CED-9 is capable of protecting cells that die normally, as well as cells that die ectopically.

The CED-9 protein is similar in sequence to the BCL2 family of proteins and contains the BH1 and BH2[25] interaction domains conserved among members of this family[23]. BCL2 family members have been implicated in both the positive and negative regulation of programmed cell death[26-32]. The *bcl-2* gene has been shown to prevent programmed cell death in a number of mammalian systems[30-32] and in *C. elegans*[23,24]. A knockout of *bcl-2* in mice results in excess cell death in the immune system[33]. Furthermore, *bcl-2* can functionally substitute for *ced-9* in worms, suggesting that these two proteins act in a similar manner[23]. Oncogenic forms of *bcl-2* result in overexpression of the wild-type protein in B cells and lead to increased cell survival[30-32,34]. This result is similar to the prevention of cell death by overexpressing the *ced-9* gene. However, *ced-9* and *bcl-2* are different in at least one respect. A unique gain-of-function missense mutation[35] in the BH1 domain of *ced-9* is capable of preventing cell death in *C. elegans* but does not seem to result in overexpression of wild-type *ced-9*. When this mutation was introduced into *bcl-2* not only did it not enhance its activity, but completely abolished its death-preventing capacity[25]. The possible nature of this mutation is discussed below, however it suggests that *ced-9* and *bcl-2* can act differently.

ced-3* and *ced-4*S act genetically downstream of *ced-9

A fundamental approach to understanding how *ced-3*, *ced-4* and *ced-9* function is to assess possible interactions among them. A number of interaction studies suggest

that these genes interact in a pathway illustrated in figure 1. Two sets of experiments suggest that *ced-3* and *ced-4* act genetically downstream of *ced-9*. First, mutations in the *ced-3* or *ced-4* genes can act to prevent the lethality and ectopic cell deaths associated with loss-of-function mutations in *ced-9*[10] (figure 2). Second, ectopic cell death induced by overexpression of *ced-3* or *ced-4* in cells that normally survive using the *mec-7* or *unc-30* promoters can be enhanced by introducing a chromosomal *ced-9*(lf) mutation into transgenic animals carrying the overexpression constructs (S. Shaham and H. R. Horvitz, manuscript in preparation; figure 2). These results suggests that *ced-9* is normally required to prevent the killing activities of *ced-3* and *ced-4*, and places *ced-9* upstream of these genes. Biochemically, this interpretation means that *ced-9* could act to directly inhibit the activities of *ced-3* and *ced-4*, or to inhibit processes which result from the activities of *ced-3* and *ced-4*[3].

ced-9 protection from _ced-3_-killing requires _ced-4_

Killing of mechanosensory neurons using $P_{mec\text{-}7}$*ced-3* transgenes can be enhanced by loss-of-function mutations in the endogenous *ced-9* gene in transgenic animals as described above. Interestingly, mutating *ced-9* only seems to have an effect when a chromosomally wild-type *ced-4* gene is present (S. Shaham and H. R. Horvitz, manuscript in preparation; figure 3). Killing by the $P_{mec\text{-}7}$*ced-3* transgenes does not get enhanced if null mutations in both *ced-9* and *ced-4* are introduced in the endogenous genes. This observation suggests that *ced-4* is required to transduce a protecting signal from *ced-9*. It is likely that this transduction is mediated by *ced-4*S (see below) and not *ced-4*L, since null alleles of *ced-4* result in the prevention of cell death[6], suggesting that the killing activity of *ced-4* has been disrupted. However, the activity of *ced-4*L in this transgenic system has not been assessed. These observations suggest that *ced-4*S acts genetically between *ced-9* and *ced-3*, although other possibilities exist, and those are discussed below. Also, it is not clear if *ced-3* is required to mediate *ced-9* protection of *ced-4*-induced killing in this system, a possibility which if true would require additional interactions in the pathway presented in figure 1.

ced-3 might act genetically downstream of _ced-4_

Overexpression of a $P_{mec\text{-}7}$*ced-3* transgene resulted in similar killing of mechanosensory neurons regardless of the state of the endogenous *ced-4* gene. Thus, killing was similar in animals carrying a chromosomally wild-type *ced-4* gene and animals carrying a *ced-4* null mutation. However, killing by overexpression of a $P_{mec\text{-}7}$*ced-4*S trangene was greatly reduced if a mutation in the endogenous *ced-3* gene was introduced (S. Shaham and H. R. Horvitz, manuscript in preparation). None of the *ced-3*

mutations, including a mutation which results in the most severe cell death phenotype, was capable of completely preventing $P_{mec\text{-}7}$*ced-4*S-induced killing. These results can be interpreted in a number of ways. If the strongest *ced-3* mutation used represents a null allele, then *ced-4*S must have the ability to bypass *ced-3* and must be able to act both upstream and in parallel to *ced-3*, conveying the reduction, but not elimination of cell-killing by the endogenous *ced-3* mutation, respectively. If the strongest *ced-3* mutation used is not a null, then the above possibility still holds, but it is also possible that a strictly linear genetic pathway exists, and that the reduced, yet significant killing results from residual *ced-3* activity. In any case, it is clear that in either model a linear component in which *ced-4* acts genetically upstream of *ced-3* must exist and is thus drawn in figure 1.

Many of the above conclusions are based on ectopic killing of mechanosensory neurons and thus, might not be generally applicable to all cells in *C. elegans*. Further work using other cell-specific promoters should help in generalizing the interactions of *ced-3*, *ced-4*S and *ced-9* to other cells.

_ced-4_L might be negatively regulated by _ced-9_

As described above, overexpression of *ced-4*L can prevent normally occuring programmed cell deaths (S. Shaham and H. R. Horvitz, manuscript in preparation). Interestingly, ubiquitous overexpression of *ced-4*L can also prevent the lethality associated with animals carrying *ced-9*(lf) mutations (S. Shaham and H. R. Horvitz, manuscript in preparation; figure 4). This observation suggests that *ced-4*L can bypass wild-type *ced-9* activity, and suggests that *ced-4*L acts genetically downstream or parallel to *ced-9*. Experiments using the *n2273* mutation of *ced-4* which has been postulated to be defective primarily in *ced-4*L function (see above) suggest that *ced-9* acts to negatively regulate the activity of *ced-4*L (S. Shaham and H. R. Horvitz, manuscript in preparation). This model relies on the assumption that the two opposing genetic functions uncovered by *n2273* represent the actions of the two *ced-4* transcripts and represent the simplest presentation of the data.

ced-9 negatively regulates both _ced-4_L and _ced-4_S

Experiments described above suggest that *ced-9* acts to negatively regulate both death-preventing (*ced-4*L) and death-causing (*ced-4*S) functions. That overexpression of *ced-9* in mechanosensory neurons can protect from killing induced by *ced-4*S overexpression in the same cells, in the presence of a complete null allele of *ced-4* suggests that *ced-9* negatively regulates *ced-4*S in parallel to negatively regulating *ced-4*L (S. Shaham and H. R. Horvitz, manuscript in preparation). This experiment suggests

that *ced-9*(lf) mutations could cause either protection or enhancement of cell death. Recently both of these observations have been demonstrated[35], supporting the pathway displayed in figure 1. This experiment also demonstrated that *ced-9* can act in a cell-autonomous fashion. Figure 1 represents *ced-4*L blocking the activity of the earliest known cell death causing component *ced-4*S. However, the genetic evidence suggests that *ced-4*L could be acting at any place downstream of *ced-4*S.

Could the *ced-9*(gf) mutation be a loss-of function mutation?

Introducing the change present in the *ced-9*(gf) mutation into *bcl-2* completely abolished the ability of *bcl-2* to protect from programmed cell death, and to interact with *bax*[26], another family member which can induce cell death[25]. The model elaborated in the previous sections suggests a mode for *ced-9*(gf) action which would reconcile the molecular and functional similarity of *ced-9* and *bcl-2*, with their dissimilar response to a given mutation. If *ced-9*(gf) abolished *ced-9*'s ability to inhibit *ced-4*L but maintained the interaction with *ced-4*S, then enhanced cell survival would be predicted in *ced-9*(gf) mutants (as is seen). This model suggests that the *ced-9*(gf) actually causes a loss of interaction with *ced-4*L, consistent with the *bcl-2* results, however, other models are certainly possible.

***C. elegans* can be used to understand general principles of cell death**

Can the work on programmed cell death in worms be applied to the study of mammalian cell death? A number of similarities exist between cell death in worms and in mammals. Both processes are morphologically very similar, showing cell shrinkage, nuclear condensation, and darkening of the cytoplasm. Some of the molecules involved in cell death in both systems are similar in both sequence and function- *ced-9* is similar to *bcl-2* and related family members, *ced-3* is similar to ICE and other family members. The pathway in *C. elegans* suggests that *ced-9* inhibits the activity of *ced-3*. Work in mammalian culture has demonstrated that *bcl-2* can prevent killing induced by ICE or ICH-1/NEDD2 overexpression[17,18], consistent with a pathway in which *bcl-2* negatively regulates ICE-like proteins just as *ced-9* regulates *ced-3*. These similarities in aspects of the cell death pathways suggest that the broader pathway in worms might exist in mammals as well.

Work in *C. elegans* has also suggested that the activities of *ced-3*, *ced-4* and *ced-9* are likely to be present in many if not all cells in a post-transcriptional state (S. Shaham and H. R. Horvitz, manuscript in preparation). This observations parallels experiments showing that anucleated mammalian cells can still die by apoptosis[36]. The latter experiment has led Raff[37] to propose that all mammalian cells are post-transcriptionally

primed to die. The *C. elegans* experiments suggest a modification of this hypothesis suggesting that cells in metazoans can regulate the decision to die in a post-transcriptional manner by interactions of death-promoting and death-preventing components.

In addition to post-transcriptionally regulating cell death, other modes of regulating cell death in *C. elegans* and mammals are similar. In both systems genes causing or protecting from cell death have to be expressed- suggesting transcriptional control. In addition, alternative splicing yielding oppositely acting products has been shown for *ced-4*, and for the human genes *Ich-1*[18] and *bcl-x*[27], suggesting that splicing is a key level of control in both these organisms.

Exploring apparent dissimilarities between worms and mammals might reveal the extent to which worms can be used to model the mammalian process. Although work in mammals has revealed multiple family members for both *bcl-2* and ICE, only one member of each family has so far been described in worms. The existence or non-existence of other family members in worms will, in part, determine if the *C. elegans* system is as complex as the mammalian one.

A number of issues remain concerning the similarity between *bcl-2* and *ced-9*. Only *ced-9*(lf) homozygotes derived from *ced-9*(lf) parents are embryonically lethal. For the *bcl-2* knockout example only *bcl-2*(lf) animals derived from heterozygous parents have been described[33]. It is possible that if these animals are bred, dead embryos would be produced as well. Also, *ced-9* seems to genetically interact with both positive and negative regulators of cell death and has a function in both protecting and killing. The current model for *bcl-2* suggests that it only has a protective function. Could *bcl-2* have a yet undetected killing function? Will a closer observation of *bcl-2*(lf) animals reveal excess cell survival? The answers to these questions will help to assess the similarity between *ced-9* and *bcl-2*.

A key unanswered question is whether a mammalian homologue of *ced-4* exists. Currently no candidates have been demonstrated, however it might be possible that only functional *ced-4* homologues exist which share little sequence similarity with *ced-4*.

Finally, one of the most important questions for the understanding of disease involves the nature and mechanisms of upstream signals that trigger the generalized cell death machinery. In mammals genes like Fas[38], p53[39], myc[40], nur77[41] and others have been described. In worms a number of upstream "specification" genes have been described genetically[6,7]. The molecular characterization of these genes should reveal if they have known mammalian counterparts.

In conclusion, continued analysis of programmed cell death in *C. elegans* is likely to yield a wealth of information about cell death processes in general, and should help

facilitate our understanding of mammalian cell death.

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Figure 1. A model for the regulation of programmed cell death in *C. elegans*.

The *ced-9* gene product can inhibit the activity of either *ced-4*S or *ced-4*L gene products. *ced-3* activity can be enhanced by CED-4S or inhibited by CED-4L, although we can not rule out a model in which CED-4L inhibits CED-4S, thus indirectly inhibiting CED-3, or a model in which CED-4L inhibits a target downstream of CED-3. See text for additional details.

Figure 1

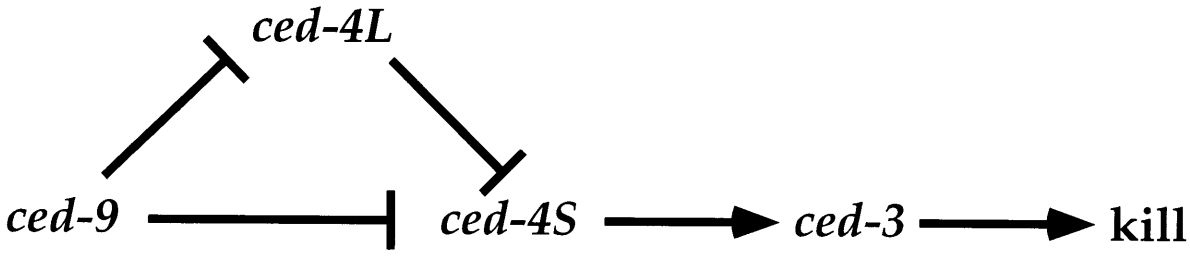


Figure 2. Loss-of-function mutations in *ced-3* and *ced-4* inhibit programmed cell death, whereas loss-of-function mutations in *ced-9*, and overexpression of *ced-3* and *ced-4* cause cell death.

Rows indicate a given genotype, lf, loss-of-function, o/e, overexpression. Model is based on figure 1. X represents loss of activity. Thicker arrows indicate higher activity. +, cell death, -, no cell death.

Figure 2

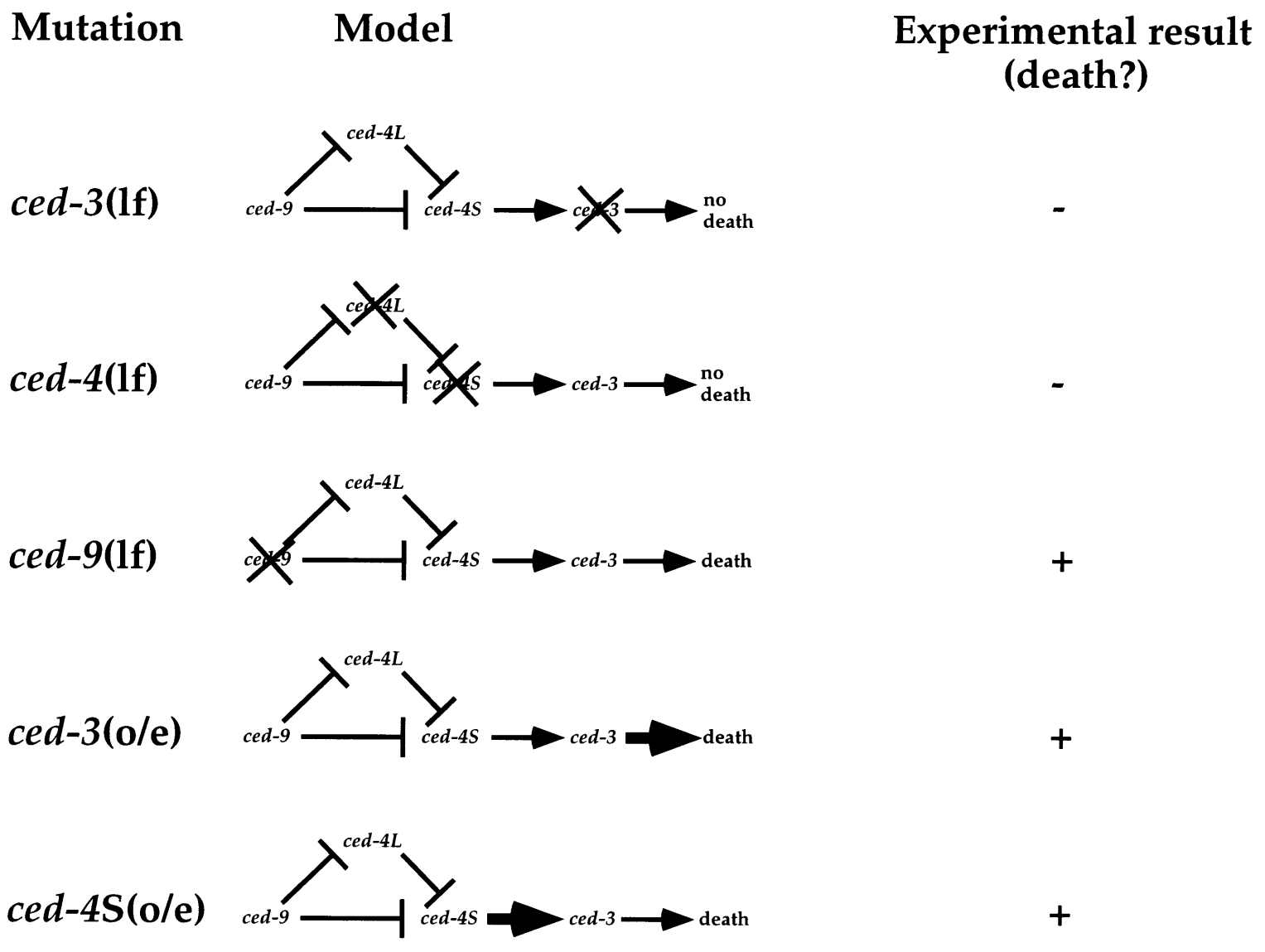

Mutation
Model
Experimental result (death?)
ced-3(lf)
ced-4L
ced-9
ced-4S
no death
-
ced-4(lf)
ced-4L
ced-9
ced-3
no death
-
ced-9(lf)
ced-4L
ced-4S
ced-3
death
+
ced-3(o/e)
ced-4L
ced-9
ced-4S
ced-3
death
+
ced-4S(o/e)
ced-4L
ced-9
ced-4S
ced-3
death
+

Figure 3. *ced-9* requires *ced-4* activity to protect from *ced-3*-induced death.

Rows indicate a given genotype, lf, loss-of-function, o/e, overexpression. Model is based on figure 1. X represents loss of activity. Thicker arrows indicate higher activity. +, cell death, ++, enhanced cell death.

Figure 3

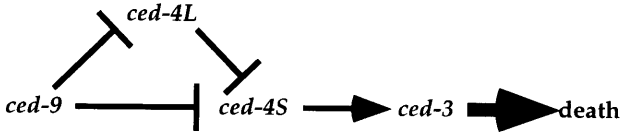
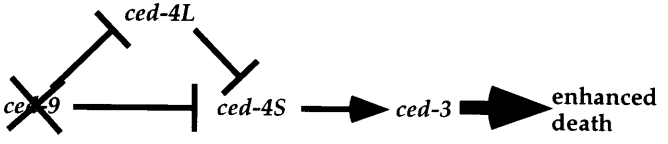
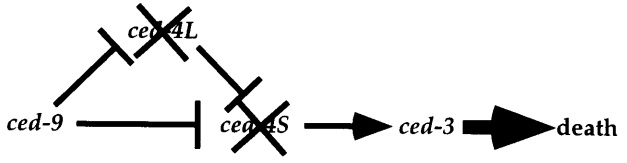
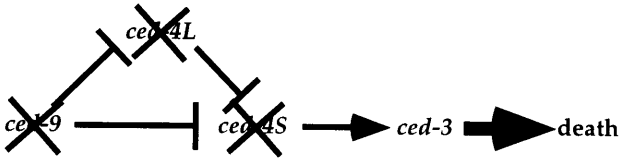
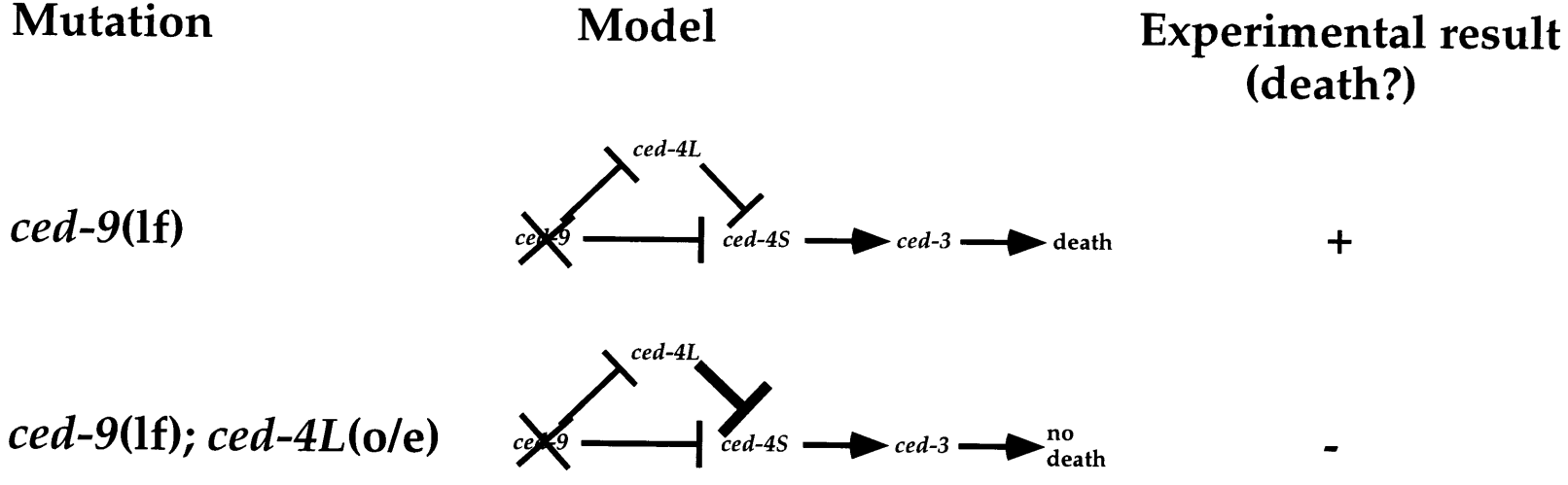
Mutation	Model	Experimental result (death?)
ced-3(o/e)		+
ced-9(lf); *ced-3*(o/e)		++
ced-4(lf); *ced-3*(o/e)		+
ced-4(lf) *ced-9*(lf); *ced-3*(o/e)		+

Figure 4. *ced-4*L can prevent cell death induced by *ced-9*(lf) mutations.

Rows indicate a given genotype, lf, loss-of-function, o/e, overexpression. Model is based on figure 1. X represents loss of activity. Thicker arrows indicate higher activity. +, cell death, -, no cell death.

Figure 4


Mutation
Model
Experimental result (death?)
ced-9(lf)
ced-4L
ced-9
ced-4S
ced-3
death
+
ced-9(lf); ced-4L(o/e)
ced-4L
ced-9
ced-4S
ced-3
no death
-

Chapter 2

The C. elegans cell death gene *ced-3* encodes a protein similar to mammalian interleukin-1β converting enzyme

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My contribution to this paper was the genomic sequence, frameshift mutation to define the open reading frame, definition of *ced-3*'s 5' end, sequencing of mutant alleles, and showing that CED-3 is similar to ICE and NEDD2.

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Summary

We have cloned the C. elegans cell death gene *ced-3*. A *ced-3* transcript is most abundant during embryogenesis, the stage during which most programmed cell deaths occur. The predicted CED-3 protein shows similarity to human and murine interleukin-1β converting enzyme and to the product of the mouse *nedd-2* gene, which is expressed in the embryonic brain. The sequences of 12 *ced-3* mutations as well as the sequences of *ced-3* genes from two related nematode species identify sites of potential functional importance. We propose that the CED-3 protein acts as a cysteine protease in the initiation of programmed cell death in C. elegans and that cysteine proteases also function in programmed cell death in mammals.

Introduction

Cell death occurs as a normal aspect of animal development as well as in tissue homeostasis and aging (Glucksman, 1950; Ellis et al., 1991a). Naturally occurring or programmed cell death can act to regulate cell number, to facilitate morphogenesis, to remove harmful or otherwise abnormal cells, and to eliminate cells that have already performed their functions. In many cases, gene expression within dying cells is thought to be required for these cells to die, since the cell death process can be blocked by inhibitors of RNA and protein synthesis (Stanisic et al., 1978; Cohen and Duke, 1984; Martin et al., 1988).

During the development of the nematode Caenorhabditis elegans, 131 cells undergo programmed cell death (Sulston and Horvitz, 1977; Sulston et al., 1983). Fourteen genes have been identified that function in different steps of the genetic pathway of programmed cell death in C. elegans (Hedgecock et al., 1983; Ellis and Horvitz, 1986; Ellis and Horvitz, 1991; Ellis et al., 1991b; Hengartner et al., 1992; reviewed by Ellis et al., 1991a). Two of these genes, *ced-3* and *ced-4*, play essential roles in either the initiation or execution of the cell death program, since recessive mutations in these genes prevent almost all of the cell deaths that normally occur during C. elegans development. Genetic mosaic analysis indicates that *ced-3* and *ced-4* most likely function within cells that die or within their close relatives to cause cell death (Yuan and Horvitz, 1990). The *ced-4* gene encodes a novel protein that is expressed primarily during embryogenesis, the period during which most programmed cell deaths occur (Yuan and Horvitz, 1992).

To understand how the *ced-3* gene acts to cause cell death, we have cloned this gene. As deduced from the sequence of a *ced-3* cDNA clone, the CED-3 protein is 503 amino acids in length and contains a serine-rich middle region of about 100 amino acids. We compared the sequences of the CED-3 protein of C. elegans with the inferred CED-3 protein sequences from the related nematode species C. briggsae and C. vulgaris. This comparison revealed that the carboxy-terminal portions of these proteins are most conserved. The non-serine-rich portions of the CED-3 protein are similar to human interleukin-1β (IL-1β) converting enzyme (ICE), a cysteine protease that can cleave the inactive 31 kD precursor of IL-lβ to generate the active cytokine (Cerretti et al., 1992; Thornberry et al., 1992). In addition, the C-terminal portions of both the CED-3 and ICE proteins are similar to the mouse Nedd-2 protein, which is encoded by an mRNA expressed during mouse embryonic brain development and is down-regulated in adult brain (Kumar et al., 1992). We suggest that CED-3 acts as a cysteine protease in

controlling the onset of programmed cell death in C. elegans and that members of the *ced-3*/ICE/nedd-2 gene family might function in programmed cell death in vertebrates.

Results

ced-3 is not essential for viability

All previously described *ced-3* alleles were isolated in screens designed to detect viable mutants in which programmed cell death did not occur (Ellis and Horvitz, 1986). Such screens might systematically have missed classes of *ced-3* mutations that result in inviability. Since animals of the genotype *ced-3/deficiency* are viable (Ellis and Horvitz, 1986), we designed a screen that would allow us to isolate recessive lethal alleles of *ced-3*, if such alleles could exist (see Experimental Procedures). We obtained four new *ced-3* alleles (*n1163*, *n1164*, *n1165*, *n1286*) in this way. All four of these new mutants are viable as homozygotes. These new alleles were isolated at a frequency of about 1 in 2500 mutagenized haploid genomes, approximately the frequency expected for the generation of loss-of-function mutations in an average C. elegans gene (Brenner, 1974; Meneely and Herman, 1979; Greenwald and Horvitz, 1980).

These observations suggest that animals that lack *ced-3* gene activity are viable. Supporting this hypothesis, we have shown by molecular analysis that three *ced-3* mutations are nonsense mutations that seem likely to eliminate *ced-3* activity (see below). Based upon these considerations, we conclude that *ced-3* gene activity is not essential for viability.

ced-3 is contained within a 7.5 kb genomic fragment

To clone the *ced-3* gene we used the approach of Ruvkun et al. (1988). Briefly (for further details see Experimental Procedures), we identified a 5.1 kb *Eco*RI restriction fragment that contained Tc1 (Emmons et al., 1983), that was present in the C. elegans Bristol strain N2 but not in the C. elegans Bergerac strain EM1002 and that was closely linked to *ced-3*. We named this restriction fragment length polymorphism (RFLP) *nP35*. Using Tc1 to probe Southern blots of cosmids derived from N2 genomic DNA and known to contain Tc1 (G. Ruvkun, personal communication), we identified two cosmids (MMM-C1 and MMM-C9) that contained this 5.1 kb *Eco*RI fragment. These cosmids overlapped an existing cosmid contig that had been defined as part of the C. elegans genome project (Coulson et al., 1986, 1988, and personal communication). We used cosmids from this contig to identify four additional Bristol-Bergerac RFLPs (*nP33*, *nP34*, *nP36*, *nP37*). By mapping these RFLPs between the Bristol and Bergerac strains with respect to the genes *unc-30*, *ced-3* and *unc-26*, we oriented the contig with respect to the

genetic map. These experiments narrowed the region containing the *ced-3* gene to an interval spanned by the three cosmids C48D1, W07H6 and C43C9 (Fig. 1a).

These three cosmids were microinjected (Fire, 1986; J. Sulston, personal communication) into *ced-3* mutant animals to test for rescue of the mutant phenotype. Specifically, a candidate cosmid and cosmid C14G10, which contains the wild-type *unc-31* gene (R. Hoskins, personal communication), were coinjected into *ced-1(e1735); unc-31(e928) ced-3(n717)* hermaphrodites, and non-Unc progeny were isolated and observed to see if the non-Unc phenotype was transmitted to the next generation, thus establishing a line of transgenic animals. Young first larval stage (L1) progeny of such transgenic lines were examined for the presence of cell deaths using Nomarski optics to see whether the Ced-3 phenotype was rescued. Cosmid C14G10 alone does not confer wild-type *ced-3* activity when injected into a *ced-3* mutant (data not shown). *ced-1* was used to facilitate scoring of the Ced-3 phenotype (see Experimental Procedures), and *unc-31* was used as a marker for co-transformation (R. Hoskins, personal communication; Kim and Horvitz, 1990).

As indicated in Fig. 1a, of the three cosmids tested, only C48D1 rescued the Ced-3 mutant phenotype. Two non-Unc transgenic lines were obtained, the insertion line *nIs1* and the extrachromosomal line *nEx2*. Both were rescued. Specifically, L1 *ced-1* animals contain an average of 23 cell corpses in the head, and L1 *ced-1; ced-3* animals contain an average of 0.3 cell corpses in the head (Ellis and Horvitz, 1986). By contrast, L1 *ced-1; unc-31 ced-3; nIs1* and L1 *ced-1; unc-31 ced-3; nEx2* animals contained an average of 16.4 (n=20) and 14.5 (n=20) cell corpses in the head, respectively. From these results, we concluded that C48D1 contains the *ced-3* gene.

To locate *ced-3* more precisely within cosmid C48D1, we subcloned this cosmid and tested the subclones for their abilities to rescue the Ced-3 mutant phenotype (Fig. 1b). From these experiments, we localized *ced-3* to a DNA fragment of 7.5 kb (pJ7.5).

ced-3 is transcribed primarily during embryogenesis and independently of *ced-4* function

We used the 7.6 kb pJ107 subclone of C48D1 (Fig. 1b) to probe a northern blot of polyA$^+$ RNA derived from the wild-type C. elegans strain N2. This probe hybridized to a 2.8 kb transcript (data not shown; also see Fig. 2). Although this transcript was present in 11 different EMS-induced *ced-3* mutant strains (data not shown; the mutant *n1164* was not tested), subsequent analysis revealed that all 11 mutants contain mutations in the genomic DNA that encodes this mRNA (see below), thus establishing this RNA as a *ced-3* transcript. The *ced-3* transcript was most abundant during

embryogenesis, when most programmed cell deaths occur (Sulston and Horvitz, 1977; Sulston et al., 1983) and was also detected at later stages (Fig. 2).

Since *ced-3* and *ced-4* are both required for programmed cell death in C. elegans, and since both are highly expressed during embryonic development (Yuan and Horvitz, 1992; see above), it is possible that one of these genes regulates mRNA levels of the other. Previous studies showed that *ced-3* does not regulate *ced-4* mRNA levels (Yuan and Horvitz, 1992). To determine if *ced-4* regulates *ced-3* mRNA levels, we probed a northern blot of RNA prepared from *ced-4* mutant embryos with the *ced-3* cDNA subclone pJ118. The abundance and size of *ced-3* transcript was normal in the *ced-4* mutants *n1162* (glutamine to ochre nonsense mutation at codon 40), *n1416* (Tc4 insertion into exon 5), *n1894* (tryptophan to opal nonsense mutation at codon 401) and *n1920* (G to A mutation in the intron 3 splice donor site)(Figure 3a). None of the *ced-4* mutants tested has detectable *ced-4* RNA (Figure 3b; Yuan and Horvitz, 1992). Thus, *ced-4* does not seem to affect the steady-state levels of *ced-3* mRNA.

ced-3 cDNA and genomic sequences

To isolate *ced-3* cDNA clones, we used the *ced-3* genomic DNA clone pJ40 (Fig. 1b), which rescued the Ced-3 mutant phenotype when microinjected into *ced-3* mutant animals, as a probe to screen a cDNA library prepared from the C. elegans wild-type strain N2 (Kim and Horvitz, 1990). The 2.5 kb cDNA clone pJ87 was isolated in this way. On northern blots pJ87 hybridized to a 2.8 kb transcript, and on Southern blots it hybridized only to bands to which pJ40 also hybridized (data not shown). Thus, pJ87 was derived from an mRNA transcribed entirely from pJ40. To confirm that pJ87 corresponds to a *ced-3* cDNA clone, we made a frameshift mutation in the unique *Sal*I site of pJ40, which corresponds to the unique *Sal*I site in the pJ87 cDNA clone and disrupts the putative *ced-3* open reading frame (see Experimental Procedures; also, Fig. 4a). Constructs containing this frameshift mutation failed to rescue the *ced-3* phenotype when microinjected into Ced-3 mutant animals (six transgenic lines were examined; data not shown), suggesting that *ced-3* activity was eliminated by mutating the putative *ced-3* open reading frame.

We determined the DNA sequence of pJ87 (Fig. 4). pJ87 contains an insert of 2482 bp that can encode a protein of 503 amino acids. pJ87 contains 953 bp of 3' untranslated sequence, not all of which is essential for *ced-3* function, since genomic constructs that lack the last 380 bp of the 3'-most region (pJ107 and its derivatives, see Fig. 1b) rescued the Ced-3 mutant phenotype. The pJ87 cDNA clone ends with a poly-A sequence (data not shown), suggesting that the 3' end of pJ87 corresponds to the extreme 3' end of the *ced-3* transcript. The 5' end of pJ87 does not contain *trans*-spliced

sequences (Bektesh et al., 1988; Huang et al., 1989) and therefore might or might not include the 5' end of the *ced-3* transcript.

To determine the 5' end of the *ced-3* transcript, we performed primer extension experiments (Fig. 5). Two primers containing sequences separated by 177 bp in the genomic DNA sequence (see below) and by 123 bp in the cDNA sequence were used for the primer extension reactions: Pex1, starting at position 2305 of the genomic sequence; and Pex2, starting at position 2482 of the genomic sequence. The Pex2 reaction yielded two major products of 283 nucleotides and 409 nucleotides, whereas the Pex1 reaction gave one product of 160 nucleotides. The 160 nucleotide product of the Pex1 reaction corresponds to the 283 nucleotide product of the Pex2 reaction, since these products differ in size by 123 nucleotides. Products of these lengths are consistent with the presence of a *ced-3* transcript that is *trans*-spliced to a 22 bp C. elegans spliced leader (Bektesh et al., 1988) at a consensus splice acceptor site at position 2166 of the genomic sequence. The larger Pex2 product might be a result of priming by the Pex2 primer from an mRNA other than the *ced-3* mRNA or might identify a second *ced-3* transcript. We failed to amplify a *ced-3* transcript using primers located between positions 1 and 2166 of the genomic sequence (figure 4a) and primers located in the cDNA (data not shown), indicating that if an alternate *ced-3* transcript exists it is not entirely encoded by DNA within the minimal rescuing fragment.

If a *trans*-spliced *ced-3* mRNA exists, it should be possible to use the polymerase chain reaction to amplify a *ced-3* product from total C. elegans RNA using primers specific for spliced leader sequences and *ced-3* cDNA sequences. We therefore prepared cDNA from total C. elegans RNA using reverse transcriptase and amplified the cDNAs using the primers SL1 (which contains the SL1 spliced leader sequence; Bektesh et al., 1988) and log-5 (which starts at position 2897 of the genomic sequence). The products of this reaction were reamplified using the primers SL1 and oligo10 (which starts at position 2344 of the genomic sequence). A product of the expected length (~200 bp) was cloned into the PCR1000 vector (Invitrogen, San Diego, CA), and its sequence was determined (data not shown). This sequence confirmed that at least some *ced-3* transcripts are *trans*-spliced to the C. elegans spliced leader SL1 at a consensus splice acceptor at position 2166 of the genomic sequence. Similar experiments using a primer containing the SL2 spliced leader sequence (Huang and Hirsh, 1989) failed to identify an SL2 *trans*-spliced mRNA. Since the primer extension experiments identified a major *ced-3* mRNA that probably is *trans*-spliced, it seems likely that a large proportion of *ced-3* mRNA is SL1-*trans*-spliced. Based upon these observations, we propose that the translational start codon of *ced-3* is the first in-frame ATG downstream of the SL1 splice-

acceptor site at position 2232 of the genomic sequence and that the CED-3 protein is 503 amino acids in length (Fig. 4a).

To define the structure of the *ced-3* gene, we determined the genomic sequence of the *ced-3* gene from the plasmid pJ107. The insert in pJ107 is 7653 bp in length (Fig. 4a). Comparison of the *ced-3* genomic and cDNA sequences revealed that the *ced-3* gene has seven introns that range in size from 54 bp to 1195 bp. The four largest introns, as well as sequences 5' of the start codon (see below), contain repetitive elements, some of which have been previously identified in non-coding regions of other C. elegans genes, such as *fem-1* (Spence et al., 1990), *lin-12* (Yochem and Greenwald, 1989, and personal communication), *glp-1* (Yochem and Greenwald, 1989) and *hlh-1* (Krause et al., 1990) as well as in the cosmids ZK643 and B0303 (Sulston et al., 1992) (Fig. 4b). Genomic sequence analysis of a *ced-3* homolog from the related nematode C. briggsae (J. Yuan and S. Ledoux, unpublished results) revealed that these repeats are not present in this nematode species, suggesting that the repeats do not have a role in regulating *ced-3* expression. It is possible that such repeats represent active or inactive transposable elements.

The predicted CED-3 protein is hydrophilic (256/503 residues are charged or polar) and does not contain any obvious potential transmembrane domains. One region of the CED-3 protein is rich in serines: from amino acid 107 to amino acid 205, 32 of 99 amino acids are serines.

We determined the sequences of 12 EMS-induced *ced-3* mutations (Fig. 4a; Table 1). Eight are missense mutations, three are nonsense mutations, and one alters a conserved G at the presumptive splice-acceptor site of intron 6.

To identify functionally important regions of the CED-3 protein, we cloned and determined the genomic sequences of the *ced-3* genes from the related nematode species C. briggsae and C. vulgaris. Sequence comparisons showed that amino acids corresponding to residues 1-205 of the C. elegans CED-3 protein are less conserved among the three nematodes (68% identical) than are amino acids corresponding to residues 206-503 of the C. elegans CED-3 protein (84% identical) (Fig. 7a). All eight EMS-induced missense mutations in *ced-3* (see above) altered residues that are conserved among the three species (Fig. 7a). Interestingly, six of these eight mutations alter residues within the last 100 amino acids of the protein, and none affects the serine-rich region (Figs. 4a and 6). These results suggest that the carboxy region is important for *ced-3* function and that the serine-rich region might be unimportant or that different residues within it might be functionally redundant.

CED-3 protein is similar to the mammalian ICE and Nedd-2 proteins

A search of the GenBank, PIR and SWISS-PROT databases revealed that the non-serine-rich regions of the CED-3 protein are similar to the human and murine interleukin-1β (IL-1β) convertases (ICE) (Fig. 7a). ICE is a cysteine protease that cleaves the inactive 31 kD precursor of IL-1β between Asp^{116} and Ala^{117}, releasing a carboxy-terminal 153 amino-acid polypeptide known as mature IL-1β (Kostura et al., 1989; Black et al., 1989). The CED-3 proteins from the three Caenorhabditis species and the human ICE protein share 29% amino acid identity. The most highly conserved region consists of amino acids 246-360 of the CED-3 protein and amino acids 166-287 of the human ICE protein: 49 of 115 residues are identical (43% identity). Cysteine 285 is thought to be an essential component of the active site of ICE (Thornberry et al., 1992). The five-amino-acid peptide QACRG containing this active cysteine is the longest peptide conserved among the murine and human ICE proteins and the CED-3 proteins of the three nematode species.

Active human ICE is composed of two subunits (p20 and p10) that appear to be proteolytically cleaved from a single proenzyme by the mature enzyme (Thornberry et al., 1992). Four cleavage sites in the proenzyme have been defined. Only p20 and p10 are necessary for the *in vitro* enzymatic activity of ICE, suggesting that the three additional fragments resulting from ICE cleavage are not required for ICE function. Two of these cleavage sites, Asp-Ser dipeptides at positions 103-104 and 297-298 of ICE, are conserved in CED-3 (positions 131-132 and 371-372, respectively) (Fig. 7).

The carboxy-terminal portion of the CED-3 protein and the p10 subunit of ICE are similar to the protein product of the murine gene *nedd-2* (Fig. 7a), which is highly expressed during embryonic brain development and is down-regulated in adult brain (Kumar et al., 1992). The C. elegans CED-3 protein and the Nedd-2 protein are 27% identical, as are the ICE and Nedd-2 proteins (Fig. 7a). The Nedd-2 protein apparently does not contain the QACRG peptide found at the active site of ICE (Fig. 7a). Six of the eight known *ced-3* missense mutations (*n718*, *n1040*, *n1129*, *n1164*, *n2426* and *n2433*) alter amino acids that are identical among the three nematode CED-3 proteins and human ICE. For example, the mutation *n2433* introduces a glycine to serine substitution at an absolutely conserved glycine near the putative active-site cysteine (Fig. 4a & 7a). Four mutations (*n1129*, *n1163*, *n1164*, *n2426*) alter amino acids that are identical among the nematode CED-3 proteins and the Nedd-2 protein (Fig. 7a).

Discussion

The genes *ced-3* and *ced-4* are the only genes known to be required for programmed cell death to occur in C. elegans (Ellis and Horvitz, 1986). Our genetic and molecular studies of the *ced-3* gene have revealed that this gene shares a number of features with *ced-4*: like *ced-4* (see Yuan and Horvitz, 1992), *ced-3* is not required for viability and is expressed mostly during embryogenesis, the stage during which 113 of the 131 programmed cell deaths occur (Sulston et al., 1983). Furthermore, just as *ced-3* gene function is not required for *ced-4* gene expression (Yuan and Horvitz, 1992), *ced-4* gene function is not required for *ced-3* gene expression. Thus, these two genes do not appear to control the onset of programmed cell death by acting sequentially in a transcriptional regulatory cascade.

The CED-4 protein is novel in sequence, and the only hint concerning its function is that two regions of the protein show some similarity to the EF-hand motif, which binds calcium (Yuan and Horvitz, 1992). For this reason we have suggested that the CED-4 protein and hence programmed cell death in C. elegans might be regulated by calcium. However, no direct evidence for this hypothesis has yet been obtained. The CED-3 protein similarly contains a region that offers a clue about possible function: a region of 99 amino acids contains 32 serines. Since serines are common phosphorylation sites (Edelman et al., 1987), it is possible that the CED-3 protein and hence programmed cell death in C. elegans are regulated by phosphorylation. Phosphorylation has previously been suggested to function in cell death (McConkey et al. 1990). Although the precise sequence of the serine-rich region varies among the three Caenorhabditis species studied, the relatively high number of serines is conserved (32, 31, and 33 in C. elegans, C. briggsae and C. vulgaris, respectively). None of the mutations in *ced-3* affects the serine-rich region. These observations are consistent with the hypothesis that the presence of serines is more important than the precise amino acid sequence within this region.

Much more striking than the presence of the serine-rich region in the CED-3 protein is the similarity between the non-serine-rich regions of CED-3 and the human and murine interleukin-1β converting enzymes (ICE). Human ICE was identified as a substrate-specific protease that cleaves the 31 kD pro-interleukin-1β between Asp^{116} and Ala^{117} to produce the mature 17.5 kD interleukin-1β (IL-1β). IL-1β is a cytokine involved in mediating a wide range of biological responses, including inflammation, septic shock, wound healing, hematopoiesis and the growth of certain leukemias (Dinarello, 1991; diGiovine and Duff, 1990). A specific inhibitor of ICE, the crmA gene product of Cowpox virus, prevents the proteolytic activation of interleukin-1β (Ray et al.,1992) and inhibits the host inflammatory response (Ray et al., 1992). Cowpox virus carrying a deleted crmA gene is unable to suppress the inflammatory response of chick

embryos, resulting in a reduction in the number of virus-infected cells and less damage to the host (Palumbo et al., 1989). These observations indicate the importance of ICE in bringing about the inflammatory response.

A region of 115 amino acids (residues 246-360 of CED-3) shows the highest identity (43%) between the C. elegans CED-3 protein and the human ICE protein. This region contains a conserved pentapeptide QACRG (positions 356-360 of the CED-3 protein), which contains a cysteine known to be essential for ICE function. Specific modification of this cysteine in human ICE results in a complete loss of activity (Thornberry et al., 1992). The *ced-3* mutation *n2433* alters the conserved glycine in this pentapeptide and eliminates *ced-3* function, suggesting that this glycine is important for *ced-3* activity and might be an integral part of the active site of ICE. Six of the other seven identified *ced-3* missense mutations also affect highly conserved residues that are likely to be important for the actions of both CED-3 and ICE. Interestingly, the mutations *n718* (position 65 of CED-3) and *n1040* (position 27 of CED-3) eliminate *ced-3* function *in vivo* yet alter conserved residues that are not contained in either the mature p10 or p20 subunits of ICE (Thornberry et al., 1992). It is possible that these residues have a non-catalytic role in both CED-3 and ICE function, for example, in maintaining a proper conformation for proteolytic activation. The human ICE proenzyme (p45) can be proteolytically cleaved at four sites (Asp^{103}, Asp^{119}, Asp^{297} and Asp^{316} of ICE) to generate two peptides (p20 and p10) necessary for *in vitro* activity (Thornberry et al., 1992) and three other peptides with as yet undefined functions. At least two of these cleavage sites are conserved in CED-3, indicating that the CED-3 protein might be processed as well.

The similarity between the CED-3 and ICE proteins strongly suggests that CED-3 functions as a cysteine protease in controlling programmed cell death by proteolytically activating or inactivating a substrate protein or proteins. A potential substrate for CED-3 might be the product of the *ced-4* gene. The CED-4 protein contains six aspartate residues that might be targets of the CED-3 protein. Four of these aspartates are followed by a serine (Asp^{151}, Asp^{184}, Asp^{192} and Asp^{541}), and two are followed by an alanine (Asp^{25} and Asp^{459}); of the four ICE cleavage sites in the ICE proenzyme, two are Asp-Ser and one is Asp-Ala. Alternatively, the CED-3 protein might directly cause cell death by proteolytically cleaving proteins that are crucial for cell viability.

The similarity between CED-3 and ICE defines a new protein family. Thornberry et al. (1992) suggested that the sequence GDSPG at position 287 of ICE resembles a GX(S/C)XG motif found in serine and cysteine protease active sites. In the three nematode CED-3 proteins, however, only the first glycine of this sequence is conserved

and in mouse ICE the S/C is missing, suggesting that the CED-3/ICE family shares little sequence similarity with known protease families.

The similarity between CED-3 and ICE suggests not only that CED-3 might function as a cysteine protease but also that ICE might function in programmed cell death in vertebrates. Consistent with this hypothesis, after murine peritoneal macrophages were stimulated with lipopolysaccharide and induced to undergo programmed cell death by exposure to extracellular ATP, mature active IL-1β was released into the culture supernatant; by contrast, when cells were injured by scraping, IL-1β was released exclusively as the inactive proenzyme (Hogoquist et al., 1991). These results suggest that ICE might be activated upon induction of programmed cell death. A role for ICE in programmed cell death need not be mediated by IL-1β but rather could be mediated by another ICE substrate. ICE transcripts have been detected in cells that do not make IL-1β (Cerretti et al., 1992), suggesting that other ICE substrates might well exist. Alternatively, members of the CED-3/ICE family other than ICE might function in vertebrate programmed cell death.

The p10 subunit of ICE and the carboxy-terminal portions of the CED-3 protein are similar to the protein encoded by the murine *nedd-2* gene, which is expressed during early embryonic brain development (Kumar et al., 1992). Since the Nedd-2 protein apparently lacks the QACRG active site region and is similar to ICE primarily in the region of the p10 subunit of ICE, *nedd-2* might function non-catalytically to regulate an ICE or ICE-like p20 subunit. Interestingly, three *ced-3* missense mutations alter residues conserved among the Nedd-2 and CED-3 proteins. *nedd-2* gene expression is high during embryonic brain development (Kumar et al., 1992), when much programmed cell death occurs (Oppenheim, 1981). These observations suggest that Nedd-2 might function in programmed cell death.

The C. elegans gene *ced-9* protects cells from undergoing programmed cell death by directly or indirectly antagonizing the activities of *ced-3* and *ced-4* (Hengartner et al., 1992). The vertebrate gene *bcl-2* acts functionally similarly to *ced-9*: overexpression of *bcl-2* protects or delays the onset of apoptotic cell death in a variety of vertebrate cell types as well as in C. elegans (Vaux et al., 1988; Nunez et al. 1990; Garcia et al., 1992; Sentman et al., 1992; Strasser et al., 1992; Vaux et al., 1992; M. Hengartner and H. R. Horvitz, unpublished results). Thus, if ICE or another CED-3/ICE family member is involved in vertebrate programmed cell death, an intriguing possibility is that *bcl-2* could act by modulating its activity. Furthermore, since *bcl-2* is a dominant oncogene (overexpression of *bcl-2* as a result of chromosomal translocation occurs in 85% of follicular and 20% of diffuse B cell lymphomas; Fukuhara et al., 1979; Levine et al. 1985; Yunis et al., 1987), ICE and other CED-3/ICE family members might be recessive oncogenes: the elimination of

such cell death genes could prevent normal cell death and promote malignancy, just as does overexpression of *bcl-2*.

Experimental Procedures

General Methods and Strains

The techniques used for culturing C. elegans were as described by Brenner (1974). All strains were grown at 20°C. The wild-type strains were C. elegans variety Bristol strain N2, Bergerac strain EM1002 (Emmons et al., 1983), C. briggsae and C. vulgaris (V. Ambros, personal communication). Genetic nomenclature follows the standard C. elegans system (Horvitz et al., 1979). The mutations used have been described by Brenner (1974) and by Hodgkin et al. (1988) or were isolated by us. These mutations are listed below.

LG I: *ced-1(e1735)*
LG IV: *unc-31(e928), unc-30(e191), ced-3(n717, n718, n1040, n1129, n1163, n1164, n1165, n1286, n1949, n2426, n2430, n2433), unc-26(e205)*
LG V: *egl-1(n487, n986)*
LG X: *dpy-3(e27)*

Isolation of additional alleles of *ced-3*

A non-complementation screen was designed to isolate new alleles of *ced-3*. Because animals carrying *ced-3(n717)* in *trans* to a deficiency are viable (Ellis and Horvitz, 1986), we expected animals carrying a complete loss-of-function *ced-3* allele generated by mutagenesis to be viable in *trans* to *ced-3(n717)*, even if the new allele caused inviability in homozygotes. We used an *egl-1* mutation in our screen. Dominant mutations in *egl-1* cause the two hermaphrodite-specific neurons known as the HSNs to undergo programmed cell death (Trent et al., 1983). The HSNs are required for normal egg-laying, and *egl-1* hermaphrodites, which lack HSNs, are egg-laying defective. The mutant phenotype of *egl-1* is suppressed in a *ced-3; egl-1* strain because mutations in *ced-3* block programmed cell deaths. *egl-1(n986)* males were mutagenized with ethyl methanesulfonate (EMS) (20 mM for four hours; Sigma, St. Louis, MO) and crossed with *ced-3(n717) unc-26(e205); egl-1(n487); dpy-3(e27)* hermaphrodites. Most cross progeny hermaphrodites were egg-laying defective because they were heterozygous for *ced-3* and homozygous for *egl-1*. Rare egg-laying competent animals were picked as candidates for carrying new alleles of *ced-3*. Four such animals were isolated from about 10,000 hermaphrodite F1 cross progeny of EMS-mutagenized animals. These animals could have carried either a dominant suppressor of the egg-laying defect of *egl-*

1 or a recessive mutation in *ced-3*. To distinguish between the two possibilities, *ced-3*(new allele); *egl-1* males were mated with *egl-1* hermaphrodites, and the progeny (*ced-3/+;egl-1*) were scored for suppression of the Egl phenotype. In each case, all of the progeny were egg-laying defective, suggesting that the new mutation was recessive and thus likely to be a *ced-3* allele.

Cosmid libraries

Two cosmid libraries were used extensively in this work. A *Sau*3A I partial digest genomic library of 7000 clones in the vector pHC79 was a gift from G. Benian (personal communication) and was used to isolate the cosmids MMM-C1 and MMM-C9. A *Sau*3A I partial digest genomic library of 6000 clones in the vector pJB8 (Ish-Horowicz and Burke, 1981) was a gift from A. Coulson and J. Sulston (Coulson et al., 1986) and was the source of cosmids Jc8, C48D1, and C43C9. W07H6 is in the vector Lorist 6.

Identification and mapping of RFLPs

To place *ced-3* on the physical map we sought to identify Tc1 elements closely linked to *ced-3* and specific to either the Bristol N2 or Bergerac EM1002 strains. We mated Bristol *ced-3 unc-26* /++ males or Bristol *unc-30 ced-3*/++ males with Bergerac hermaphrodites. We isolated recombinants of phenotypes Ced-3 non-Unc-26, Unc-26 non-Ced-3, Unc-30 non-Ced-3, and Ced-3 non-Unc-30 from the progeny of the *ced-3 unc-26* (Bristol)/++(Bergerac) and *unc-30 ced-3* (Bristol)/++(Bergerac) animals, respectively. In this way, we established 15 inbred lines containing copies of chromosome IV derived from both the Bristol and Bergerac strains and recombinant in the region of *ced-3*.

By probing DNA from these strains with the Tc1 insert of plasmid pCe2001 (Emmons et al., 1983), we identified a 5.1 kb *Eco*RI Tc1-containing restriction fragment specific to the Bristol strain and closely linked to *ced-3*. We named this RFLP *nP35*. *nP35* was localized to cosmids MMM-C1 and MMM-C9, which were used to identify a cosmid contig in the *ced-3* region (see text for details).

To identify additional RFLPs in the *ced-3* region DNAs from the recombinant inbred Bristol and Bergerac strains were digested with various restriction enzymes and probed with different cosmids to look for RFLPs between these strains. *nP33* is a *Hin*dIII RFLP detected by the "right" end of cosmid Jc8, which is from the Coulson and Sulston library. The "right" end of Jc8 was made by digesting Jc8 with *Eco*RI and self-ligating. *nP34* is a *Hin*dIII RFLP detected by the "left" end of Jc8. The "left" end of Jc8 was made by digesting Jc8 with *Sal*I and self-ligating. *nP36* and *nP37* are *Hin*dIII RFLPs detected by the cosmids T10H5 and B0564, respectively.

We mapped the RFLPs *nP33*, *nP34*, *nP35*, *nP36* and *nP37* with respect to the genes *unc-30*, *ced-3* and *unc-26*. The location of *unc-30* on the physical map was determined by R. Hoskins (personal communication). Southern blots using DNA from the Ced-3 non-Unc-26 and Unc-26 non-Ced-3 inbred recombinant strains described above were used to map *nP33*, *nP34* and *nP35* (data not shown). Three of three Unc-26 non-Ced-3 recombinants carried the Bristol RFLP *nP33*, while two of two Ced-3 non-Unc-26 recombinants showed the Bergerac pattern; thus, *nP33* maps very close to or to the right of *unc-26*. For *nP34*, two of two Ced-3 non-Unc-26 recombinants and two of three Unc-26 non-Ced-3 recombinants showed the Bergerac pattern; the remaining Unc-26 non-Ced-3 recombinant showed the Bristol pattern. The genetic distance between *ced-3* and *unc-26* is about 0.3 map units (mu). Thus, *nP34* maps between *ced-3* and *unc-26*, about 0.2 mu to the right of *ced-3*. Similar experiments mapped *nP35*, defined by the 5.1 kb restriction fragment containing a Bristol-specific Tc1 element, to about 0.2 mu to the right of *ced-3*. Southern blots of the Unc-30 non-Ced-3, and Ced-3 non-Unc-30 inbred strains described above were used to map *nP36* and *nP37* (data not shown). *nP36* mapped very close to or to the left of *unc-30*, since two of two Unc-30 non-Ced-3 recombinants showed the Bristol pattern on Southern blots, and two of two Ced-3 non-Unc-30 recombinants showed the Bergerac pattern. Similarly, *nP37* mapped very close to or to the left of *unc-30* since four of the four Ced-3 non-Unc-30 recombinants showed the Bergerac pattern on Southern blots, and six of six Unc-30 non-Ced-3 recombinants showed the Bristol pattern.

Germline transformation

Our procedure for microinjection and germline transformation basically followed that of A. Fire (1986) with modifications by J. Sulston (personal communication). Cosmid DNA was CsCl-gradient purified twice. Miniprep DNA was used when cosmid subclones were injected. Miniprep DNA was prepared from 1.5 ml of an overnight bacterial culture grown in superbroth (Sambrook et al., 1989). DNA was extracted by the alkaline lysis method, as described by Sambrook et al. (1989), and was treated with RNase A (37^{o}C, 30 min) and then with proteinase K (55^{o}C, 30 min), phenol- and then chloroform-extracted, precipitated twice (first in 0.3 M sodium acetate pH 5.2 and second in 0.1 M potassium acetate, pH 7.2), and resuspended in 5 ul injection buffer as described by Fire (1986). The DNA concentrations used in injections were between 100 ug and 1 mg per ml.

Subclones of C48D1 were generated as follows. C48D1 was digested with *Bam*HI and self-ligated to generate subclone C48D1-28. C48D1-43 was generated by partial

digestion of C48D1-28 with *Bgl*II. pJ40 was generated by digestion of C48D1-43 with *Apa*I and *Bam*HI. pJ107 was generated by partial digestion of pJ40 with *Bgl*II. pJ7.5 and pJ7.4 were generated by *Exo*III deletion of pJ107.

All transformation experiments used a *ced-1(e1735); unc-31(e928) ced-3(n717)* strain. The *ced-1* mutation was present to facilitate scoring of the Ced-3 phenotype. Mutations in *ced-1* block the engulfment of corpses generated by programmed cell death, causing the corpses of dead cells to persist much longer than in the wild type (Hedgecock et al., 1983). Thus, the presence of a corpse indicates a cell that has undergone programmed cell death. The Ced-3 phenotype was scored by counting the number of corpses present in the head of young L1 animals. Cosmid C48D1 or plasmid subclones were mixed with C14G10 (which contains the *unc-31(+)* gene) at a weight ratio of 2:1 or 3:1 to increase the chances that an Unc-31(+) transformant would contain the cosmid or plasmid being tested. Usually, 20-30 animals were injected in one experiment. Non-Unc F1 progeny of injected animals were isolated three to four days later. About 1/2 to 1/3 of the non-Unc progeny transmitted the non-Unc phenotype to the F2 generation and could be used to establish a line of transgenic animals. The young L1 progeny of such non-Unc transgenics were checked for the number of dead cells present in the head using Nomarski optics, as described by Ellis and Horvitz (1986). Animals of the transgenic line *nIs1* transmitted the non-Unc phenotype to 100% of their progeny, implying that the *ced-3* and *unc-31* transgenes had integrated into the genome. Non-Unc animals of the transgenic line *nEx2* transmitted the non-Unc phenotype to 50% of their progeny, implying that the *ced-3* and *unc-31* transgenes were present on an extra-chromosomal array that is maintained as an unstable free duplication (Way and Chalfie, 1988).

Isolation of *ced-3* cDNAs

To isolate *ced-3* cDNA clones, the insert of pJ40 was used as a probe to screen a cDNA library from the wild-type strain N2 (Kim and Horvitz, 1990). Seven cDNA clones were isolated, of which four were 3.5 kb in length (e.g., pJ85) and three were 2.5 kb in length (e.g., pJ87). One cDNA clone of each size class was subcloned and analyzed further. Two experiments showed that pJ85 contained DNA derived from a *ced-3* transcript fused to DNA derived from an unrelated transcript: first, on a northern blot containing N2 RNA, the pJ85 insert hybridized to two transcripts, one of which did not hybridize to the pJ40 insert; second, on a Southern blot containing N2 DNA, the pJ85 insert hybridized to one band in addition to those to which pJ40 hybridized (data not shown). Plasmid pJ87 contained a 2.5 kb cDNA clone and was determined to contain the complete coding region for *ced-3* (see text). The 5' end of pJ87 contains 25 bp

of poly-A/T sequence (not shown), which is probably a cloning artifact since it is not present in the genomic sequence. The cDNA subclone pJ118 was generated by digesting pJ87 with *Eco*RI and ligating the resulting 2.2 kb fragment to the pBluescript SKII+ vector (Stratagene, La Jolla, CA).

Construction of a *ced-3* frameshift mutation

The *Sal*I site in pJ40 is located at position 5850 of the genomic sequence (Figs. 1b and 4a). The construct PSA was obtained by cleaving pJ40 with *Sal*I and filling in the staggered termini with the Klenow enzyme. By determining the sequence of PSA we confirmed that it contained the sequence GTCGATCGAC instead of GTCGAC at the *Sal*I site and so had a frameshift mutation that should result in the premature termination of protein synthesis at a UGA codon at position 6335.

Determination of the *ced-3* transcription initiation site

Two primers, Pex1 (5'TCATCGACTTTTAGATGACTAGAGAACATC3') and Pex2 (5'GTTGCACTGCTTTCACGATCTCCCGTCTCT3'), were used for primer extension experiments, which were performed as described by Triezenberg (1987) with minor modifications. The primers we used to amplify total RNA with the polymerase chain reaction were SL1 (5'GTTTAATTACCCAAGTTTGAG3') and log-5 (5'CCGGTGACATTGGACACTC3'). Amplification was performed using the GeneAmp kit (Perkin Elmer Cetus, Norwalk, CT). The products were reamplified using the primers SL1 and oligo10 (5'ACTATTCAACACTTG3'). See text for additional details.

DNA sequence analysis

For DNA sequencing, serial deletions were made according to the procedure of Henikoff (1984). DNA sequences were determined using the Sequenase kit (US Biochemical Corp., Cleveland, Ohio) and protocols obtained from the manufacturer.

The CED-3 protein sequence was compared with protein sequences in the GenBank, PIR and SWISS-PROT databases at the National Center for Biotechnology Information using the BLAST network service.

Cloning of *ced-3* genes from other nematode species

The C. briggsae and C. vulgaris *ced-3* genes were isolated from corresponding phage genomic libraries (C. Link, personal communication) using the *ced-3* cDNA subclone pJ118 as a probe under low-strigency hybridization conditions (5xSSPE (Sambrook, 1989), 20% formamide, 0.02% ficoll, 0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone, 1% sodium dodecyl sulfate (SDS) at 40°C overnight) and washed

in 1xSSPE and 0.5% SDS twice at room temperature and twice at 42°C for 20 min for each wash.

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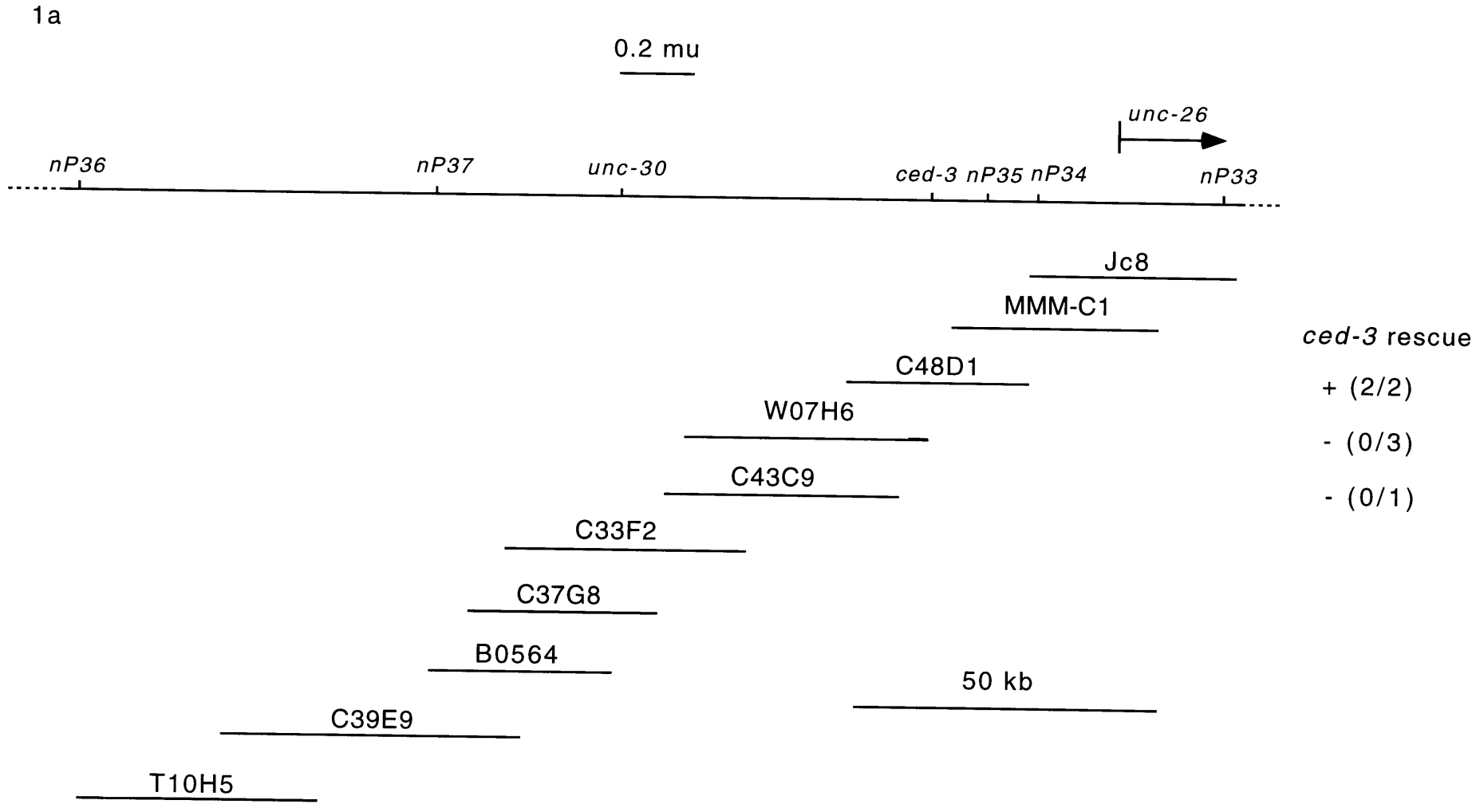
Figure 1. Genetic and physical maps of the *ced-3* region of chromosome IV.
(a) Alignment of the genetic and physical maps in the *ced-3* region. *nP33*, *nP34*, *nP35*, *nP36*, and *nP37* are RFLPs between the Bristol N2 and Bergerac EM1002 wild-type C. elegans strains. The three cosmid clones C43C9, W07H6 and C48D1 were tested for their abilities to rescue the Ced phenotype of *ced-3(n717)* animals. The ability of each cosmid clone to rescue *ced-3* mutants and the fraction of independently obtained transgenic lines that were rescued are indicated on the right. +, rescue; -, no rescue. See text for data. The results indicate that *ced-3* is contained in cosmid C48D1. mu, map units.
(b) A restriction map of subclones of cosmid C48D1. Bold lines represent cosmid vector sequences. Subclones were assayed for rescue of the Ced phenotype of *ced-3(n717)* animals as in (a). +, rescue; -, no rescue; -/+, weak rescue (fewer than five corpses on average); the numbers in parentheses indicate the fraction of independently obtained transgenic lines that were rescued. The smallest fragment that fully rescued the *ced-3* mutant phenotype was the 7.5 kb pJ7.5 subclone.


1a
0.2 mu
unc-26
nP36
nP37
unc-30
ced-3 nP35 nP34
nP33
Jc8
MMM-C1
C48D1
W07H6
C43C9
C33F2
C37G8
B0564
C39E9
T10H5
50 kb
ced-3 rescue
+ (2/2)
- (0/3)
- (0/1)

1b

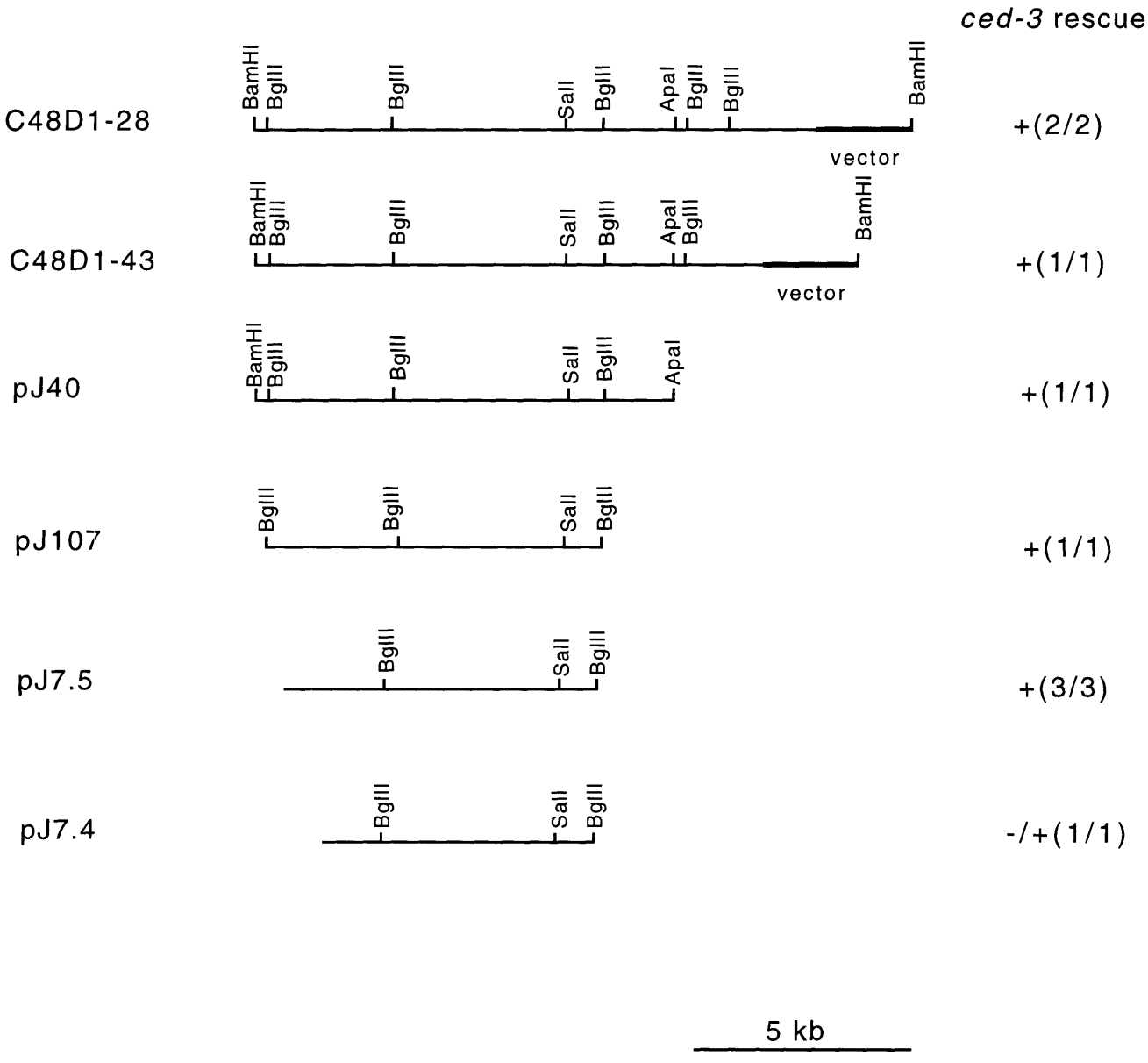

ced-3 rescue
C48D1-28
BamHI
BglII
BglII
SalI
BglII
ApaI
BglII
BglII
BamHI
vector
+(2/2)
C48D1-43
BamHI
BglII
BglII
SalI
BglII
ApaI
BglII
BamHI
vector
+(1/1)
pJ40
BamHI
BglII
BglII
SalI
BglII
ApaI
+(1/1)
pJ107
BglII
BglII
SalI
BglII
+(1/1)
pJ7.5
BglII
SalI
BglII
+(3/3)
pJ7.4
BglII
SalI
BglII
-/+(1/1)
5 kb

Figure 2. *ced-3* RNA.
A northern blot of polyA+ RNA from mixed stages, embryos, L1 through L4 larvae, and young adults, probed with the *ced-3* cDNA subclone pJ118 (see Experimental Procedures). pJ118 did not detectably hybridize to RNA derived from *glp-1(q231)* adults (which lack a germ line; Austin and Kimble, 1987) (data not shown), suggesting that the *ced-3* RNA detected in the young adults in this experiment was derived from embryos within these animals. The level of RNA in each lane can be estimated based upon hybridization to a control actin 1 probe (Krause and Hirsh, 1984).

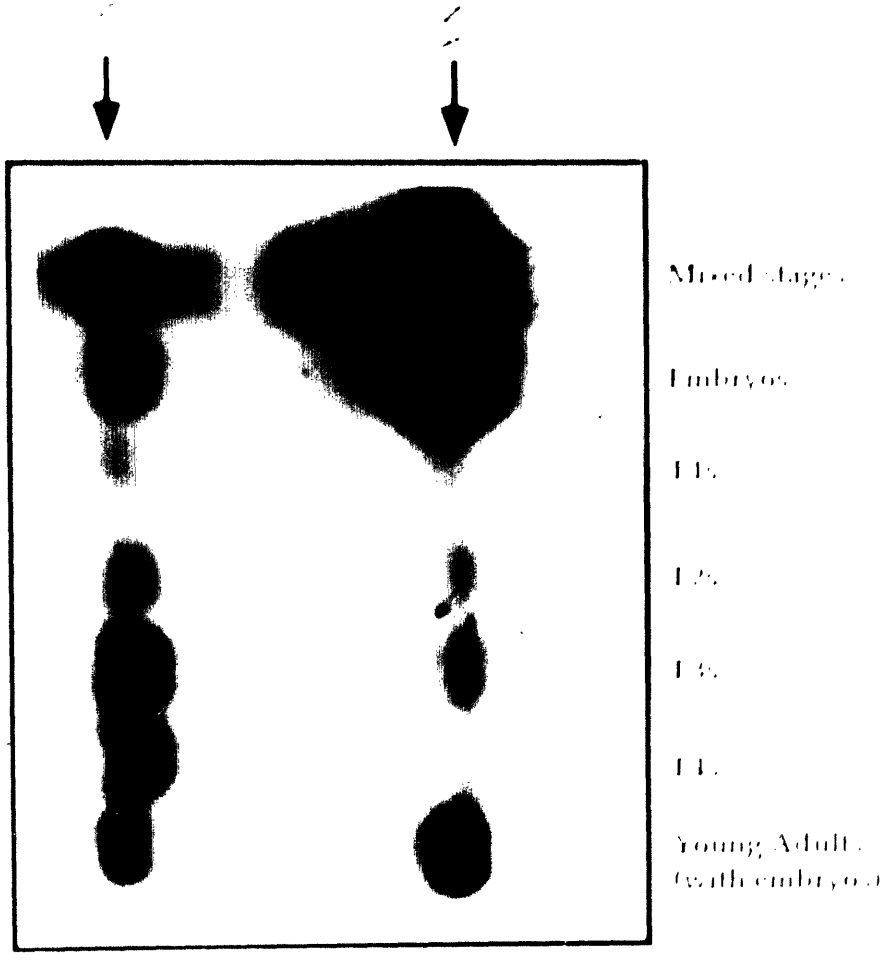
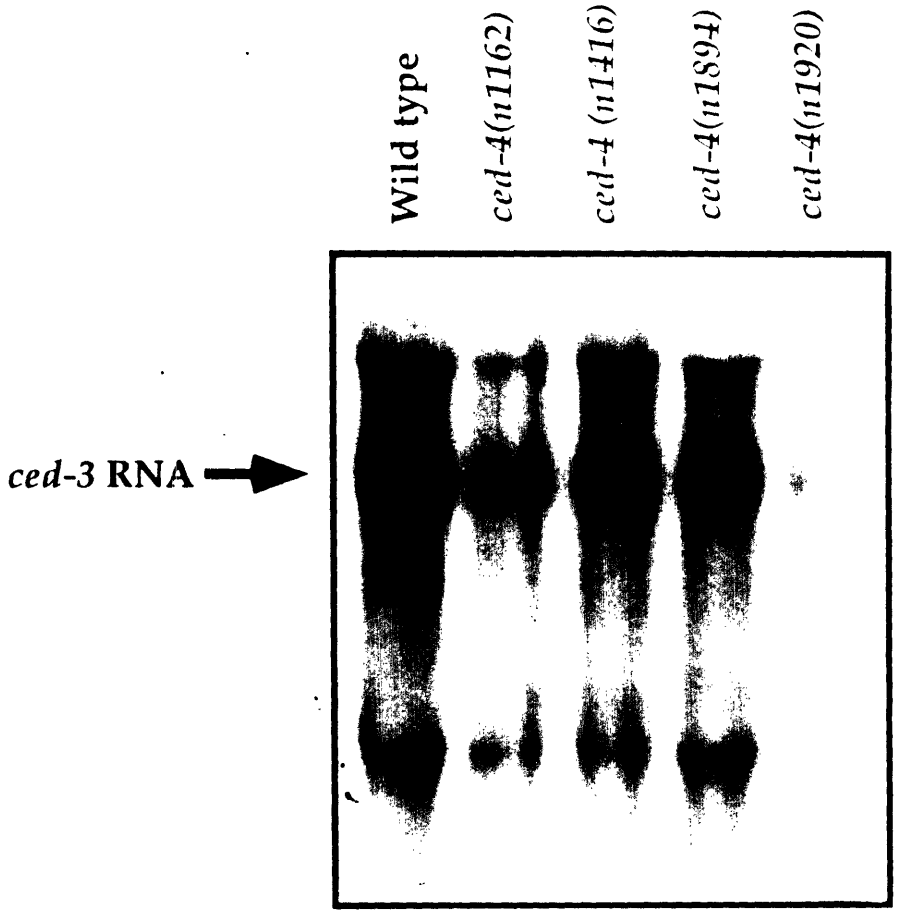

Mixed stages
Embryos
L1s
L2s
L3s
L4s
Young Adult
(with embryos)

Figure 3. Northern blots of total RNA from wild-type embryos and *ced-4*(*n1162*, *n1416*, *n1894*, *n1920*) embryos probed with (a) the *ced-3* cDNA subclone pJ118 (see Experimental Procedures) or (b) the *ced-4* probe SK2-1 and a control actin 1 probe (Krause and Hirsh, 1984). Unlabelled bands in (a) and (b) correspond to ribosomal RNA bands. (b) is reprinted from Yuan and Horvitz (1992).


Wild type
ced-4(n1162)
ced-4 (n1416)
ced-4(n1894)
ced-4(n1920)
ced-3 RNA

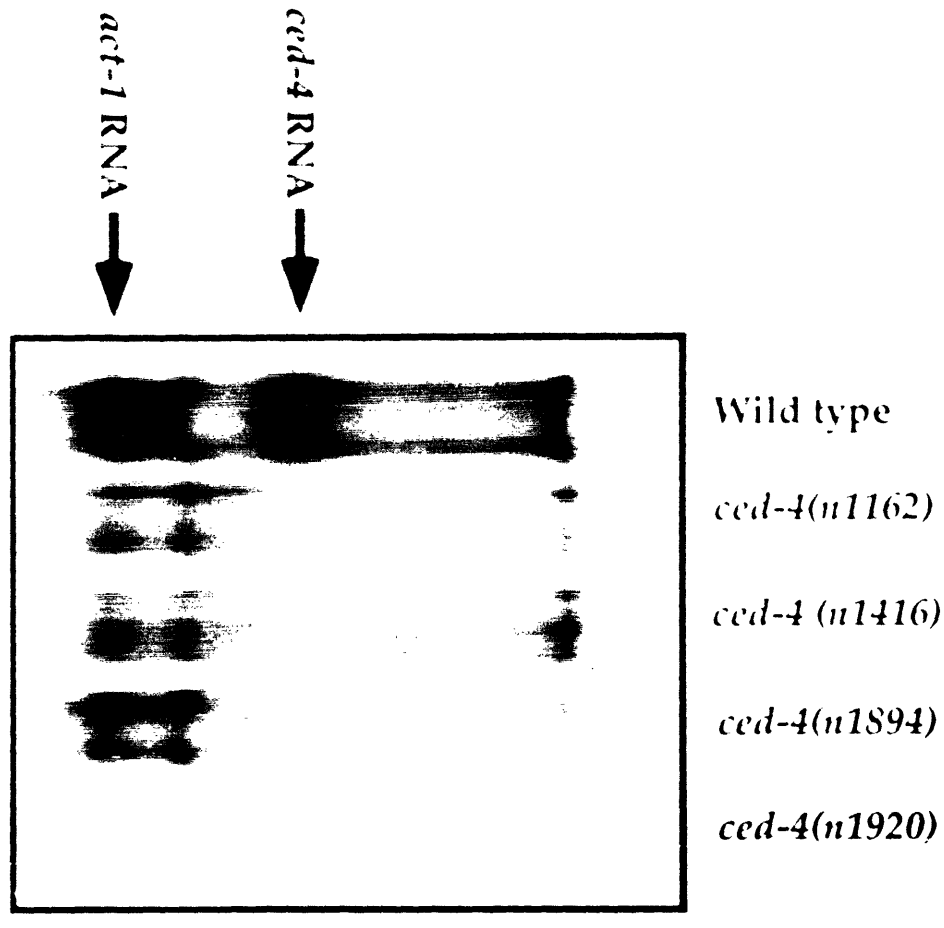
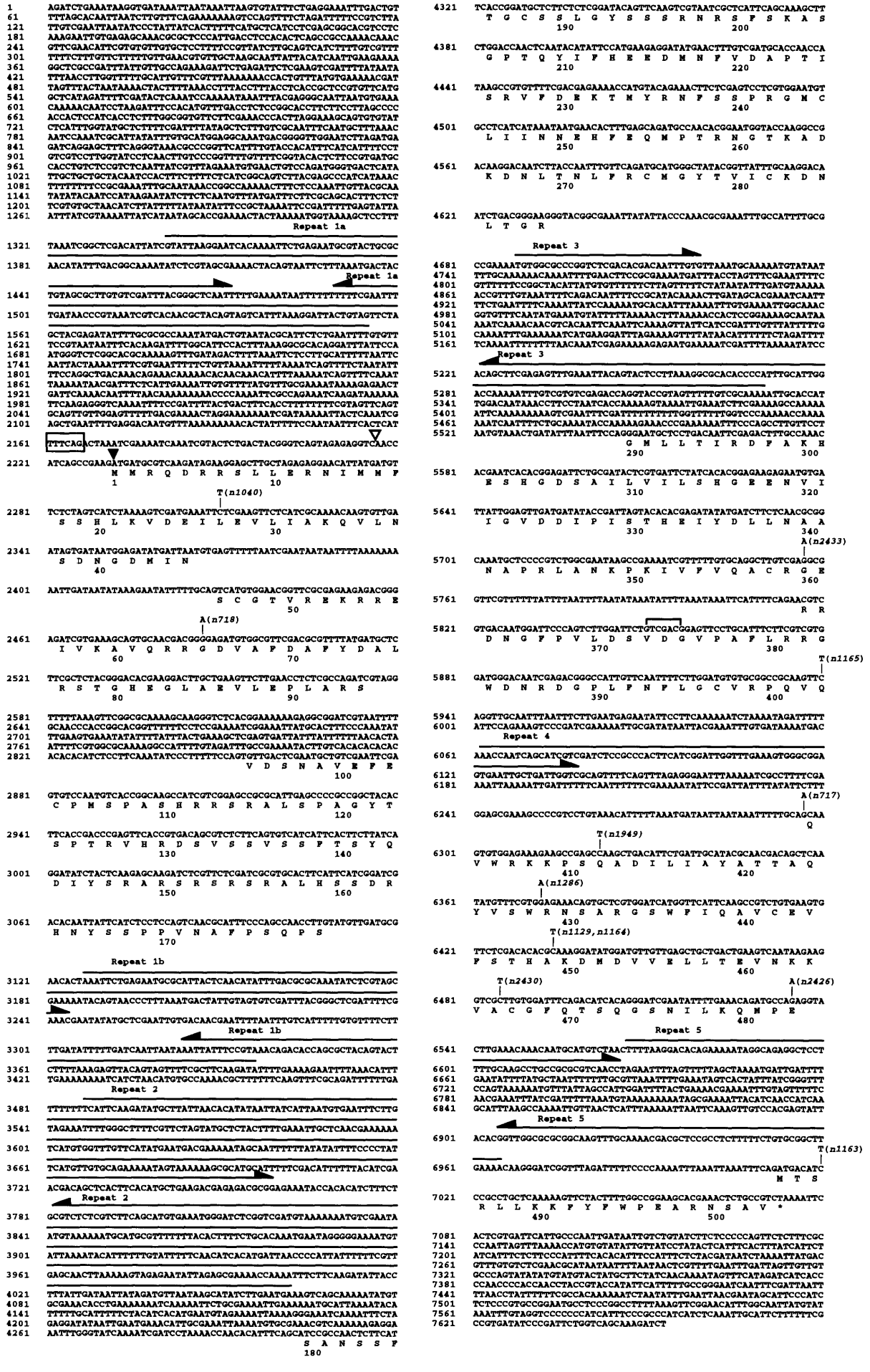

act-1 RNA
ced-4 RNA
Wild type
ced-4(n1162)
ced-4 (n1416)
ced-4(n1894)
ced-4(n1920)

Figure 4. *ced-3* genomic organization, nucleotide sequence, and deduced amino acid sequence.
(a) The genomic sequence of the *ced-3* region, as obtained from plasmid pJ107, which rescued the Ced-3 mutant phenotype. The deduced amino acid sequence of the CED-3 protein is derived from the DNA sequence of the *ced-3* cDNA clone pJ87 and from other experiments described in the text and in the Experimental Procedures. The likely start site of translation is marked with a black arrowhead. The 5' end of pJ87 is indicated by a white arrowhead. The SL1 splice acceptor site of the *ced-3* transcript is boxed. The *Sal*I site is represented by a bracket. The positions of 12 *ced-3* mutations are indicated. Repetitive elements are indicated as arrows above the relevant sequences. Numbers on the left indicate nucleotide positions, begining with the start of pJ107. Numbers below the amino acid sequence indicate amino acid positions. (b) Comparison of repetitive elements in *ced-3* with repetitive elements in the genes *fem-1*, *hlh-1*, *lin-12*, *glp-1*, and the cosmids B0303 and ZK643 (see text for references). In the case of inverted repeats, each arm of a repeat ("for" or "rev" for "forward" or "reverse," respectively) was compared to both its partner and to individual arms of the other repeats. Dashes represent gaps in the sequence to allow optimal alignment except in the consensus sequence where dashes indicate non-identical residues.

4a


Repeat 1a
Repeat 1a
T(n1040)
A(n718)
Repeat 1b
Repeat 1b
Repeat 2
Repeat 2
Repeat 3
Repeat 3
A(n2433)
T(n1165)
Repeat 4
A(n717)
T(n1949)
A(n1286)
T(n1129,n1164)
T(n2430)
A(n2426)
Repeat 5
Repeat 5
T(n1163)

(4b)

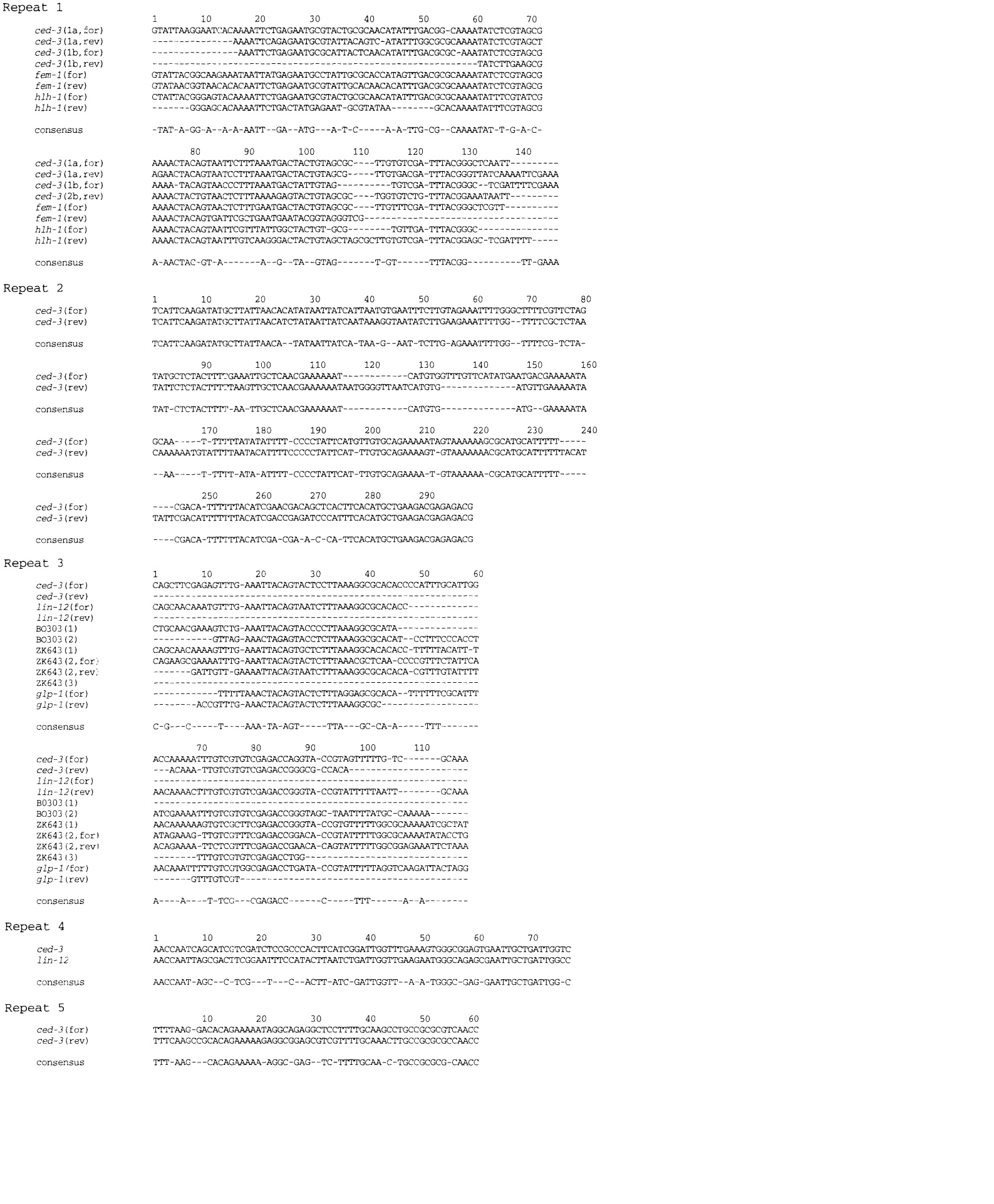

Repeat 1
1 10 20 30 40 50 60 70
ced-3(1a,for) GTATTAAGGAATCACAAAATTCTGAGAATGCGTACTGCGCAACATATTTGACGG-CAAAATATCTCGTAGCG
ced-3(1a,rev) ----------AAAATTCAGAGAATGCGTATTACAGTC-ATATTTGGCGCGCAAAATATCTCGTAGCT
ced-3(1b,for) ----------AAATTCTGAGAATGCGCATTACTCAACATATTTGACGCGC-AAATATCTCGTAGCG
ced-3(1b,rev) ----------TATCTTGAAGCG
fem-1(for) GTATTACGGCAAGAAATAATTATGAGAATGCCTATTGCGCACCATAGTTGACGCGCAAAATATCTCGTAGCG
fem-1(rev) GTATAACGGTAACACACAATTCTGAGAATGCGTATTGCACAACACATTTGACGCGCAAAATATCTCGTAGCG
hlh-1(for) CTATTACGGGAGTACAAAATTCTGAGAATGCGTACTGCGCAACATATTTGACGCGCAAAATATTTCGTATCG
hlh-1(rev) -------GGGAGCACAAAATTCTGACTATGAGAAT-GCGTATAA--------GCACAAAATATTTCGTAGCG
consensus -TAT-A-GG-A--A-A-AATT--GA--ATG---A-T-C-----A-A-TTG-CG--CAAAATAT-T-G-A-C-
80 90 100 110 120 130 140
ced-3(1a,for) AAAACTACAGTAATTCTTTAAATGACTACTGTAGCGC----TTGTGTCGA-TTTACGGGCTCAATT---------
ced-3(1a,rev) AGAACTACAGTAATCCTTTAAATGACTACTGTAGCG-----TTGTGACGA-TTTACGGGTTATCAAAATTCGAAA
ced-3(1b,for) AAAA-TACAGTAACCCTTTAAATGACTATTGTAG----------TGTCGA-TTTACGGGC--TCGATTTTCGAAA
ced-3(2b,rev) AAAACTACTGTAACTCTTTAAAAGAGTACTGTAGCGC----TGGTGTCTG-TTTACGGAAATAATT---------
fem-1(for) AAAACTACAGTAACTCTTTGAATGACTACTGTAGCGC----TTGTTTCGA-TTTACGGGCTCGTT----------
fem-1(rev) AAAACTACAGTGATTCGCTGAATGAATACGGTAGGGTCG----------
hlh-1(for) AAAACTACAGTAATTCGTTTATTGGCTACTGT-GCG--------TGTTGA-TTTACGGGC----------
hlh-1(rev) AAAACTACAGTAATTTGTCAAGGGACTACTGTAGCTAGCGCTTGTGTCGA-TTTACGGAGC-TCGATTTT-----
consensus A-AACTAC-GT-A-------A--G--TA--GTAG-------T-GT------TTTACGG----------TT-GAAA
Repeat 2
1 10 20 30 40 50 60 70 80
ced-3(for) TCATTCAAGATATGCTTATTAACACATATAATTATCATTAATGTGAATTTCTTGTAGAAATTTTGGGCTTTTCGTTCTAG
ced-3(rev) TCATTCAAGATATGCTTATTAACATCTATAATTATCAATAAAGGTAATATCTTGAAGAAATTTTGG--TTTTCGCTCTAA
consensus TCATTCAAGATATGCTTATTAACA--TATAATTATCA-TAA-G--AAT-TCTTG-AGAAATTTTGG--TTTTCG-TCTA-
90 100 110 120 130 140 150 160
ced-3(for) TATGCTCTACTTTTGAAATTGCTCAACGAAAAAAT----------CATGTGGTTTGTTCATATGAATGACGAAAAATA
ced-3(rev) TATTCTCTACTTTTTAAGTTGCTCAACGAAAAAATAATGGGGTTAATCATGTG----------ATGTTGAAAAATA
consensus TAT-CTCTACTTTT-AA-TTGCTCAACGAAAAAAT----------CATGTG----------ATG--GAAAAATA
170 180 190 200 210 220 230 240
ced-3(for) GCAA-----T-TTTTTATATATTTT-CCCCTATTCATGTTGTGCAGAAAAATAGTAAAAAAGCGCATGCATTTTT-----
ced-3(rev) CAAAAAATGTATTTTAATACATTTTCCCCCTATTCAT-TTGTGCAGAAAAGT-GTAAAAAAACGCATGCATTTTTTACAT
consensus --AA-----T-TTTT-ATA-ATTTT-CCCCTATTCAT-TTGTGCAGAAAA-T-GTAAAAAA-CGCATGCATTTTT-----
250 260 270 280 290
ced-3(for) ----CGACA-TTTTTTACATCGAACGACAGCTCACTTCACATGCTGAAGACGAGAGACG
ced-3(rev) TATTCGACATTTTTTTACATCGACCGAGATCCCATTTCACATGCTGAAGACGAGAGACG
consensus ----CGACA-TTTTTTACATCGA-CGA-A-C-CA-TTCACATGCTGAAGACGAGAGACG
Repeat 3
1 10 20 30 40 50 60
ced-3(for) CAGCTTCGAGAGTTTG-AAATTACAGTACTCCTTAAAGGCGCACACCCCATTTGCATTGG
ced-3(rev) ----------
lin-12(for) CAGCAACAAATGTTTG-AAATTACAGTAATCTTTAAAGGCGCACACC----------
lin-12(rev) ----------
BO303(1) CTGCAACGAAAGTCTG-AAATTACAGTACCCCTTAAAGGCGCATA----------
BO303(2) ----------GTTAG-AAACTAGAGTACCTCTTAAAGGCGCACAT--CCTTTCCCACCT
ZK643(1) CAGCAACAAAAGTTTG-AAATTACAGTGCTCTTTAAAGGCACACACC-TTTTTACATT-T
ZK643(2,for) CAGAAGCGAAAATTTG-AAATTACAGTACTCTTTAAACGCTCAA-CCCCGTTTCTATTCA
ZK643(2,rev) -------GATTGTT-GAAAATTACAGTAATCTTTAAAGGCGCACACA-CGTTTGTATTTT
ZK643(3) ----------
glp-1(for) ----------TTTTTAAACTACAGTACTCTTTAGGAGCGCACA--TTTTTTCGCATTT
glp-1(rev) --------ACCGTTTG-AAACTACAGTACTCTTTAAAGGCGC----------
consensus C-G---C-----T----AAA-TA-AGT-----TTA---GC-CA-A-----TTT-------
70 80 90 100 110
ced-3(for) ACCAAAAATTTGTCGTGTCGAGACCAGGTA-CCGTAGTTTTTG-TC-------GCAAA
ced-3(rev) ---ACAAA-TTGTCGTGTCGAGACCGGGCG-CCACA----------
lin-12(for) ----------
lin-12(rev) AACAAAACTTTGTCGTGTCGAGACCGGGTA-CCGTATTTTTAATT--------GCAAA
BO303(1) ----------
BO303(2) ATCGAAAATTTGTCGTGTCGAGACCGGGTAGC-TAATTTTATGC-CAAAAA-------
ZK643(1) AACAAAAAAGTGTCGCTTCGAGACCGGGTA-CCGTGTTTTTGGCGCAAAAATCGCTAT
ZK643(2,for) ATAGAAAG-TTGTCGTTTCGAGACCGGACA-CCGTATTTTTGGCGCAAAATATACCTG
ZK643(2,rev) ACAGAAAA-TTCTCGTTTCGAGACCGAACA-CAGTATTTTTGGCGGAGAAATTCTAAA
ZK643(3) --------TTTGTCGTGTCGAGACCTGG----------
glp-1(for) AACAAATTTTTGTCGTGGCGAGACCTGATA-CCGTATTTTTAGGTCAAGATTACTAGG
glp-1(rev) -------GTTTGTCGT----------
consensus A----A----T-TCG---CGAGACC------C-----TTT------A--A--------
Repeat 4
1 10 20 30 40 50 60 70
ced-3 AACCAATCAGCATCGTCGATCTCCGCCCACTTCATCGGATTGGTTTGAAAGTGGGCGGAGTGAATTGCTGATTGGTC
lin-12 AACCAATTAGCGACTTCGGAATTTCCATACTTAATCTGATTGGTTGAAGAATGGGCAGAGCGAATTGCTGATTGGCC
consensus AACCAAT-AGC--C-TCG---T---C--ACTT-ATC-GATTGGTT--A-A-TGGGC-GAG-GAATTGCTGATTGG-C
Repeat 5
10 20 30 40 50 60
ced-3(for) TTTTAAG-GACACAGAAAAATAGGCAGAGGCTCCTTTTGCAAGCCTGCCGCGCGCGTCAACC
ced-3(rev) TTTCAAGCCGCACAGAAAAAGAGGCGGAGCGTCGTTTTGCAAACTTGCCGCGCGCCAACC
consensus TTT-AAG---CACAGAAAAA-AGGC-GAG--TC-TTTTGCAA-C-TGCCGCGCG-CAACC

Figure 5. A primer extension experiment to determine the *ced-3* transcription initiation site (see text for details). Lanes 1-4, DNA sequencing reaction products using primer Pex1 and pJ40 as the template. Lanes 5-8, sequencing reaction products using the primer Pex2 and pJ40 as the template. Lane 9, primer extension reaction products using primer Pex1 and N2 total RNA as template. Lane 10, primer extension reaction products using primer Pex2 and N2 total RNA as template. Sizes of the primer extension products are indicated to the right of lane 10.

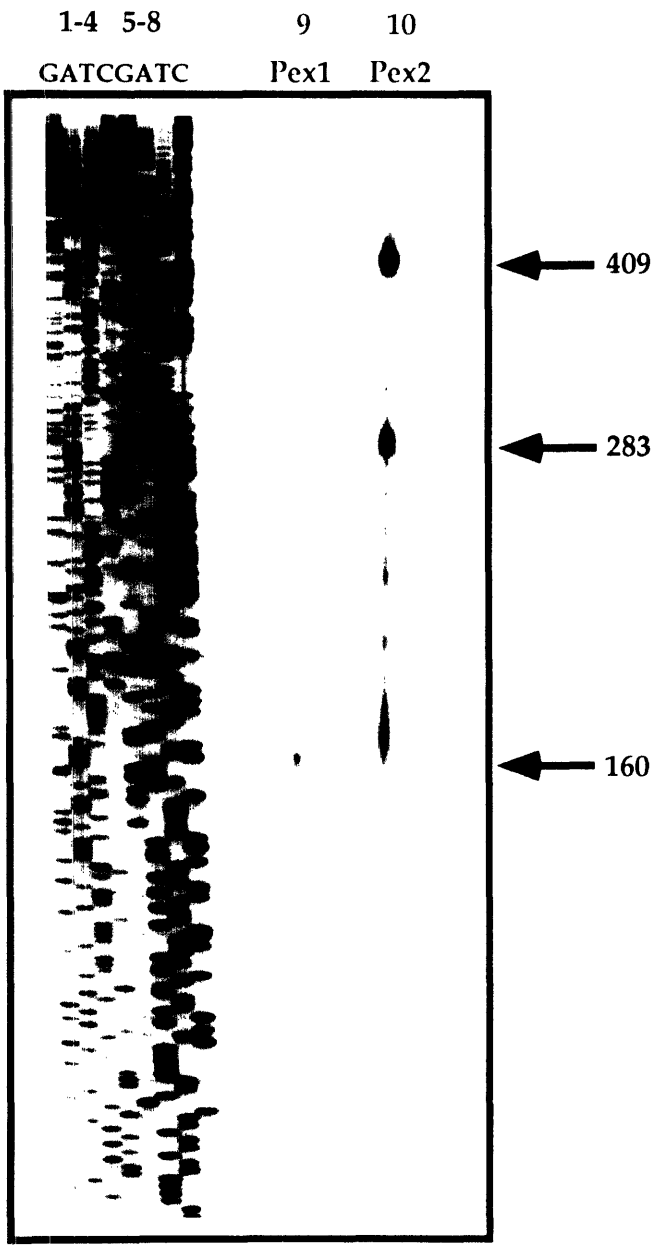

1-4
5-8
9
10
GATCGATC
Pex1
Pex2
409
283
160

Figure 6 Intron-exon structure (represented by lines and boxes, respectively) of the *ced-3* gene and positions of 12 *ced-3* mutations. The *trans*-spliced SL1 leader, the serine-rich region, the mutations, and the presumptive translational start site (ATG), termination site (TAA), and polyadenlyation site (AAA) are indicated.

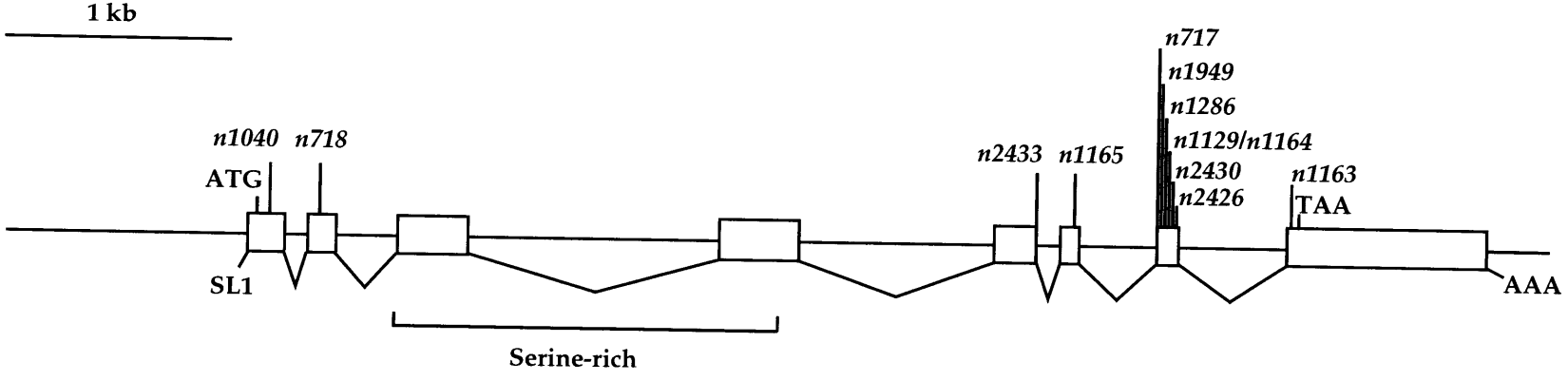
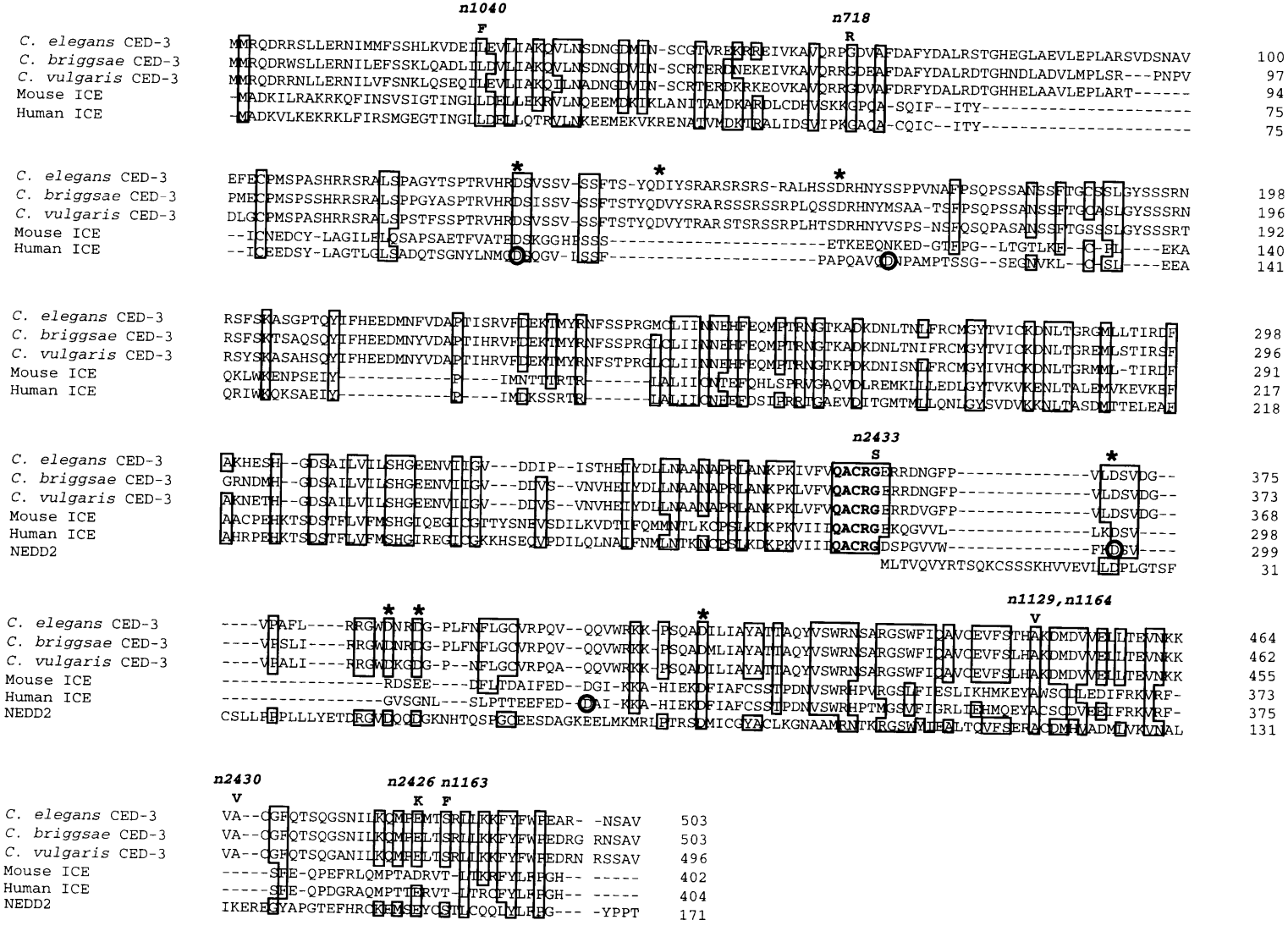

1 kb
n1040
n718
ATG
SL1
n2433
n1165
n717
n1949
n1286
n1129/n1164
n2430
n2426
n1163
TAA
AAA
Serine-rich

Figure 7. CED-3 protein.

(a) Comparison of the CED-3 protein sequences from C. elegans, C. briggsae, and C. vulgaris with the human and mouse interleukin-1β converting enzymes (ICE) and with the mouse Nedd-2 protein. Amino acids are numbered to the right of each sequence. Dashes indicate gaps in the sequence to allow optimal alignment. Residues that are identical among more than half of the proteins and between nematode and mammalian sequences are boxed. Missense *ced-3* mutations are indicated above the comparison blocks showing the residue in the mutant CED-3 protein and the allele name. Asterisks indicate potential aspartate self-cleavage sites in the CED-3 protein. Circles indicate known aspartate self-cleavage sites in human ICE. Residues indicated in boldface correspond to the highly conserved pentapeptide containing the active cysteine in ICE.

(b) Comparison of structural features of the CED-3 protein and human ICE. The predicted proteins corresponding to the ICE proenzyme and CED-3 are represented. The active site in ICE and the predicted active site in CED-3 are indicated by the black rectangles. The four known cleavage sites in ICE flanking the processed ICE subunits (p24, which was detected in low quantities when ICE was purified (Thornberry et al., 1992), p20, and p10) and two conserved presumptive cleavage sites in the CED-3 protein are indicated with solid lines and linked with dotted lines. Five other potential cleavage sites in the CED-3 protein are indicated with dashed lines. The positions of the aspartate (D) residues at potential cleavage sites are indicated below each diagram. The carboxy terminus of p24 has not been determined and is indicated by a dotted arrow.


n1040
F
n718
R
C. elegans CED-3
C. briggsae CED-3
C. vulgaris CED-3
Mouse ICE
Human ICE
n2433
S
QACRG
NEDD2
n1129,n1164
V
n2430
V
n2426 n1163
K F

7b

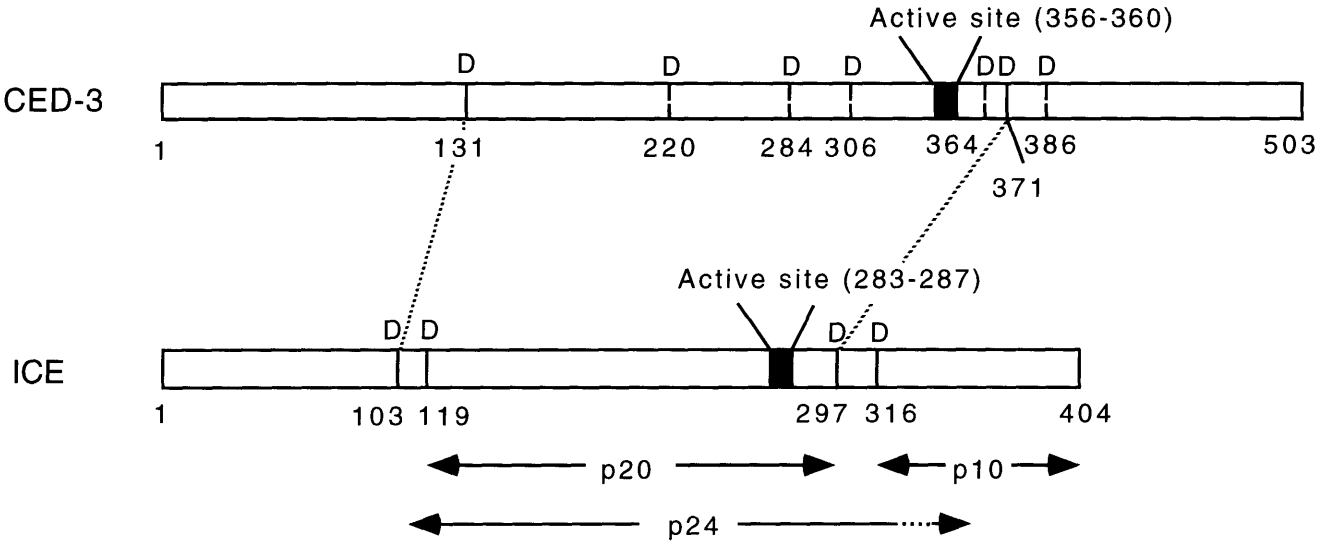

Active site (356-360)
CED-3
D
D
D
D
DD
D
1
131
220
284
306
364
386
503
371
Active site (283-287)
ICE
D
D
D
D
1
103
119
297
316
404
p20
p10
p24

TABLE 1. Sequences of *ced-3* Mutations

Allele	Wild-type sequence	Mutant sequence	Substitution or splice site change
n717	ttttgcag ! CAA	ttttgcaa ! CAA	Exon 7 acceptor
n718	GGA	AGA	G65R
n1040	CTC	TTC	L27F
n1129, n1164	GCA	GTA	A449V
n1163	TCC	TTC	S486F
n1165	CAG	TAG	Q404amber
n1286	TGG	TGA	W428opal
n1949	CAA	TAA	Q412ochre
n2426	GAG	AAG	E483K
n2430	GCT	GTT	A466V
n2433	GGC	AGC	G360S

Amino acid positions correspond to the numbering in Fig. 4a.

Chapter 3

Overexpression of either *ced-3* or *ced-4*, two C. elegans cell death genes, can cause cells that normally live to undergo programmed cell death

Running title: Cell killing by C. elegans cell death genes

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Summary

Programmed cell death in the nematode Caenorhabditis elegans requires the actions of the genes *ced-3* and *ced-4*. We show that overexpression of either *ced-3* or *ced-4* in specific neurons is sufficient to cause those neurons, which normally live, to undergo a process morphologically and kinetically similar to programmed cell death. The efficacy of cell killing by *ced-3* or *ced-4* is enhanced if the activity of the cell survival gene *ced-9* is eliminated. Overexpression of *ced-3* can kill cells in the absence of endogenous *ced-4*, but overexpression of *ced-4* kills at most poorly in the absence of endogenous *ced-3*. Protection by *ced-9* against *ced-3*-induced killing requires *ced-4* function. Our observations indicate that *ced-3* and *ced-4* can act cell autonomously to cause programmed cell death and are consistent with a pathway for programmed cell death in which *ced-9* inhibits *ced-4* function and *ced-4* enhances *ced-3* function.

Introduction

Programmed cell death is a major and apparently universal aspect of metazoan development and tissue homeostasis (Glücksman, 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; reviewed by 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 specify the fate of programmed cell death for 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). By 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, 1994), 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; reviewed by 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 mammalian interleukin-1β (IL-1β) converting enzyme (ICE) (Yuan et al., 1993), a cysteine protease isolated based on its ability to cleave a 31 kD pro-IL-1β 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 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 would suffice to cause a cell that would otherwise survive instead to undergo programmed cell death. Nor does this observation reveal whether *ced-3* and *ced-4* act together, perhaps encoding two components of a heteromeric protein complex, or sequentially, with the activity of one needed only to cause the expression or activation of the other. To resolve 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 if 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, personal communication) (see Figure 1A). The $P_{mec\text{-}7}$*ced-3* and $P_{mec\text{-}7}$*ced-4* fusion constructs were separately injected into wild-type animals, and lines containing integrated copies of the constructs were established. We obtained three lines ($P_{mec\text{-}7}$*ced-3*-1,-2,-3) containing integrated copies of $P_{mec\text{-}7}$*ced-3* and four lines ($P_{mec\text{-}7}$*ced-4*-1, -2, -3, -4) containing integrated copies of $P_{mec\text{-}7}$*ced-4*.

To determine if cells that normally express *mec-7* were absent in animals carrying the $P_{mec\text{-}7}$*ced-3* or $P_{mec\text{-}7}$*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 9/46 (20%) of sides scored in line $P_{mec\text{-}7}$*ced-3*-3 and on only 4/39 (10%) of sides scored in line $P_{mec\text{-}7}$*ced-4*-1. We established two lines containing integrated arrays harboring both a $P_{mec\text{-}7}$*ced-3* and a $P_{mec\text{-}7}$*ced-4* fusion construct (lines $P_{mec\text{-}7}$*ced-3/4*-1 and $P_{mec\text{-}7}$*ced-3/4*-2). Wild-type animals carrying these arrays showed a slight loss of ALM neurons: ALMs were present on 35/37 (95%) and 45/46 (98%) of sides scored, respectively. Wild-type animals not carrying these arrays always contained both ALMs (n=31), and animals expressing a $P_{mec\text{-}7}$*lacZ* construct (*jeIs1*, J. Way, personal communication) had ALMs on 40/40 (100%) of 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.

Killing by overexpression of *ced-3* or *ced-4* is better in the presence of a loss-of-function mutation in the endogenous *ced-9* gene

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. Since 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 $P_{mec\text{-}7}$*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 $P_{mec\text{-}7}$*ced-3*-3 transgene, ALMs were present on 0/29 (0%) of sides scored in a *ced-9; ced-3* background, yet were present on 16/34 (47%) of sides scored in a *ced-3* background. Similarly, lines containing a $P_{mec\text{-}7}$*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 $P_{mec\text{-}7}$*ced-4*-1 transgene, ALMs were present on 0/30 (0%) of sides scored in a *ced-4 ced-9* background, yet were present on 12/28 (43%) of 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 and 2B). In line $P_{mec\text{-}7}$*ced-3/4*-1, for example, ALMs were present on only 16/37 (43%) of the sides scored in a *ced-9; ced-3* background, but were present on 40/40 (100%) of 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 a mutant *ced-9* background 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). We also tested the effect of the *ced-9* partial loss-of-function allele *n1950 n2161* on ALM cell death in strains containing the $P_{mec\text{-}7}$*ced-3*-3 transgene and again observed enhanced killing: ALMs were present on 1/15 (7%) of 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 point mutation that activates the CED-9 protein and causes it to prevent the normal cell deaths that occur during C. elegans development (Hengartner et al., 1992; Hengartner and Horvitz, 1994). We tested the ability of *ced-9(n1950)* to prevent the deaths of ALM neurons in animals carrying $P_{mec\text{-}7}$*ced-3* or $P_{mec\text{-}7}$*ced-4* transgenes. However, as shown in Table 3, the *n1950* mutation did not consistently decrease the abilities of these trangesnes 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 if these neurons were missing because they underwent programmed cell death, we directly observed the cell divisions leading to the formation of the *mec-7*-expressing neuron PVM (Chalfie, 1993) in animals carrying transgenes $P_{mec\text{-}7}$*ced-3*-1, $P_{mec\text{-}7}$*ced-3*-3, and $P_{mec\text{-}7}$*ced-3/4*-1 in a *ced-9(n2812); ced-3(n717)* background or transgene $P_{mec\text{-}7}$*ced-4*-1 in a *ced-4(n1162) ced-9(n2812)* background. As illustrated in Figure 2A, presumptive PVM neurons in these lines showed the characteristic refractile appearance of programmed cell deaths viewed using Nomarski optics (12 animals observed); these deaths were morphologically indistinguishable from normal programmed cell deaths. We observed four PVMs through the entire cell death process. Two PVMs died and disappeared within an hour of their initial refractility, one after approximately an hour and a half and one after approximately two hours. These kinetics are similar to those of some normal programmed cell deaths that occur during the L1 stage (Sulston and Horvitz, 1977). Thus, these ectopic cell deaths were both morphologically and kinetically similar to normal cell deaths as well.

We also analyzed the ultrastructure of a cell corpse resulting from a PVM cell death. Figure 2C 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 cells that normally die by programmed cell death, suggesting that the deaths we observed were ultrastructurally similar to normal programmed cell deaths.

To establish further that the deaths caused by overexpression of *ced-3* or *ced-4* were similar to programmed cell deaths, we examined L1 *ced-1(e1735); ced-4(n1162) ced-9(n2812)* animals containing the $P_{mec\text{-}7}$*ced-4*-1 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 (Hedgecock et al., 1983), causing these corpses to persist for hours or even days. We observed corpses on 11/40 sides near the BDU cell (the sister cell of the ALM), and we observed corpses in the normal location of the ALM on 2/40 sides, suggesting that the ALMs often died before migrating posteriorly, yet somtimes died after migration was complete. 27/40 sides scored did not have observable corpses probably because the penetrence of *ced-1*(*e1735*) can be quite low (1/100 sides scored for the NSM sister had an NSM sister corpse; Ellis et al., 1991b). We obtained similar results using the $P_{mec\text{-}7}$*ced-3*-3 transgene in a *ced-3*(*n3002*) background (data not shown). These findings suggest that the gene *ced-1* which is

required for engulfment of normal programmed cell death corpses, is also required for the engulfment of the ectopic ALM cell deaths.

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 $P_{mec\text{-}7}$*ced-3* that were stained with the DNA stain 4,6-Diamidino-2-phenylindole (DAPI). Near the position of the BDU cell, 7/10 sides had DAPI-positive structures that did not correspond to visible nuclei when viewed using Nomarski optics. These findings suggest that the gene *nuc-1* which is required for the degradation of DNA of normally dying cells, is also required for the degradation of DNA of ectopic ALM cell deaths.

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

To determine if 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. Since we were not interested in scoring a given array in a number of different genetic backgrounds where the array must remain identical for accurate results, the above constructs were maintained as extrachromosomal arrays (which are easier to generate than integrated arrays; Fire, 1986; Way and Chalfie, 1988) and were compared with 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 $P_{mec\text{-}7}$*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 for the enzymatic activity of ICE (Cerretti et al., 1992; Thornberry et al., 1992) and presumably for the activity of CED-3 as well. By contrast, only about 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 $P_{mec\text{-}7}$*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 about 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 if 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 three other proteins under the control of the *mec-7* promoter: E. coli β-galactosidase (using a worm strain that carries the insertion *jeIs1*; J. Way, personal communication); murine ICE; and C. elegans NCC-1 protein (which is similar to cdc-2 kinase; P. Sternberg, personal communication). (ICE and CDC-2 have been implicated in mammalian cell death; Miura et al., 1994; Shi et al., 1994.) The presence of these constructs in *ced-9*; *ced-3* or *ced-4 ced-9* worms did not cause ALM cell death (data not shown), suggesting that it is not simply excess or foreign protein that killed the ALMs in the experiments described above.

Ectopic killing by overexpression of *ced-4* may require *ced-3* function

To examine the requirement for endogenous *ced-3* in the killing of ALMs by $P_{mec\text{-}7}$*ced-4* transgenes, we introduced these transgenes into *ced-3* mutant strains. Table 5 shows that killing of ALMs in all four $P_{mec\text{-}7}$*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 $P_{mec\text{-}7}$*ced-4*-1 ALMs survived on 4/39 (10%) of sides scored in a wild-type background but survived on 27/38 (71%) of sides scored in a *ced-3* background. Similarly, in line $P_{mec\text{-}7}$*ced-4*-1 ALMs survived on 0/30 (0%) of sides scored in a *ced-4 ced-9* background but survived on 27/38 (71%) of 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 $P_{mec\text{-}7}$*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 which is likely to have little if any *ced-9* function based on both genetic and molecular criteria (Hengartner et al., 1994; S. Shaham, unpublished data).

To assess whether only the *ced-3* allele *n717* used in the above experiments could prevent killing by $P_{mec\text{-}7}$*ced-4* constructs, and to assess if more penetrant alleles of *ced-3* could block killing more efficiently we examined the abilities of several *ced-3* alleles to inhibit killing by *ced-4* overexpression. As shown in Table 6, the better the ability of a *ced-3* mutation to cause survival of cells that normally die, the better was its ability to prevent killing of ALM neurons by $P_{mec\text{-}7}$*ced-4*-1. Animals carrying the *ced-3* allele *n1040*, for example, contained an average of 7.6 extra cells in the anterior pharynx. These cells would have normally died in wild-type animals. ALMs survived on 35/64

(55%) of sides scored in line $P_{mec\text{-}7}$*ced-4*-1 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 33/41 (80%) of sides scored in line $P_{mec\text{-}7}$*ced-4*-1 containing this mutation. Although *n2433* is the most severe *ced-3* allele known (S. Shaham, unpublished observations; M. Hengartner, personal communication), neither *n2433* nor any other *ced-3* allele characterized to date has been shown to completely eliminate *ced-3* function. None of the currently characterized *ced-3* alleles is clearly a null allele by both molecular and genetic criteria (S. Shaham and H. R. Horvitz, unpublished data; Yuan et al., 1993). 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* trangene absolutely requires *ced-3* function.

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

Similarly, to examine the requirement for endogenous *ced-4* in the killing of ALMs by $P_{mec\text{-}7}$*ced-3* trangenes 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, unpublished results), and thus this allele is an excellent candidate for being a molecular null allele. Columns 1 and 2 of Table 7 show that ALM survival in $P_{mec\text{-}7}$*ced-3*- containing lines is not consistently greater in a *ced-4(n1162)* mutant background than in a wild-type background. For example, in line $P_{mec\text{-}7}$*ced-3*-2 ALMs survived on 16/38 (42%) of sides scored in a wild-type background and on 18/61 (30%) of sides scored in a *ced-4(n1162)* background. These results suggest that killing by *ced-3* overexpression might be different from killing by *ced-4* overexpression. Supporting this notion, eventhough killing in line $P_{mec\text{-}7}$*ced-4*-1 is more efficient in a wild-type background than lines $P_{mec\text{-}7}$*ced-3*-2 and $P_{mec\text{-}7}$*ced-3*-3, killing in this line is much weaker in a *ced-3* mutant background than lines $P_{mec\text{-}7}$*ced-3*-2 and $P_{mec\text{-}7}$*ced-3*-3 in a *ced-4* mutant background. Similarly, in line $P_{mec\text{-}7}$*ced-3*-3 ALMs survived on 9/46 (20%) of sides scored in a wild-type background and on 8/30 (27%) of sides scored in a *ced-4 ced-9; ced-3* background (Table 7), whereas in the same two backgrounds in line $P_{mec\text{-}7}$*ced-4*-1, ALMs survived on 4/39 (10%) and 27/38 (71%) of sides scored, respectively (Table 5). This observation suggests that killing by a *ced-4* construct might be more dependent on endogenous *ced-3* than killing by *ced-3* is dependent on endogenous *ced-4*.

When we compared the extent of ALM survival in $P_{mec\text{-}7}$*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 had reduced killing, although this effect was still smaller than the effect a *ced-3* mutant has on $P_{mec\text{-}7}$*ced-4*-induced 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 $P_{mec\text{-}7}$*ced-3*-containing lines. In line $P_{mec\text{-}7}$*ced-3*-2, for example, ALMs survived on 0/37 (0%) of sides scored in a *ced-9; ced-3* background, but on 12/32 (37%) of sides scored in a *ced-4 ced-9; ced-3* background. These results suggest that in the absence of endogenous *ced-9*, *ced-4* can, to some extent, influence killing by *ced-3* overexpression, suggesting that endogenous *ced-4* can help killing by overexpression of *ced-3*. (see Discussion).

Protection by *ced-9* against *ced-3*-induced ALM killing requires *ced-4* function

As described above, endogenous *ced-9* function inhibited killing by overexpression of *ced-3* and *ced-4*. Specifically, ALM survival in $P_{mec\text{-}7}$*ced-3* and $P_{mec\text{-}7}$*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 8A shows that whereas for $P_{mec\text{-}7}$*ced-3*-containing *ced-4*(+) lines ALMs survive better if *ced-9*(+) function is present, ALMs in $P_{mec\text{-}7}$*ced-3*-containing *ced-4*(-) lines survive to similar extents in *ced-9*(+) and *ced-9* mutant backgrounds. For example, in line $P_{mec\text{-}7}$*ced-3*-2 ALMs survived on 5/29 (17%) of sides scored in a *ced-4; ced-3* background and on 12/32 (37%) of sides scored in a *ced-4 ced-9; ced-3* background, i.e. *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 the *ced-3* activity in *ced-3*-overexpression strains by acting at least in part via *ced-4*. A similar analysis using $P_{mec\text{-}7}$*ced-4* transgenes is presented in Table 8B. However, since killing by a $P_{mec\text{-}7}$*ced-4* transgene in a *ced-3*(-) background was inefficient, we cannot assess whether *ced-3* is required for protection from *ced-4* killing by *ced-9*.

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

Since endogenous *ced-9* could protect against cell death induced by *ced-4*-overexpression we wanted to assess whether overexpression of *ced-9* would have a similar effect. 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 $P_{mec\text{-}7}$*ced-9* fusion (carried as an extrachromosomal array) on survival of ALMs in line $P_{mec\text{-}7}$*ced-4*-3 in a *ced-4 ced-9* background. We found that in lines transgenic for both $P_{mec\text{-}7}$*ced-4* and $P_{mec\text{-}7}$*ced-9*, more ALMs survived than in the

absence of $P_{mec\text{-}7}$*ced-9* or in the presence of a $P_{mec\text{-}7}$*ced-9* construct containing a frameshift mutation in the *ced-9* gene (see Table 9 and Experimental Procedures). These results suggest that overexpression of *ced-9* is sufficient to protect *mec-7*-expressing cells from killing by *ced-4* overexpression. We were not able to protect against killing by overexpression of $P_{mec\text{-}7}$*ced-3*-3 with a $P_{mec\text{-}7}$*ced-9* transgene (data not shown).

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

To see if 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, personal communication) (Figure 1B) and obtained lines containing integrated copies of either $P_{unc\text{-}30}$*ced-3* or $P_{unc\text{-}30}$*ced-4*. As Table 10 shows, we observed that $P_{unc\text{-}30}$*ced-3* and $P_{unc\text{-}30}$*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 $P_{unc\text{-}30}$*ced-3* and $P_{unc\text{-}30}$*ced-4* ($P_{unc\text{-}30}$*ced-3/4*-1). 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 eight of the 13 VD neurons in the ventral cord of a transgenic animal carrying both *unc-30* fusion constructs. Figure 2B 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 a $P_{unc\text{-}30}$*ced-3* transgene in *ced-9*(-) animals and that an endogenous *ced-3* mutation inhibited killing by a $P_{unc\text{-}30}$*ced-4* transgene in *ced-9*(-) animals. These results parallel our findings with the *mec-7* promoter fusion constructs. None of our lines 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 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 requirements for *ced-3* and *ced-4* in killing cells by programmed cell death and to examine the interactions between these genes and with the gene *ced-9* we

expressed *ced-3* and *ced-4* 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., 1995). Expression of *ced-3* or *ced-4* killed both ALMs and DDs. 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 allows the ready killing of any cell, relatively few cells and animals can be analyzed using this approach. The use of *ced-3* or *ced-4* transgenes for cell ablations could 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. If a strongly expressing promoter is used, the ablation can be done in a wild-type background (as in the case of the *mec-7* promoter). If a weaker promoter is used, the ablation should be done in either a *ced-9; ced-3* or a *ced-4 ced-9* background (as with the *unc-30* promoter).

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 a characteristic refractile appearance when viewed with Nomarski optics, as do normal programmed cell deaths. Second, the kinetics of the cell death process, from the initial appearance of a refractile body to the disappearance of the cell were similar to the kinetics of normal cell deaths that occur during the same developmental stage. Third, 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. Fourth, mutations in a gene required for the engulfment of corpses resulting from normal cell deaths prevented the engulfment of corpses from ectopic cell deaths, suggesting that the ectopic cell deaths resembled normal cell deaths. Fifth, a mutation that prevents the degradation of the DNA of cells that normally die also prevented the degradation of the DNA of ALM cells killed ectopically. Sixth, the extent of killing by *ced-3* or *ced-4* overexpression was influenced by endogenous mutations in genes (*ced-3*, *ced-4* and *ced-9*) known to affect normal programmed cell deaths, strongly suggesting

that the molecular components responsible for the ectopic cell deaths correspond to those involved in normal programmed cell deaths.

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 strongly suggest 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, since overexpression of *ced-9* in *mec-7*-expressing cells rescued killing by overexpression of *ced-4* in the same cells.

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). Nonetheless, *ced-4* trangenes cause the deaths of ALMs in wild-type animals (see Table 1). Since *ced-3* acts cell autonomously, together these findings suggest 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, 2B), and *ced-9* acts 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 required *ced-4* function (see Table 8), and yet ALMs did not require the expression of a *ced-4* transgene to be protected by *ced-9* (see Table 2A). Since *ced-4* also acts cell autonomously, together these findings suggest that wild-type ALMs 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*.

Presumably these killing functions are inhibited in the ALMs directly or indirectly by the protective function of *ced-9*. Since 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 cells, and perhaps more generally in all C. elegans cells, there is a

competition between functions that activate (e.g., *ced-3* and *ced-4*) and functions that inhibit (e.g., *ced-9*) programmed cell death. 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 for the insertions $P_{mec\text{-}7}$*ced-3*-2, $P_{mec\text{-}7}$*ced-3*-3, and $P_{mec\text{-}7}$*ced-4*-1 showed little ALM death. For example, ALMs survived on 23/25 (92%) of sides scored in animals heterozygous for the $P_{mec\text{-}7}$*ced-4*-1 transgene in a wild-type background, and ALMs survived on 19/20 (95%) sides scored in animals heterozygous for the $P_{mec\text{-}7}$*ced-3*-3 transgene in a wild-type background, 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 (reviewed by Vaux and Weissman, 1993), suggesting that the protein components needed for cell death are present in living cells. Similarly, that many, and perhaps all, mammalian cells are protected by exogenous growth factors from dying by programmed cell death has led Raff (1992) to propose that all cells contain cell-death killing factors and thus are "poised for death."

A 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* may require endogenous *ced-3* function. These results suggest either that (1) *ced-4* acts upstream of *ced-3* and *ced-4* function can be bypassed by high levels of *ced-3* activity, or that (2) *ced-3* and *ced-4* act in parallel, with *ced-3* perhaps having a greater ability to kill. The former model is supported by the observation that *ced-9* acts via *ced-4* to protect against cell death mediated by *ced-3*. Taken together, these findings suggest that programmed cell death in C. elegans involves a linear pathway in which *ced-9* antagonizes the function of *ced-4*, which in turn potentiates or activates *ced-3*.

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? 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 position of *ced-4* in our proposed pathway suggests a similarity between the action of the CED-4 protein and the action of the mammalian Bax protein. Overexpression of Bax results in cell death just as does overexpression of *ced-4*, and *bcl-2* overexpression blocks this death (Oltavi et al., 1993) just as *ced-9* overexpression blocks death caused by *ced-4* overexpression. Although the CED-4 and Bax proteins do not share obvious sequence similarity, they might similarly mediate signaling between a negative regulator of cell death (CED-9/Bcl-2) and a cysteine-protease activator of cell death (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 might exist in mammals, be negatively regulated by *bcl-2*, and positively activate an ICE-like cysteine protease to cause programmed cell death.

Experimental Procedures

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 mutations used have been described by Ellis and Horvitz (1986), Hedgecock et al. (1983), Hengartner et al. (1992), Sulston (1976), or were isolated by us. These mutations are listed below:

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: $P_{mec\text{-}7}$*ced-3*-1 is *nIs33*, $P_{mec\text{-}7}$*ced-3*-2 is *nIs38*, $P_{mec\text{-}7}$*ced-3*-3 is *nIs50*, $P_{mec\text{-}7}$*ced-4*-1 is *nIs31*, $P_{mec\text{-}7}$*ced-4*-2 is *nIs44*, $P_{mec\text{-}7}$*ced-4*-3 is *nIs47*, $P_{mec\text{-}7}$*ced-4*-4 is *nIs45*, $P_{mec\text{-}7}$*ced-3*/*4*-1 is *nIs32*, $P_{mec\text{-}7}$*ced-3*/*4*-2 is *nIs29*, $P_{unc\text{-}30}$*ced-4*-1 is *nIs46*, and $P_{unc\text{-}30}$*ced-4*-2 is *nIs48*.

The integrated *mec-7-lacZ* fusion construct is designated as allele *jeIs1* (J. Way, personal communication).

Plasmid Constructions

$P_{mec\text{-}7}$*ced-3*: the vector pPD52.102 (A. Fire, M. Hamelin, and J. Culotti, personal communication) 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. $P_{mec\text{-}7}$*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. $P_{unc\text{-}30}$*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, personal communication). $P_{unc\text{-}30}$*ced-4*: plasmid pS125 was digested with the enzyme SpeI as above and ligated to the same XbaI fragment used to construct $P_{unc\text{-}30}$*ced-3*. $P_{mec\text{-}7}$*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. $P_{mec\text{-}7}$*ced-9(lf)*: plasmid $P_{mec\text{-}7}$*ced-9* was digested with BamHI, the overhangs were re-polymerized 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' GCTCCTGAGCCCAACGATTTGTTTCTTCTTGAACT 3') was used to introduce the mutation, and plasmid pS125 was used as the template.

Germline transformation and integration of extrachromosomal arrays

Our procedure for microinjection and germline 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 50-100 µg/ml. All constructs were co-injected 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 N2. 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 $P_{mec\text{-}7}ced\text{-}9$ and $P_{mec\text{-}7}ced\text{-}9(lf)$ 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, personal communication). 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 $P_{mec\text{-}7}ced\text{-}3$, $P_{mec\text{-}7}ced\text{-}4$, $P_{unc\text{-}30}ced\text{-}3$, or $P_{unc\text{-}30}ced\text{-}4$ 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. 30-50 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 shown to segregate homozygous Rol animals at a frequencey of approximately 1 in 3 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 approximately 30,000 F2 animals to obtain three independent $P_{mec\text{-}7}ced\text{-}3$ integrants, 30,000 F2 animals to obtain four independent $P_{mec\text{-}7}ced\text{-}4$ integrants, 10,000 F2 animals to obtain two independent $P_{unc\text{-}30}ced\text{-}3$ integrants, 10,000 F2 animals to obtain 2 independent $P_{unc\text{-}30}ced\text{-}4$ integrants, 5,000 F2 animals to obtain two independent integrants containing both the $P_{mec\text{-}7}ced\text{-}3$ and $P_{mec\text{-}7}ced\text{-}4$ constructs, and 1,000 F2 animals to obtain one integrant containing both $P_{unc\text{-}30}ced\text{-}3$ and $P_{unc\text{-}30}ced\text{-}4$ constructs. None of the integrated lines used in our experiments exhibited any obvious phenotype 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. Occasionally, we scored both sides of an individual animal; however, we avoided scoring sides that were not easily visible. When scoring ALM survival in lines containing extrachromosomal arrays of a given construct we had to address the fact that not all L1 animals we scored would contain the transgene. After scoring L1s for the presence or absence of ALMs, we therefore allowed these animals 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) for an integrated line were scored using Nomarski optics for the presence of 15 neuronal nuclei located between the retro-vesicular ganglion and the pre-anal ganglion. Four of these 15 nuclei are DD nuclei (Sulston and Horvitz, 1977), some of which are missing in strains containing $P_{unc\text{-}30}$*ced-3* or $P_{unc\text{-}30}$*ced-4* constructs.

We also directly observed the deaths of PVM neurons in *ced-9(n2812); ced-3(n717)* $P_{mec\text{-}7}$*ced-3/4*-1 and in *ced-4(n1162) ced-9(n2812)*; $P_{mec\text{-}7}$*ced-4*-1 animals by following the QL cell lineage 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 weakly expressed in this cell (M. Chalfie, personal communication), supporting our hypothesis that the level of overexpression of *ced-3* or *ced-4* is important for the penetrence of cell killing. We also directly observed the deaths of the VD neurons in one *ced-9(n2812); ced-3(n717)*; $P_{unc\text{-}30}$*ced-3/4*-1 animal by following the P5-P12 cell lineages in that animal.

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

	# ALMs / # sides scored
	Wild type
–	31/31
$P_{mec\text{-}7}lacZ$	40/40
$P_{mec\text{-}7}ced\text{-}3\text{-}1$	48/48
$P_{mec\text{-}7}ced\text{-}3\text{-}2$	16/38
$P_{mec\text{-}7}ced\text{-}3\text{-}3$	9/46
$P_{mec\text{-}7}ced\text{-}4\text{-}1$	4/39
$P_{mec\text{-}7}ced\text{-}4\text{-}2$	33/38
$P_{mec\text{-}7}ced\text{-}4\text{-}3$	39/40
$P_{mec\text{-}7}ced\text{-}4\text{-}4$	40/41
$P_{mec\text{-}7}ced\text{-}3/4\text{-}1$	35/37
$P_{mec\text{-}7}ced\text{-}3/4\text{-}2$	45/46

Each row represents an independently derived line containing a given integrated transgene or pair of transgenes. Fractions indicate the number of ALMs per number of animal sides scored. Each animal normally has two ALMs, one on the left and one on the right side. Only sides that were easily visible were scored for the presence of an ALM. Line *jeIs1* contains a *lacZ* fusion to the *mec-7* promoter (J. Way, personal communication). For each line identified as $P_{mec\text{-}7}ced\text{-}x$-y, *x* is either 3 or 4 (for *ced-3* or *ced-4*) and y identifies the specific independent line; in rows 10 and 11, *x* is 3/4 indicating the presence of both *ced-3* and *ced-4* transgenes.

Table 2A. ALM killing by *ced-3* overexpression is better in a *ced-9*(lf) background

	# ALMs / # sides scored	
	ced-9; ced-3	*ced-3*
$P_{mec\text{-}7}$*ced-3-1*	9/43	34/34
$P_{mec\text{-}7}$*ced-3-2*	0/37	8/27
$P_{mec\text{-}7}$*ced-3-3*	0/29	16/34
$P_{mec\text{-}7}$*ced-3/4-1*	16/37	40/40
$P_{mec\text{-}7}$*ced-3/4-2*	18/27	46/46

The 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 $P_{mec\text{-}7}$*ced-3-3*, in which *ced-3(n3002)* was used.

Table 2B. ALM killing by *ced-4* overexpression is better in a *ced-9(lf)* background

	# ALMs / # sides scored	
	ced-4 ced-9	*ced-4*
$P_{mec\text{-}7}$*ced-4-1*	0/30	12/28
$P_{mec\text{-}7}$*ced-4-2*	18/34	32/34
$P_{mec\text{-}7}$*ced-4-3*	15/36	36/37
$P_{mec\text{-}7}$*ced-4-4*	4/27	36/36
$P_{mec\text{-}7}$*ced-3/4-1*	35/50	41/41
$P_{mec\text{-}7}$*ced-3/4-2*	37/50	30/30

The 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***

	# ALMs / # sides scored	
	Wild type	***ced-9(n1950)***
$P_{mec\text{-}7}$***ced-3-1***	48/48	42/42
$P_{mec\text{-}7}$***ced-3-2***	16/38	10/37
$P_{mec\text{-}7}$***ced-3-3***	9/46	18/31
$P_{mec\text{-}7}$***ced-4-1***	4/39	2/47
$P_{mec\text{-}7}$***ced-4-2***	33/38	39/41
$P_{mec\text{-}7}$***ced-4-3***	39/40	41/41
$P_{mec\text{-}7}$***ced-4-4***	40/41	42/42
$P_{mec\text{-}7}$***ced-3/4-1***	36/37	46/46
$P_{mec\text{-}7}$***ced-3/4-2***	45/46	34/35

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

Table 4A. ALM killing by *ced-3* overexpression requires a functional CED-3 protein

	# ALMs / # sides scored
	ced-9; ced-3
$P_{mec\text{-}7}$*ced-3*(+)-1	5/12
$P_{mec\text{-}7}$*ced-3*(+)-2	4/13
$P_{mec\text{-}7}$*ced-3*(+)-3	9/15
$P_{mec\text{-}7}$*ced-3*(C358A)-1	18/18
$P_{mec\text{-}7}$*ced-3*(C358A)-2	24/24
$P_{mec\text{-}7}$*ced-3*(C358A)-3	17/17

Each 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 (+, figure 1) or a *ced-3* cDNA encoding a C358A mutation in the CED-3 protein.

Table 4B. ALM killing by *ced-4* overexpression requires functional CED-4 protein

	# ALMs / # sides scored	
	ced-4	*ced-9*
$P_{mec\text{-}7}$*ced-4*(+)-1	7/14	
$P_{mec\text{-}7}$*ced-4*(+)-2	9/16	
$P_{mec\text{-}7}$*ced-4*(+)-3	9/21	
$P_{mec\text{-}7}$*ced-4*(I258N)-1	17/17	
$P_{mec\text{-}7}$*ced-4*(I258N)-2	9/9	
$P_{mec\text{-}7}$*ced-4*(I258N)-3	17/17	

Each 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 (+, figure 1) or a *ced-3* cDNA encoding an I258N mutation in the CED-4 protein.

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

	# ALMs / # sides scored			
	Wild type	***ced-3***	***ced-4 ced-9***	***ced-4 ced-9; ced-3***
$P_{mec\text{-}7}$***ced-4*-1**	**4/39**	**27/38**	**0/30**	**27/38**
$P_{mec\text{-}7}$***ced-4*-2**	**33/38**	**20/20**	**18/34**	**27/32**
$P_{mec\text{-}7}$***ced-4*-3**	**39/40**	**37/37**	**15/36**	**39/40**
$P_{mec\text{-}7}$***ced-4*-4**	**40/41**	**36/36**	**4/27**	**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	No. extra cells in anterior of pharynx	# ALMs / #sides scored
Wild type	**NA**	**0.1 ± 0.4 (40)**	**4/39**
ced-3 (n1040)	**L27F**	**7.6 ± 1.2 (11)**	**35/64**
ced-3 (n1129)	**A449V**	**8.7 ±1.2 (10)**	**20/30**
ced-3 (n717)	**Exon 7 splice acceptor G to A**	**11.2 ± 2.0 (10)**	**27/38**
ced-3 (n718)	**G65R**	**11.8 ± 1.0 (10)**	**47/64**
ced-3 (n2433)	**G360S**	**12.4 ± 0.9 (10)**	**33/41**

Rows indicate the genotype of the strain examined. Numbers in column 2 are the average number of extra cells in the anterior region of the pharynx in a given mutant ± SD. The number of animals examined is in parentheses. Numbers in column 3 are the number of ALMs per number of sides scored in a $P_{mec\text{-}7}$*ced-4*-1 background in a given mutant line. NA, not applicable.

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

	# ALMs / # sides scored			
	Wild type	***ced-4***	***ced-9; ced-3***	***ced-4 ced-9; ced-3***
$P_{mec\text{-}7}$*ced-3*-1	**48/48**	**35/39**	**9/43**	**28/33**
$P_{mec\text{-}7}$*ced-3*-2	**16/38**	**18/61**	**0/37**	**12/32**
$P_{mec\text{-}7}$*ced-3*-3	**9/46**	**24/56**	**0/29**	**8/30**

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 $P_{mec\text{-}7}$*ced-3*-3, in which the *ced-3* allele *n3002* was used.

Table 8A. ***ced-9*** **inhibition of ALM killing by** ***ced-3*** **overexpression requires** ***ced-4*** **function**

	# ALMs / # sides scored					
	ced-3	*ced-9; ced-3*	*ced-4*	*ced-4 ced-9*	*ced-4; ced-3*	*ced-4 ced-9; ced-3*
$P_{mec\text{-}7}$*ced-3*-1	34/34	9/43	35/39	73/78	40/41	28/33
$P_{mec\text{-}7}$*ced-3*-2	8/27	0/37	18/61	9/47	5/29	12/32
$P_{mec\text{-}7}$*ced-3*-3	16/34	0/29	24/56	10/39	13/27	8/30

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 $P_{mec\text{-}7}$*ced-3*-3 in which the *ced-3* allele *n3002* was used.

Table 8B. Effects of *ced-3* on *ced-9* inhibition of ALM killing by *ced-4*

 # ALMs / # sides scored

	ced-4	*ced-4 ced-9*	*ced-4; ced-3*	*ced-4 ced-9; ced-3*
$P_{mec\text{-}7}$*ced-4-1*	12/28	0/30	32/40	27/38
$P_{mec\text{-}7}$*ced-4-2*	32/34	18/34	39/40	27/32
$P_{mec\text{-}7}$*ced-4-3*	36/37	15/36	37/38	39/40
$P_{mec\text{-}7}$*ced-4-4*	36/36	4/27	ND	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)*, ND, not determined.

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

	# ALMs / # sides scored
	ced-4 ced-9
$P_{mec\text{-}7}$*ced-4*-4	4/27
$P_{mec\text{-}7}$*ced-4*-4; $P_{mec\text{-}7}$*ced-9*-1	14/20
$P_{mec\text{-}7}$*ced-4*-4; $P_{mec\text{-}7}$*ced-9*-2	17/27
$P_{mec\text{-}7}$*ced-4*-4; $P_{mec\text{-}7}$*ced-9*-3	20/24
$P_{mec\text{-}7}$*ced-4*-4; $P_{mec\text{-}7}$*ced-9(lf)*-1	3/24
$P_{mec\text{-}7}$*ced-4*-4; $P_{mec\text{-}7}$*ced-9(lf)*-2	1/20
$P_{mec\text{-}7}$*ced-4*-4; $P_{mec\text{-}7}$*ced-9(lf)*-3	4/21

Each row indicates an independently derived transgenic line containing the integrated transgene $P_{mec\text{-}7}$*ced-4*-4 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); nIs45; lin-15(n765)* background. $P_{mec\text{-}7}$*ced-9* arrays contain a wild-type *ced-9* cDNA. $P_{mec\text{-}7}$*ced-9(lf)* arrays contain a frameshift mutation in an otherwise wild-type *ced-9* cDNA. Each array was coinjected with a plasmid containing the *lin-15* gene (see Experimental Procedures).

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

	# DDs scored/# expected			
	ced-9; ced-3	*ced-3*	*ced-4 ced-9*	*ced-4*
$P_{unc\text{-}30}$*ced-3-1*	11/60	38/40	88/88	39/40
$P_{unc\text{-}30}$*ced-3-2*	7/40	39/40	40/40	39/40
$P_{unc\text{-}30}$*ced-4-1*	40/40	40/40	6/40	40/40
$P_{unc\text{-}30}$*ced-4-2*	40/40	40/40	1/40	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)*.

Figure 1. Overexpression constructs.

(A) $P_{mec\text{-}7}$*ced-3* and $P_{mec\text{-}7}$*ced-4* constructs. (B) $P_{unc\text{-}30}$*ced-3* and $P_{unc\text{-}30}$*ced-4* constructs. Open boxes indicate the *mec-7* or *unc-30* promoters, gray boxes indicate *ced-4* sequences, hatched boxes indicate *ced-3* sequences, black boxes indicate the *unc-54* 3' untranslated region (UTR). See Experimental Procedures for details.

Figure 1

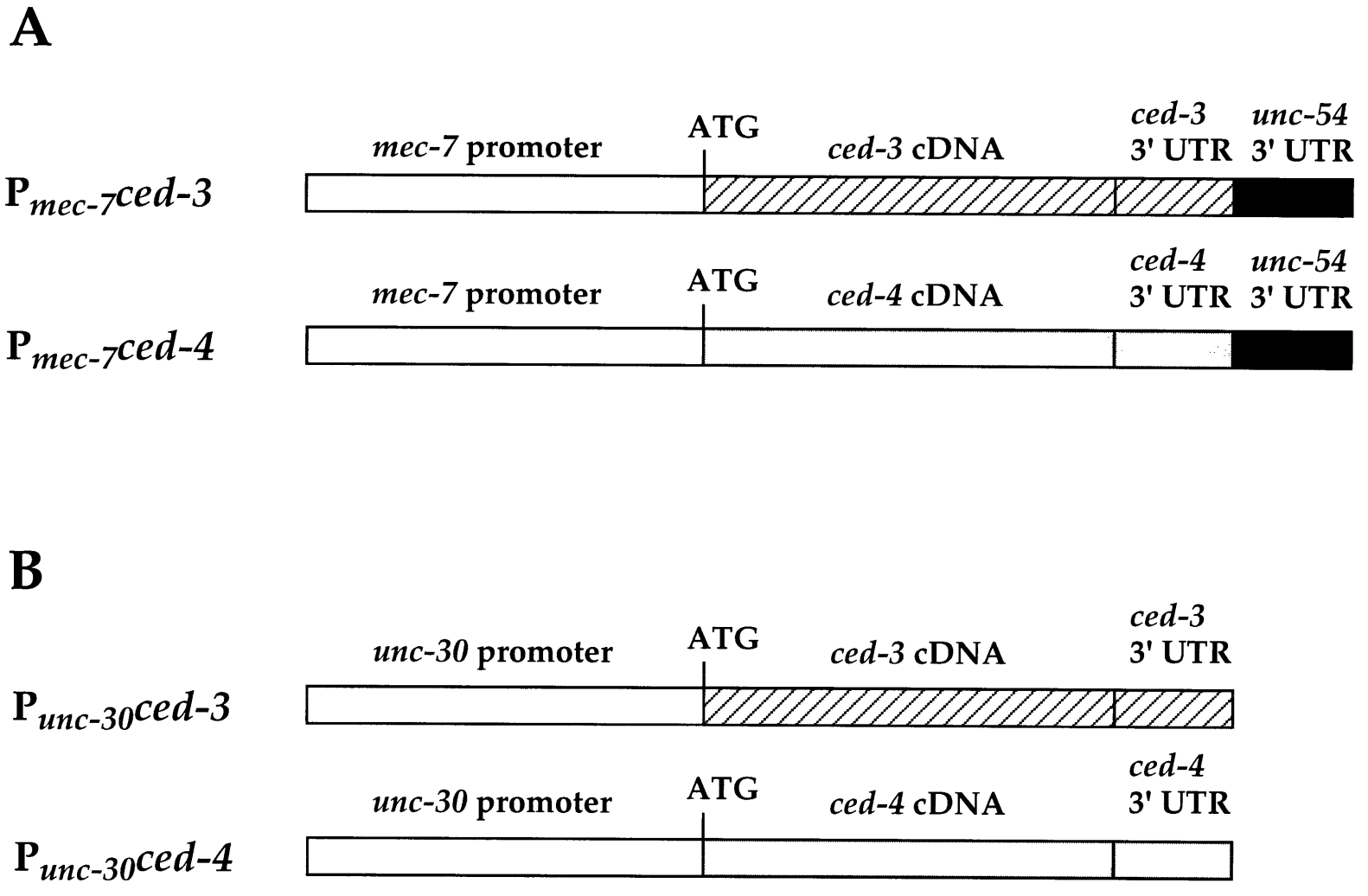
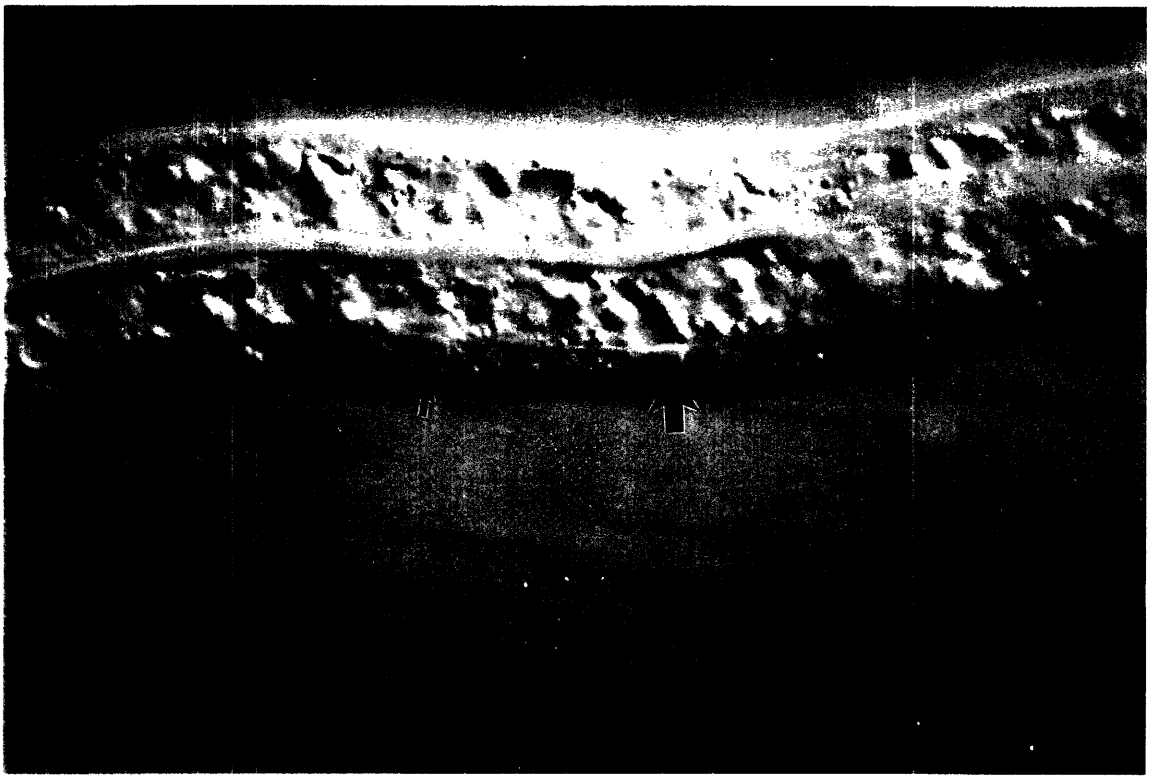

A
P mec-7 ced-3
mec-7 promoter
ATG
ced-3 cDNA
ced-3 3' UTR
unc-54 3' UTR
P mec-7 ced-4
mec-7 promoter
ATG
ced-4 cDNA
ced-4 3' UTR
unc-54 3' UTR
B
P unc-30 ced-3
unc-30 promoter
ATG
ced-3 cDNA
ced-3 3' UTR
P unc-30 ced-4
unc-30 promoter
ATG
ced-4 cDNA
ced-4 3' UTR

Figure 2. 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 $P_{mec\text{-}7}$*ced-3/4*-1 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 $P_{unc\text{-}30}$*ced-3/4* transgene. Anterior is to the left; dorsal is on top.

(C) Electron microscope photomicrograph 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).



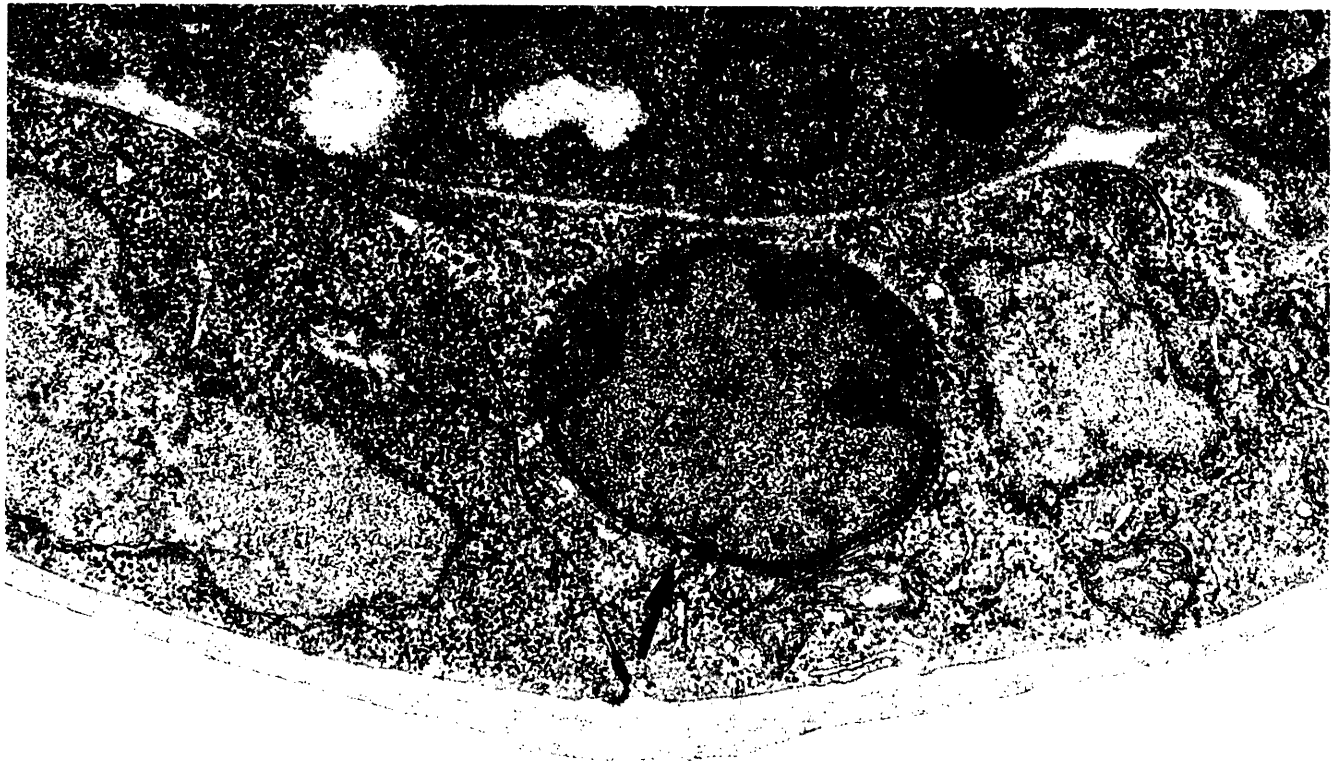
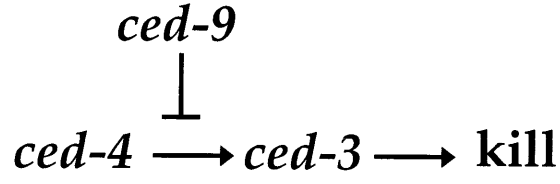


Figure 3. Model for programmed cell death in C. elegans

ced-4 acts upstream of *ced-3* to activate it and *ced-9* is genetically upstream of *ced-4*, suggesting that *ced-9* can negatively regulate *ced-4* by acting biochemically upstream (left) or downstream (right) of *ced-4*. See text for additional details.

Figure 3

OR



Chapter 4

The *C. elegans* cell death gene *ced-4* encodes both death-promoting and death-preventing transcripts

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This chapter will be submitted for publication

MUTATIONS in the gene *ced-4* prevent programmed cell death in *C. elegans*. Sequence comparison of *ced-4* genes from the related nematodes *C. briggsae* and *C. vulgaris* revealed that the third intron of these genes is conserved at its 3' end. We have shown that this region can serve as an alternatively spliced exon resulting in a novel *ced-4* transcript which is 72 nucleotides larger than the one previously described. Overexpression of this transcript can prevent programmed cell death and can suppress the lethality associated with ectopic cell deaths in *ced-9(lf)* mutants, suggesting that this transcript has an opposite role to that of the previously described transcript. Genetic analysis of the *ced-4* mutation *n2273*, as well as an analysis of the interactions of *ced-4* mutations with other cell death mutations suggest that two functions are encoded by *ced-4 in vivo*. We propose that these functions correspond to the two transcripts described above and that the balance between these functions can influence the decision of a cell to live or die.

Programmed cell death is a common aspect of metazoan development and is used, among other things, to regulate cell number, to rid organisms of harmful or unwanted cells, and to shape tissues and organs[1,2]. In the nematode *Caenorhabditis elegans*, of the 1090 somatic cells generated during hermaphrodite development 131 undergo programmed cell death[3,4]. Genes involved in several aspects of the death process have been previously defined[2,5-9]. Loss-of-function (lf) mutations in the genes *ced-3* and *ced-4* as well as gain-of-function (gf) mutations in the gene *ced-9* prevent programmed cell death[5,9]. Loss-of-function mutations in the gene *ced-9* cause ectopic cell deaths to occur, and result in lethality[9]. These, and other results, suggest that *ced-3* and *ced-4* are necessary for proper execution of the cell killing program in *C. elegans*, and that *ced-9* is necessary to inhibit this program[9,10]. The CED-9 protein is similar in sequence to the mammalian proto-oncoprotein Bcl-2 that can function to prevent programmed cell death[11-15]. The CED-3 protein is similar to the Interleukin-1β (IL-1β) converting enzyme (ICE) class of cysteine proteases which have been shown to induce programmed cell death in culture[16-21]. These sequence and function similarities between the nematode and mammalian cell-death proteins suggest that the molecular mechanism of programmed cell death has been conserved from worm to man. No mammalian protein similar to the *C. elegans* protein CED-4 has yet been described.

To define regions of the *ced-4* gene which are important for function we cloned *ced-4* homologues from the related nematodes *C. briggsae* and *C. vulgaris*. Analysis of the genomic sequences of these clones revealed that the third intron of the *ced-4* genes (as defined by previously described *C. elegans ced-4* cDNAs[22]) was conserved at its 3' end among all three nematode species, suggesting that this region is important for *ced-4* function. Specifically, intron 3 of *C. elegans*, *C. briggsae* and *C. vulgaris* is 186 bp, 177 bp, and 205 bp in length, respectively. The 3' 72 bp of these introns are highly conserved (see figure 1b). Immediately upstream of the conserved region is a consensus splice acceptor sequence (figure 1b) suggesting that the conserved region might be used as an exon. A transcript (*ced-4*L) resulting from this alternative splice would contain an in-frame insertion of 72 nucleotides relative to the previously described transcript (*ced-4*S) and would encode a protein with a 24 amino-acid insertion relative to the previously described protein.

To confirm that the *C. elegans ced-4* gene encodes a transcript containing the conserved sequences of intron 3 we hybridized a radioactive probe consisting of the 72 bp conserved sequence to a northern blot of mixed-stage *C. elegans* polyA+ RNA prepared from wild-type animals. As shown in figure 1c, a band slightly larger than *ced-4*S can be seen. The abundance of *ced-4*L, as judged by densitometry, is 10-30 fold less than that of *ced-4*S. To confirm that *ced-4*L is produced by splicing at an acceptor

site at position 114 of intron 3 we prepared cDNAs from the RNA used for the northern blot, and amplified the region surrounding intron 3 by the polymerase chain reaction (PCR) using primers flanking intron 3 (figure 1d). In addition we used primers complementary to the conserved region with primers outside intron 3 to amplify cDNAs containing segments of intron 3 (data not shown). Sequence determination of these transcripts confirmed that they were spliced as predicted.

We have previously shown that overexpression of *ced-4*S in the *mec-7*-expressing ALM neurons[23] in animals carrying a *mec-7*-promoter::*ced-4*S-cDNA fusion construct can kill these cells by programmed cell death[10]. To determine the function of *ced-4*L we fused a *ced-4*L cDNA to the *mec-7* promoter and established transgenic animals carrying this fusion construct. None of the ALM neurons died, suggesting that *ced-4*L can not kill cells by programmed cell death (data not shown). We next fused a *ced-4*L cDNA to two *C. elegans* heat shock promoters (A. Fire and P. Candido, personal communication) and established transgenic lines containing both fusion constructs. Interestingly, extra cells accumulated in transgenic embryos subjected to a heat shock, suggesting that programmed cell death had been blocked in these animals (table 1a).

To determine if overexpression of *ced-4*L blocked programmed cell death we introduced a construct containing a *ced-4*L cDNA fused to the constitutive promoter of the *dpy-30* gene (D. Hsu and B. Meyer, personal communication) into animals containing a loss-of-function mutation in the gene *ced-9*. Animals homozygous for *ced-9(lf)* mutations die because of massive ectopic cell death[9]. *ced-9(lf)* mutants carrying the $P_{dpy\text{-}30}$*ced-4*L transgene were rescued from lethality, suggesting that *ced-4*L can prevent programmed cell death (table 1b).

The above results suggest that *ced-4* encodes two alternative transcripts, *ced-4*S and *ced-4*L, with opposing functions (figure 1a). *ced-4*S can kill cells by programmed cell death[10], whereas *ced-4*L can protect cells from undergoing programmed cell death. The mammalian gene *bcl-x* also encodes two transcripts with opposite cell-death functions[24], however, neither *ced-4* product has any significant similarity to *bcl-x*.

Ellis and Horvitz[5] showed that the egg-laying defect (resulting from the programmed cell deaths of the HSN neurons which are required for proper egg laying) in animals heterozygous for the mutation *egl-1(n487)*[25] could be enhanced by introducing a single copy of the strong *ced-4* loss-of-function mutation *n1162* (which prematurely terminates both CED-4L and CED-4S at amino acid 79, and makes no RNA[22]) into the strain. Specifically, they showed that *ced-4*/+; *egl-1*/+ animals were more egg-laying defective than *egl-1*/+ animals. Thus, eventhough *ced-4* function was reduced, killing was enhanced. This observation suggested to us that *ced-4* might encode a genetic function that prevents cell death in addition to a function known to

cause cell death. If this function were the same as the function of *ced-4*L then the enhanced death of the HSN neurons in *ced-4* /+; *egl-1* /+ animals could be explained as a reduction in the level of protective *ced-4*L function in these neurons (figure 2). To further correlate the *ced-4*S and *ced-4*L transcripts to the two genetic functions encoded by *ced-4* we examined the *ced-4* mutation *n2273*.

The *n2273* mutation can weakly prevent programmed cell death (table 3, M. Hengartner, personal communication). This mutation changes a conserved G to an A at position 186 of intron 3[22]. We examined *ced-4* transcripts in this mutation by probing a northern blot of polyA+ RNA derived from *n2273* mutant animals with either a full-length *ced-4*S cDNA probe (detecting both *ced-4*S and *ced-4*L), or a probe consisting of the 72 bp conserved region of intron 3 (detecting only *ced-4*L). As shown in figures 1c and 1d, expression of *ced-4*L is enhanced in this strain, however, the total amount of *ced-4* transcript remains the same as in wild-type animals, suggesting that *ced-4*S expression is reduced. To determine the nature of the *ced-4* transcripts encoded by *n2273* mutants we amplified both *ced-4*S and *ced-4*L transcripts from this mutant as described above. We observed three transcripts. One transcript corresponded to *ced-4*L, yet contained a mutation at the conserved G at position 186 of intron 3 causing an arginine to lysine mutation in CED-4L. One transcript (*ced-4*SD) corresponded to *ced-4*S except for a deletion of three bp immediately downstream of the splice acceptor site at position 186 of intron 3 resulting in the deletion of a single amino acid from CED-4S. The third transcript represented a *ced-4*S transcript (*ced-4*I) that was spliced at position 185 instead of 186 of intron 3, resulting in an insertion of a T which would result in a truncated CED-4S protein. No other transcripts were detected (data not shown).

The variant *ced-4*S products and the lower level of their transcripts in *n2273* mutants are consistent with the defect in cell killing defined in *n2273* animals. Because *n2273* animals produce a mutated *ced-4*L product, we surmised that these mutants might also be defective in the protective function of *ced-4*L. To test this idea we sensitized the *n2273* background by introducing a weak and viable allele of the *ced-9* gene (*n1653*)[9] into this background. Eventhough *n1653* animals or *n2273* animals produce viable progeny on their own, the doubly mutant *n2273 n1653* animals produced only dead progeny (table 2, M. Hengartner, personal communication). This synthetic lethality is identical to that seen in mutants carrying stronger mutations in *ced-9*, suggesting that *n2273* can enhance the cell-killing effect of *ced-9(lf)* mutations, and consistent with the idea that *n2273* produces a mutant *ced-4*L product. To test if this synthetic lethality resulted from inappropriate activation of the cell death pathway we introduced the *ced-3* mutations *n2427*[26], and *n2438*[26] into the synthetically lethal strain. As shown in table 2, *n2273 n1653*; *ced-3* triple mutants are alive, suggesting that the

synthetic lethality of *n2273 n1653* animals is due to inappropriate activation of the cell death pathway. Furthermore, this experiment suggests that *ced-4*L might act between *ced-9* and *ced-3* in the cell death pathway, as we have similarly proposed for *ced-4*S[10] (figure 2).

To place *ced-4*L function in the cell death pathway more precisely we examined the phenotypes of animals containing both *n2273* and other cell death mutations. Hengartner and Horvitz[26] have previously shown that animals containing a weak mutation in *ced-3* have surprisingly fewer surviving cells than animals carrying both a *ced-9*(lf) and the same *ced-3*(weak) mutations. They suggested that an explanation for this result is that *ced-9* might encode a death-promoting function in addition to its death-preventing function. It is possible, however, that this effect is due not to the loss of a death-promoting function of *ced-9*, but to an increase in the death-preventing function of *ced-4*L. Specifically, if *ced-9* could negatively regulate *ced-4*L, a reduction in *ced-9* function would cause an increase in the protective function of *ced-4*L and lead to more survival. To test this notion we examined the effect of the *n2273* mutation on the enhanced survival described above. *n1653; ced-3* mutants show enhanced cell survival relative to *ced-3* mutants alone (table 3, ref. 26). However, *n2273 n1653; ced-3* mutant animals show the same extent of cell survival as *n2273; ced-3* mutants (table 3), suggesting that *ced-4*L might be the cause of the cell-survival enhancement in *n1653; ced-3* mutants. This result supports the notion that *ced-9* normally negatively regulates the activity of *ced-4*L (figure 2).

The results described so far are consistent with a model for programmed cell killing shown in figure 2. Our model suggests that CED-9 can act to negatively regulate both CED-4L and CED-4S, and that it is the regulation of the balance between the activities of the latter two (by CED-9, by factors responsible for alternative splicing of the *ced-4* transcripts, or by other factors) which determines if a cell will live or die. A prediction of this model is that if *ced-9* can no longer negatively regulate *ced-4*L, then cell survival should be enhanced. This enhancement should be reduced by the *n2273* mutation which causes a defect in *ced-4*L function. The gain-of-function mutation *ced-9(n1950)* results in the enhancement of cell survival and changes a conserved glycine to an arginine in the BH2 domain required for Bcl-2-Bax interactions[27]. Interestingly, *n2273 n1950* double mutants contain fewer extra cells than *n1950* animals alone (table 3). This result suggests that perhaps *n1950* is defective in the inhibition of *ced-4*L, and might explain how a single-amino-acid substitution can "activate" the *ced-9* product by actually inactivating an interaction with *ced-4*L. Interestingly, *n1950* does not prevent cell death in the gonad (M. Hengartner, personal communication). If *ced-4*L is not produced by the gonad, this result would be expected since *n1950* and wild-type *ced-9*

should behave the same in the absence of *ced-4*L. A careful analysis of *ced-4*L expression could lend support for this interpretation.

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FIG. 1 *a*, A schematic drawing showing the splicing pattern of *ced-4*S and *ced-4*L. Open boxes are exons present in both *ced-4*S and *ced-4*L. The closed box is an exon fragment specific to *ced-4*L, and is an intron in the *ced-4*S transcript. V-shaped lines represent introns. The AUG start codon and UAA stop codon are indicated as well. *b*, An alignment of the terminal 72 bp sequence of intron 3 from the nematodes *C. elegans*, *C. briggsae*, and *C. vulgaris*. Numbers in the *C. elegans* sequence correspond to the positions within intron 3. Residues conserved in all species are boxed. The putative *C. elegans* peptide produced by this region is indicated below the alignment. Consensus splice acceptor sites are indicated by a horizontal bar above the alignment. Arrows above the alignment indicate positions of splicing in *ced-4*L and *ced-4*S. The arrow below the alignment indicates the change observed in the mutation *n2273*. *c*, right, top: A northern blot of wild-type and *ced-4(n2273)* polyA+ RNA probed with a full-length *ced-4*S cDNA. left, top: An identical blot of wild-type and *ced-4(n2273)* polyA+ RNA probed with a 72 bp fragment corresponding to the conserved intron shown in *b*. left and right, bottom: same blots as top panels probed with a *C. elegans* ribosomal protein probe as a loading control. *d*, A photograph of an ethidium bromide stained agarose gel showing two products generated by PCR using primers flanking intron 3 from wild-type and *ced-4(n2273)* RNA. These products were cloned into the pBluscript SK(+) vector (Stratagene, La Jolla, CA) and 20 clones of each band were sequenced. In the wild type preparation the top band corresponds to *ced-4*L and the bottom to *ced-4*S (data not shown). In the *n2273* preparation the bottom band consisted of two cDNAs (*ced-4*D and *ced-4*I, see text for details). The top band corresponds to a single mutated *ced-4*L transcript.

METHODS. *b*, *ced-4* genes from *C. briggsae* and *C. vulgaris* were cloned by low stringency hybridization from genomic libraries provided by C. Link and phage DNA was isolated as described in Sambrook et al.[28]. Clones were digested with EcoRI or HindIII, and blotted for a Southern blot as described in Sambrook et al.[28]. These blots were probed with the *C. elegans ced-4*S cDNA and positive bands were excised and ligated to a pBluscript SK(+) (Stratagene, La Jolla, CA) vector cut with EcoRI or HindIII. Insert sequences were determined by shot-gun sequencing[28] on an ABI sequencer (Applied Biosystems, Inc., Foster City, CA). *c*, RNA was prepared from wild-type or from *n2273* animals using the Fast-Track polyA+ RNA isolation system (Invitrogen, San Diego, CA). RNA was blotted as described in Sambrook et al.[28]. The *ced-4*S probe was prepared by random priming[28]. The 72 bp intron probe was prepared by amplifying the genomic *ced-4* clone C10D8-5[22] with primers located at the beginning and end of the sequence shown in *b* in the presence of ^{32}P-labelled dATP. The ribosomal RNA probe was prepared by random priming[28] of an insert provided by M. Koelle. Quantitation of band intensity was done using a phosphoimager set up (Molecular Dynamics, Inc., Sunnyvale, CA). *d*, Primers in exon 2 and exon 6 were used to generate cDNAs and then amplify products from the wild-type and *n2273* RNAs prepared in *c*. Bands were purified and sequenced using an ABI sequencer (Applied Biosystems, Inc., Foster City, CA).

Figure 1a

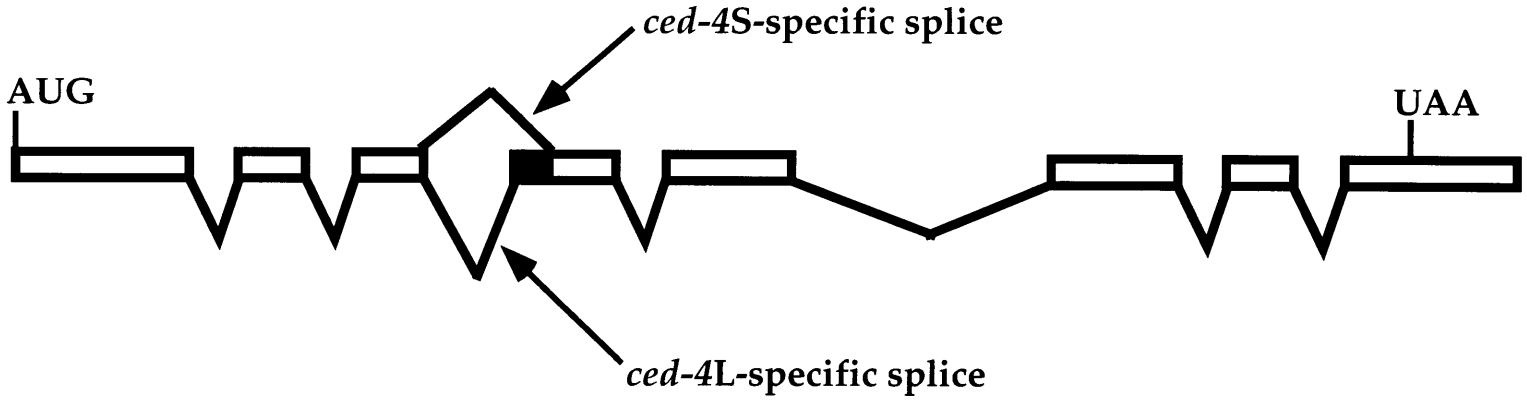

Figure 1b


*ced-4*L splice acceptor site
*ced-4*S splice acceptor site
C. briggsae TTTAAAGACGAGCCCGTGTTGTGAGCGACACGGATGACTCGCACAACATGCCCGACTTCATTAACCGTGTTCTTTCAAG
C. vulgaris TTTGAAGACGAGCCCGTGTTGTGAGCGACACGGATGACTCGCACTCCATCACCGACTTCATTAACCGTGTTCTTTCAAG
C. elegans 108 TTTGAAGACGAGCCCGTGTTGTGAGCGACACGGATGACTCGCATTCGATCACCGACTTCATTAACCGTGTTCTTTCAAG 186
ArgAlaArgValValSerAspThrAspAspSerHisSerIleThrAspPheIleAsnArgValLeuSerArg
n2273: A

Figure 1c

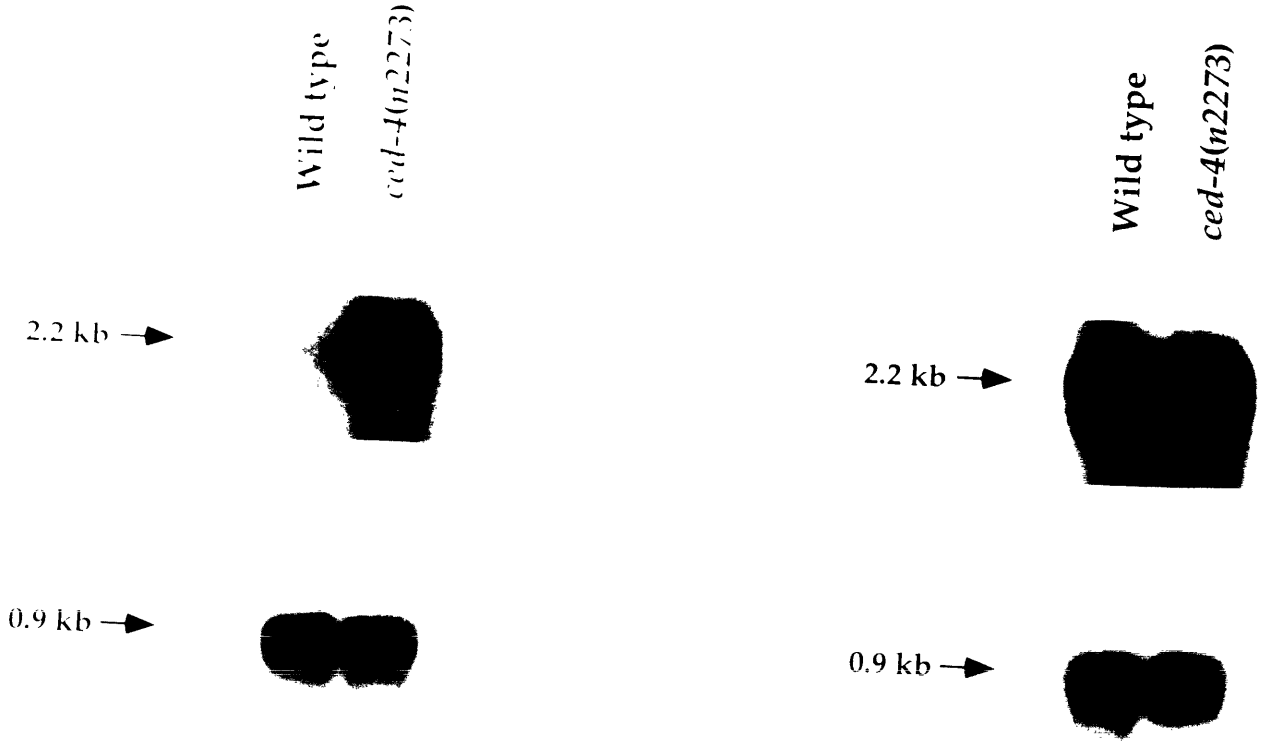
Wild type
ced-4(n2273)
2.2 kb
0.9 kb
Wild type
ced-4(n2273)
2.2 kb
0.9 kb

Figure 1d

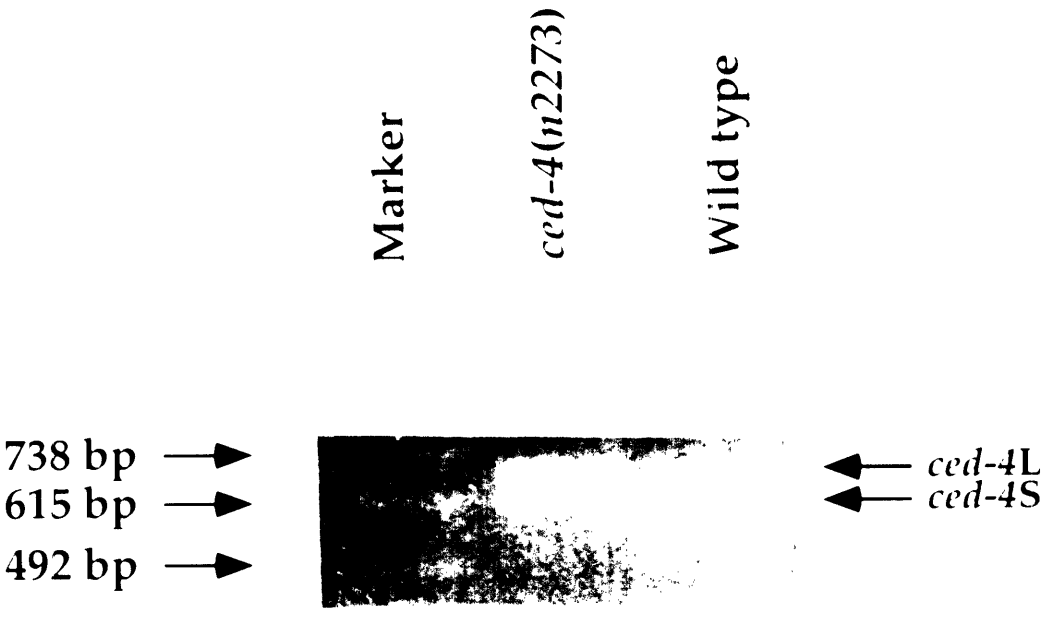

FIG. 2 A model for the regulation of programmed cell killing in *C. elegans*. The *ced-9* gene product can inhibit the activity of either *ced-4*S or *ced-4*L gene products. *ced-3* activity can be enhanced by CED-4S or inhibited by CED-4L, although we can not rule out a model in which CED-4L inhibits CED-4S, thus indirectly inhibiting CED-3, or a model in which CED-4L inhibits a target downstream of CED-3. The bottom pathway is identical to one we have previously proposed[10]. See text for additional details.

Figure 2

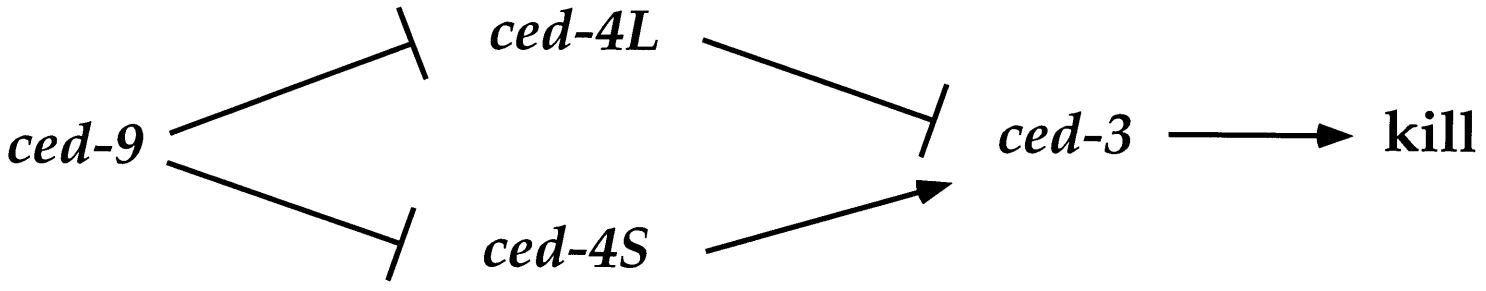

Table 1*a*. Overexpression of *ced-4*L can prevent programmed cell death.

Heat-shock constructs were made by cloning a *ced-4* cDNA into the two heat-shock promoter vectors (A. Fire, personal communication). The parental strain for all transgenics was the Bristol N2 strain. Transgenic lines transmitting the Rol-6 phenotype were obtained as previously described[R]. Adults were allowed to lay embryos for 2 hours at 20°C, subjected to a 60 minute heat shock at 33°C, allowed to lay embryos for an additional 2 hours and removed from the plate. Hatched Rol larvae were scored for extra cells in the anterior pharynx as previously described[29,30]. Numbers in column 1 indicate different transgenic lines. +, with heat shock, -, without heat shock. Numbers in column 3 are average number of extra cells ± standard error of the mean. Parentheses in column 3 indicate the number of animals observed. The numbers in column 4 indicate the range of extra cells seen in individual animals.

Table 1a

Construct	Heat shock	Average # extra cells in anterior pharynx (±SEM)	Range
HSP-*ced-4*L-1	+	7.0 ± 0.9 (21)	0-13
HSP-*ced-4*L-1	-	0.07 ± 0.07 (15)	0-1
HSP-*ced-4*L-2	+	10 ± 0.6 (15)	5-14
HSP-*ced-4*L-2	-	0.2 ± 0.1 (15)	0-1
HSP-*ced-4*L-3	+	7.7 ± 0.7 (15)	4-12
HSP-*ced-4*L-3	-	0.07 ± 0.07 (15)	0-1

Table 1*b*. Overexpression of *ced-4*L can rescue *ced-9*(lf) animals from lethality.

The mutations used have been previously described (ref 9, 11, 26) except for *n2812* which was isolated by us. Transgenic lines were obtained as in table 1*a*. Over 100 animals were scored for each non-transgenic genotype. None of these animals produced viable progeny and were scored as "-". At least 3 independent transgenic lines were isolated for each genotype, and 10-30 animals of each line were scored. Constructs which gave at least two lines that produced viable progeny were scored as "+".

Table 1b

Construct	Genotype	Viable Progeny
none	*ced-9(n1950n2161)*	-
$P_{dpy\text{-}30}$*ced-4*L	*ced-9(n1950n2161)*	+
none	*ced-9(n1950n2077)*	-
$P_{dpy\text{-}30}$*ced-4*L	*ced-9(n1950n2077)*	+
none	*ced-9(n2812)*	-
$P_{dpy\text{-}30}$*ced-4*L	*ced-9(n2812)*	+

Table 2. *ced-4(n2273)* can enhance killing by programmed cell death.

All experiments were done at 20°C. 10-20 animals were allowed to lay embryos for 3-5 days. The number of embryos that progressed past the L3 stage were scored as viable. The alleles used have been prviously described (ref 9,22,26).

Table 2

Maternal genotype	No. embryos observed	No. viable embryos	% survival
ced-4(n2273)	707	694	98
ced-9(n1653)	926	922	99.6
ced-4(n2273) ced-9(n1653)	532	2	0.4
ced-4(n2273) ced-9(n1653); ced-3(n2427)	651	636	98
ced-4(n2273) ced-9(n1653); ced-3(n2438)	583	565	97

Table 3. *ced-4*L might be negatively regulated by *ced-9*.

Numbers in column 2 indicate the average number of extra cells observed for each genotype ± the standard error of the mean. Numbers in column 3 indicate the number of animals observed for each genotype. The alleles used have been previously described (ref 9, 26).

Table 3

Genotype	Average no. extra cells in anterior pharynx (± SEM)	n
ced-3(n2427)	1.2 ± 0.2	19
ced-3(n2438)	2.1 ± 0.4	10
ced-9(n1653); ced-3(n2427)	7.4 ± 0.5	15
ced-9(n1653); ced-3(n2438)	8.2 ± 0.4	15
ced-4(n2273)	2.9 ± 0.4	15
ced-4(n2273); ced-3(n2427)	10.1 ± 0.3	15
ced-4(n2273); ced-3(n2438)	11.9 ± 0.3	15
ced-4(n2273) ced-9(n1653); ced-3(n2427)	8.7 ± 0.4	15
ced-4(n2273) ced-9(n1653); ced-3(n2438)	10.9 ± 0.3	15
ced-9(n1950)	12.5 ± 0.2	15
ced-4(n2273) ced-9(n1950)	10.1 ± 0.4	14

Chapter 5

The C. elegans cell death gene *ced-3* encodes a killing function separate from its proteolytic function

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Brian Davies sequenced many of the *ced-3* mutant alleles.

This chapter is in preparation for submission for publication.

Abstract

Mutations in the gene *ced-3* which encodes an Interleukin-1β convertase (ICE) - like protease prevent programmed cell death in the nematode *Caenorhabditis elegans*. We have characterized the phenotypes conferred by and determined the sequences of 50 mutations in *ced-3*. We distinguish five phenotypic classes of alleles based on the penetrance of the cell death defect. At least two of the mutations studied are likely to be null for CED-3 proteolytic activity. At least 14 of the recessive alleles as well as four dominant-negative alleles prevent cell death to a greater extent than the protease-null alleles, suggesting that these alleles also affect a cell death function different from the proteolytic function of CED-3. 25 of 29 sites altered by *ced-3* missense mutations are conserved with other ICE-like family members. Interestingly, we find that mutations in the non-catalytic N-terminal domain of *ced-3* also prevent its activity, suggesting that this region has functional importance *in vivo*. We found that overexpression of constructs containing point mutations in the active site cysteine, as well as overexpression of constructs containing the N-terminal portion of *ced-3* fused to a reporter gene can prevent programmed cell death in wild-type animals, suggesting that this region might be involved in protein-protein interactions.

Introduction

Programmed cell death is a process conserved throughout evolution and serves many functions (Glücksmann, 1950). For example, selective killing of cells by programmed cell death can be used to shape tissues and organs, to rid the body of harmful cells (such as in the immune system) (Cohen and Duke, 1984), and to eliminate cells whose function is no longer needed. Work on the nematode *Caenorhabditis elegans* (*C. elegans*) has revealed that the molecular mechanisms by which programmed cell death occurs have been conserved from worm to man (reviewed by Horvitz et al., 1994 and Ellis et al., 1991a). In *C. elegans* 131 of the 1090 cells born in the hermaphrodite undergo programmed cell death in a manner which is morphologically and ultrastructurally similar to the mammalian process of apoptosis (Sulston and Horvitz, 1977). Mutations in 14 genes affecting several different aspects of the death program have been isolated and ordered in a pathway (Hedgecock et al., 1983; Ellis and Horvitz, 1986; Ellis and Horvitz, 1991; Ellis et al., 1991b; Hengartner et al, 1992). Mutations in three genes, *ced-3*, *ced-4*, and *ced-9*, globally perturb the process of cell killing (Ellis and Horvitz, 1986; Hengartner et al., 1992). In particular, mutations in *ced-3* and *ced-4*, as well as a gain-of-function mutation in *ced-9* prevent programmed cell death from occuring. Loss-of-function (lf) mutations in *ced-9* result in the inappropriate activation of the cell death program in cells that should normally survive and hence lead to lethality. This result suggests that *ced-9* is normally required to prevent progrmmed cell death in cells that survive. The *ced-9*-associated lethality can be suppressed by mutations in *ced-3* or *ced-4* suggesting that the ectopic deaths observed in *ced-9*(lf) animals are a consequence of inappropriate activation of *ced-3* and *ced-4* (Hengartner et al., 1992). Studies suggest that the activities of the genes *ced-3*, *ced-4* and *ced-9* are required within cells that die for these cells' demise (Yuan and Horvitz, 1990; Shaham and Horvitz, manuscript in preparation).

The *ced-9* gene encodes a memeber of the *bcl-2* family of proteins that function in mammalian cell death (Hengartner and Horvitz, 1994). The *ced-4* gene encodes two novel transcripts, an abundant transcript that can cause cell death, and a rarer transcript that can prevent programmed cell death (Yuan and Horvitz, 1992; Shaham and Horvitz, manuscript in preparation). The *ced-3* gene encodes a member of the CED-3/ICE (Interleukin-1β convertase) family of cysteine proteases (Yuan et al., 1993). Overexpression of the mammalian members of this family can result in cell death in culture (Miura et al., 1993; Kumar et al., 1994; Fernandez-Alnemri, 1994; Wang et al., 1994). Expression of the viral inhibitor of ICE, crmA, in neurons has prevented the death of these neurons following factor deprivation (Gagliardini et al., 1994). In addition, a knock-out mutation of the ICE gene in mice reveals a defect in Fas-mediated

killing (Kuida et al., 1995). These results suggest that as with *ced-3*, overexpression of the mammalian proteases can kill, and these proteases are likely to be required for killing. Several of these proteases have the unique substrate specificity of cleavage following an aspartate residue (Thornberry et al., 1992), a specificity shared with only one other known eukaryotic protease (fragmentin 2) that is thought to function in cell death mediated by cytotoxic T cells (Shi et al., 1992a; Shi et al., 1992b; Heusel et al., 1994). The genes for the ICE-like proteases encode proteins with three major domains: an N-terminal domain that is not required for protease activity, a large subunit of the protease containing the active site cysteine, and a small subunit of the protease (Thornberry et al., 1992). The large and small subunits associate to form the protease heterodimeric unit. X-ray crystallographic studies of the ICE protease suggest that the active protease consists of two heterodimers bound to each other (Wilson et al., 1994; Walker et al., 1994). These proteases can undergo autocatalysis in which the primary translation product is cleaved at specific aspartate residues separating the various domains described above (Thornberry et al., 1994).

To better understand the function of this class of proteases in programmed cell death we have undertaken an analysis of mutations affecting the function of *ced-3 in vivo*. We charactarized the phenotypes of 50 mutant strains containing mutations in *ced-3* and determined the molecular nature of the lesion in each mutant strain. Our results show that most missense mutations affect residues conserved among CED-3 and other related family members. Some of the mutations encode products that act in a dominant-negative fashion, suggesting that *ced-3* may physically associate with itself or with other components of the cell death pathway. Interestingly, these dominant-negative alleles as well as other alleles of *ced-3* prevent programmed cell death to a greater extent than alleles lacking CED-3 proteolytic activity, suggesting that they not only eliminate the proteolytic function of CED-3 but also interfere with the cell death process in a different manner. We also find that expression of the N-terminal region of CED-3 fused to a reporter protein can prevent programmed cell death in wild type animals, suggesting that this region might be involved in protein-protein interactions.

Results

Characterization of *ced-3* mutations

The 50 mutations analyzed in this study were isolated in several different genetic screens in our lab (see Materials and Methods), however, the majority of the mutations were identified in screens intended to suppress the lethality of the weak loss-of-function mutation *ced-9(n1950n2161)* (M. Hengartner, personal communication; S. S and B. D., unpublished results). Loss-of function mutations in the *ced-9* gene result in massive

ectopic cell death and thus result in lethality. Mutations in the gene *ced-3* will prevent this lethality, suggesting that *ced-9* normally acts to negatively regulate the activity of *ced-3* (Hengartner et al., 1992, Shaham et al., manuscript in preparation). To quantitate the reduction in function of a given *ced-3* allele we counted the number of extra surviving cells present in the anterior region of the pharynx of mutant animals as has been previously described (Hengartner et al., 1992).

Five phenotypic classes of *ced-3* mutations can be recognized. Weak mutations in which none of the animals have more than one extra cell in the anterior pharynx (category I), weak mutations in which some of the animals have more than one extra cell in the anterior pharynx but a significant portion do not have any extra cells (category II), medium strength mutations in which all animals have at least one extra cell in the anterior pharynx (category III), strong mutations that have a greater number of extra cells in the anterior pharynx than the putative protease-null alleles (see below) and are fully recessive (category IV), and strong mutations that have a weakly dominant phenotype (category V). The alleles *n2445*, *n2854*, *n2426*, *n1165*, *n1286*, *n2444*, *n2859*, *n2922*, *n2442*, and *n2721* could not be reliably assigned to either category III or category IV because they resulted in a a cell-survival phenotype which was on the border between these categories, and were arbitrarily divided so that the first 8 were assigned to category III and the remaining two to category IV.

To determine the molecular nature of the *ced-3* mutations studied we used the polymerase chain reaction (PCR) to amplify coding regions and exon/intron boundaries from each mutant strain and determined the sequence of these regions (see Materials and Methods). In one case (the allele *n2452*) we could not amplify sequences downstream of position 3200 in the *ced-3* genomic sequence (Yuan et al., 1993), suggesting that a deletion might be present. All the mutations analyzed were isolated in screens using ethyl methanesulfonate (EMS) as a mutagen. 45 of the mutations analyzed resulted in GC->AT transitions which are most often induced by EMS. Two mutations resluted in TA->AT changes, one mutation resulted in a TA->CG change, two mutations (*n2854* and *n2830*) altered several nucleotides, and one mutation (*n2452*) resulted in a deletion (see below). Of the point mutations isolated 37 were missense mutations, six were nonsense mutations, and four probably affect splicing. Of the missense mutations 25 of 29 affected sites are conserved with other CED-3/ICE family members. Interestingly, four of the 29 sites affected by missense mutations in *ced-3* are in the N-terminal region which is not required for catalytic function. This observation suggests that the N-terminal region can either influence the proteolytic activity of this class of proteases, or that it has a separate cell-killing function (also see below). The non-conserved serine-rich region of *ced-3* (amino acids 93 to 205 of the CED-3 protein) is

not affected by any of the missense mutations examined and its function remains unknown. We analyzed *ced-3* RNA expression in 11 of the mutant strains representing a range of cell death defects, and a range of molecular lesions (see figure 2). The results suggest that none of the mutations examined grossly affect the size or level of the *ced-3* transcript.

Category I *ced-3* mutations can not suppress the lethality caused by strong loss-of-function mutations in *ced-9*

The weakest *ced-3* mutations consist of the alleles *n2923*, *n2446*, *n2449*, and *n2425*. Animals carrying these alleles have on average 0-0.3 extra cells and as such are indistiguishable from wild-type animals which on average have 0.13 extra cells (table 1). All of these alleles are recessive and were isolated as suppressors of the lethality of the weak *ced-9* loss-of-function allele *n1950n2161*. Interestingly, the allele *n2425* is not capable of suppressing a stronger *ced-9* mutation which results from a nonsense mutation at codon 160 of the *ced-9* open reading frame (table 2), suggesting that in animals carrying this allele wild-type *ced-3* activity is too high to allow survival in the complete absence of functional *ced-9* product. We suggest that the other alleles in this category behave in a similar manner.

We have previously argued that both cells that die and those that do not die contain *ced-3* product, and that in the absence of *ced-9* function *ced-3* is active and kills both cells that live and cells that die (Shaham and Horvitz, manuscript in preparation). That the weak *ced-3* alleles are capable of suppressing the ectopic cell deaths that occur in *ced-9(n1950n2161)* animals, but do not result in the survival of cells that normally die (no extra cells in the anterior pharynx) suggests that cells that normally live differ in their sensitivity to these *ced-3* mutations. In particular, this observation suggests that cells that normally die have more *ced-3* function or are capable of transducing a killing signal from *ced-3* in a more efficient manner than cells that die ectopically (as in the *ced-9(n1950n2161)* mutants). These observations suggest that the weakest *ced-3* alleles probably retain just enough function to allow normal programmed cell death to occur but not enough function to allow ectopic cell deaths to occur.

The sequence lesions in these mutations are shown in table 1. Two alleles, *n2923* and *n2449*, are missense mutations in residues which are not conserved among CED-3/ICE family members. The remaining two alleles, *n2446* and *n2425*, are missense mutations in residues conserved among CED-3/ICE family members suggesting that these residues are probably important for the function of these proteins (figure 1).

Category II *ced-3* alleles can suppress strong loss-of function mutations in *ced-9*

A significant proportion of animals carrying the category II mutations *n2447*, *n2427*, or *n2443* do not have any extra cells. 4/15 *n2447* animals, 5/19 *n2427* animals, and 4/15 *n2443* animals scored had no extra cells. The allele *n2438* contains the identical sequence change as *n2427* yet appears to be slightly more defective in *ced-3* function. The nature of this difference is not understood but may be related to additional modifying mutations in the *n2438* background (data not shown). The mutation *n2427* and *n2443* are capable of suppressing the lethality of strong *ced-9* alleles (table 2; data not shown). However, *ced-9; n2427* or *ced-9; n2443* animals can be severly egg-laying defective, suggesting that the HSN neurons which are required for egg-laying are dying ectopically as in *ced-9*(lf) animals (Hengartner et al., 1992, data not shown). These observations suggest that this class of mutations reduces *ced-3* function more than the category I mutations, yet does not completely eliminate *ced-3* activity. All category II alleles are recessive.

The sequence lesions in these mutations are shown in table 1. The mutations *n2447*, *n2427*, and *n2438* are missense mutations in residues conserved with other CED-3/ICE proteins. The *n2443* mutation affects a residue that is not conserved.

Category III *ced-3* alleles include alleles which are likely to lack proteolytic function

Alleles in category III are all recessive, and have an average of 6.2 - 10.9 extra cells in the anterior pharynx, representing a broad range of *ced-3* activity. 15 of the alleles in this category are missense alleles (*n2436*, *n2877*, *n2921*, *n1040*, *n2861*, *n1129*, *n2885*, *n2870*, *n1163*, *n2722*, *n2924*, *n2429*, *n2426*, *n2444*, *n2922*), six of the mutations result in nonsense codons (*n1949*, *n2998*, *n2888*, *n1165*, *n1286*, *n2859*), one mutation (*n2445*) results in the alteration of the stop codon to a lysine resulting in the addition of 26 amino acids to the carboxy-terminus of the protein, one mutation (*n2452*) is a large deletion (see below), and one affects splicing (*n2854*, see below). 14 of the 15 missense mutations alter residues conserved with other CED-3/ICE family members.

Animals carrying two of the alleles in this category are likely to lack proteolytic activity based on their molecular lesion. The allele *n2452* contains a deletion of at least 3.4 kb which completely deletes the *ced-3* coding region from amino acid 179 to the end of the open reading frame, and thus eliminates the region encoding the active *ced-3* protease (figure 3, also see Materials and Methods). The allele *n2888* changes codon 154 from an arginine to a nonsense codon, presumably truncating the *ced-3* protein upstream of the region required for proteolytic activity. As shown in table 1 these alleles have 9.5 ± 1.5 and 10.6 ± 1.7 extra cells respectively, suggesting that loss of *ced-3* proteolytic activity leads to the accumulation of about 10 extra cells in the anterior pharynx of mutant animals. The true number of extra cells for a protease-null allele is

probably reflected by the deletion mutant *n2452*. Whether this represents the phenotype of a complete *ced-3* null is unclear since it is possible that the N-terminal region of *ced-3* which remains intact in the two alleles is responsible for part of the killing function of *ced-3* (see below) and that a true *ced-3* null allele would have a stronger phenotype than an allele lacking only proteolytic activity.

Category IV *ced-3* alleles inhibit cell death to a larger extent than alleles lacking *ced-3* proteolytic activity

Animals carrying the category IV *ced-3* alleles have a greater average number of extra cells in the anterior pharynx than the alleles *n2452* and *n2888* which are likely to lack *ced-3* proteolytic activity. The number of extra cells in this categoty ranges from 11.0 to 12.2. All of these alleles are recessive. 11 of the alleles in this category are missense alleles (*n2442*, *n2721*, *n2889*, *n2439*, *n2441*, *n2720*, *n2719*, *n2454*, *n2432*, *n718*, *n2883*). One (*n2830*) contains two missense mutations, and two of the alleles affect splicing (*n717*, *n3002*, see below). Ten of the 11 missense mutations alter residues which are conserved with other CED-3/ICE family members.

The observation that category IV alleles prevent programmed cell death to a greater extent than alleles lacking the *ced-3* proteolytic domain suggests that these alleles are interfering with programmed cell death in a manner which is separate from the proteolytic activity of CED-3. This additional interaction could take one of two forms. Either these alleles are preventing another component of the cell death machinery from acting properly in addition to disrupting the proteolytic activity of CED-3, or it is possible that the N-terminal region of *ced-3* is important for its killing function, and these alleles perturb both the proteolytic aspect of the killing and the aspect which is N-terminal dependent (see previous section). Currently we cannot distiguish between these two models (see below).

Category V *ced-3* alleles are weakly dominant negative

Animals carrying category V *ced-3* alleles have a greater average number of extra cells in the anterior pharynx than the alleles *n2452* and *n2888* which are likely to lack the *ced-3* proteolytic region. This observation suggests that as with category IV alleles, category V alleles also disrupt a cell death function which is not related to the proteolytic activity of *ced-3*. The number of extra cells in this category ranges from 11.7 to 12.4. Three of the alleles in this category are missense alleles (*n2871*, *n2430*, *n2433*) and one allele results from a splicing defect (*n2440*, see below). The alleles *n2871* and *n2433* affect conserved arginine and glycine residues, respectively, in the conserved pentapeptide QACRG which surrounds the active site of CED-3 and is characteristic of

CED-3/ICE-like proteases. The remaining missense allele alters a non-conserved residue.

Animals heterozygous for these alleles show weak cell survival (0-2 extra cells per animal) which is noticably different from the other *ced-3* alleles (table 1). To confirm that these alleles are indeed dominant we examined the ability of *n2871*/+ to suppress the lethality of the weak *ced-9* allele *n1950n2161*. As shown in table 3 *ced-9; n2871*/+ animals segregated live animals which were themselves heterozygous for *n2871*. A similar experiment with the *ced-3* allele *n717* which does not show a dominant phenotype failed to produce heterozygous progeny. These results confirm the dominant nature of the *n2871* allele and suggest that other members of this category behave in a similar manner.

To test whether this dominance is due to a haploinsufficiency of the *ced-3* locus or is due to a dominant-negative effect we examined the phenotype of animals heterozygous for the deficiency *sDf21* which uncovers *ced-3*. As shown in table 1 *sDf21*/+ animals do not show significant cell survival, suggesting that the dominant phenotype of category V alleles is due to a dominant-negative interaction. This dominant-negative effect could be due to two types of interactions. One possibility is that the products of these alleles interact with another component of the cell death machinery and inhibit its activity. Another possibility is that these alleles reflect a possible multimerization of *ced-3*. Interestingly, the X-ray crystal structure of ICE suggests that this protease acts as a dimer of p10-p20 heterodimers (Wilson et al., 1994; Walker et al., 1994), thus it is possible that *ced-3* also functions as a dimer of heterodimers, and that the dominant-negative products of category V alleles result in non-productive dimer formation and therefore reduce *ced-3* activity (see Discussion).

Expression of mutant *ced-3* constructs can prevent programmed cell death in wild-type animals

The observation that some *ced-3* alleles act in a dominant-negative fashion suggested that expression of truncated *ced-3* products, or certain mutant *ced-3* products should inhibit programmed cell death in wild-type animals. We initially examined the effect of introducing into wild-type animals a *ced-3* construct containing a heat-shock promoter fused to a *ced-3* cDNA with a mutation in the active site cysteine. As shown in table 5 animals that were heat-shocked showed significant accumulation of extra cells in the anterior region of the pharynx, suggesting that programmed cell death had been inhibited. To further study the regions of *ced-3* that could confer a cell survival phenotype on wild-type animals we developed two classes of fusion constructs. The first class of constructs consists of the genomic region of *ced-3* upstream of the start

codon fused to 3' deletions of the genomic coding region of *ced-3* fused to either of the reporter genes *lacZ* or GFP (Table 5, constructs E-G). The second class of constructs consists of a *C. elegans* heat-shock promoter fused to a trucated *ced-3* cDNA fused to either the *lacZ* or GFP reporter genes (Table 5, constructs B-D). Introduction of most truncation constructs prevented programmed cell death to varying extents. In some cases we could observe surviving cells staining with the reporter construct. To confirm that the extra cells were a result of an inhibition of programmed cell death we introduced construct F (table 5) into *ced-9(n1950n2161)* or *ced-9(n2812)* animals. As table 4 shows the lethality of these strains was suppressed by construct G, suggesting that this construct is capable of preventing programmed cell death.

Interestingly construct C containing fewer than the first 95 codons of *ced-3* cannot prevent programmed cell death (table 5). This observation suggests that a region between amino acids 95 and 150 of CED-3 (constructs C and D) is necessary for this protective effect to occur. This region is not required for *ced-3* proteolytic activity suggesting that the N-terminal region of *ced-3* might be involved in interactions with other components of the cell death machinery, consistent with the observations in the previous section.

Splicing mutants of *ced-3*

We examined in more detail three (*n2854*, *n717*, *n2440*) of the four mutations (*n2854*, *n717*, *n2440*, *n3002*) that are likely to affect splicing. The allele *n2854* contains the sequence AGGCG | gattt in the donor region of intron 5 of *ced-3* (table 1) instead of AGGCG | gttcg present in the wild type. To characterize the *ced-3* transcripts made in animals carrying this *ced-3* mutation we prepared RNA from mutant animals (see Materials and Methods), prepared cDNAs from the RNA and amplified this DNA using PCR and *ced-3*- specific primers. The sequence of the resulting band was then determined. Interestingly, the only product isolated from this mutant spliced at a position upstream of the normal splice site to give a deletion of 3 bp with respect to the wild-type message resulting in the deletion of glycine 360 in the ORF of *ced-3*. Why this splicing pattern is observed is not understood. The *n717* mutation changes a conserved acceptor site G to an A in intron 7. To characterize the products made in *n717* animals we prepared RNA from mutants and used it for a northern blot probed with a *ced-3* cDNA probe (see figure 2). The size and levels of the message were not discernably different from wild type. We then prepared cDNAs from the *n717* RNA and amplified this DNA using PCR and *ced-3* specific primers. Sequence determination of the resulting bands suggested that multiple splice sites were used around the wild-type splice location (data not shown). The mutation *n2440* changes the sequence

CCGCAAGTT to CCGTAAGTT apparently changing codon 401 from a glutamine to a stop codon. However, we noticed that this change also creates a cryptic splice donor site which could be used instead of the intron 6 splice donor which is immediately down stream of the mutation site (CC | gtaagtt). To confirm this hypothesis we determined the sequence of *ced-3* cDNAs prepared from *n2440* mutant RNAs (see above and Materials and Methods). Only one class of RNAs was discernable and used the predicted cryptic donor site. The product produced by this splice is out of frame and is predicted to form a truncated protein with 13 amino acids downstream of amino acid 400. Thus this mutation is likely not to be a nonsense mutation.

Discussion

ced-3 encodes a killing function separate from its proteolytic function

Studies addressing the functional requirement of the proteolytic activity of CED-3/ICE-like proteins in programmed cell death have suggested that this activity is essential for cell killing (Miura et al., 1993; S. Shaham, manuscript in preparation; Kumar et al., 1994; Wang et al., 1994). In this work we show, however, that *ced-3* is likely to encode a separate function involved in cell killing. Among the 50 alleles characterized in this study, two are likely to completely eliminate CED-3 proteolytic function. The allele *n2854* contains a deletion which removes all sequences present in the mature protease. The allele *n2888* contains a stop codon upstream of the sequences encoding the active protease and presumably results in early truncation of the protein. These two alleles contain an average of 9.5 ± 1.5 and 10.6 ± 1.7 extra cells in the anterior pharynx, respectively, that result from the survival of cells which normally undergo programmed cell death. Interestingly, at least 18 other alleles of *ced-3* contain more surviving cells on average than the protease-null alleles (table 1). This observation suggests that these 18 alleles are perturbing cell death in a manner additional to affecting *ced-3*'s proteolytic activity. We offer two possible models which are not mutually exclusive to explain these results. First, the products of the 18 alleles with additional extra cells might interfere both with the proteolytic activity of *ced-3* and with another cell death component present in the cell. Second, since the protease-null alleles have an intact N-terminus it is possible that this region is also important for cell-killing. The alleles containing additional extra cells might perturb both the protease function of *ced-3* and the N-terminal cell-killing function. None of the existing *ced-3* alleles eliminate the N-terminal region so that we can not assess the function of such alleles to distinguish between the two models presented above. Animals carrying the strongest *ced-3* allele *n2433* make normal levels of *ced-3* RNA. This allele behaves genetically as a dominant-negative allele (figure 2, see below), suggesting that this allele produces

protein, and supporting the notion that these alleles might be actively interfering with a function seperate from the proteolytic function of *ced-3*.

Dominant-negative alleles of *ced-3* hint at protein-protein interaction

Four of the *ced-3* alleles studied act in a dominant-negative fashion. Animals heterozygous for these alleles show a significant amount of additional cell survival, whereas animals heterozygous for a deficiency uncovering *ced-3* do not. This observation confirms that the dominant cell-survival phenotype does not result from a loss-of-function of these alleles. At least one of the dominant-negative alleles produces normal levels of *ced-3* transcript (*n2433*, figure 2), consistent with the notion that this allele produces a mutated protein that acts to interfere with either *ced-3* function or with another cell death function. Interestingly, X-ray crystallographic studies of ICE suggest that this protein acts as a dimer of p10-p20 heterodimers (Wilson et al., 1994; Walker et al., 1994). If CED-3 functions in a similar manner it is possible that the dominant negative alleles of *ced-3* interfere with this multimerization. The observation that *ced-3* encodes a killing function seperate from its proteolytic activity raises the possibility that this activity is related to the mechanism of action of the dominant negative alleles. If so, it is likely that the 14 recessive alleles that inhibit programmed cell death to a greater extent than the protease-null alleles act in a manner similar to the dominant negative alleles, yet as heterozygotes produce an effect too weak to be detected.

The N-terminal region of *ced-3* is essential for function and can interact with components of the cell death machinery

Mutations in the N-terminal region of *ced-3* which is not required for proteolytic function can severly block programmed cell death (table 1). This observation suggests that this region is essential for programmed cell death to occur properly. This region can affect programmed cell death either by normally aiding in the proteolytic activation of CED-3 or by interacting with another component of the cell death machinery to induce cell death. These two possibilities are, again, not mutually exclusive.

Overexpression of a number of *ced-3* truncation constructs can prevent programmed cell death in wild-type animals (table 5). In particular, expression of only the N-terminus fused to a heterologous protein will prevent cell death. This observation suggests that these constructs are acting in a dominant-negative fashion to inhibit cell death and that the N-terminal region of *ced-3* can mediate this inhibition. Again, as with the dominant-negative alleles, this inhibition could occur by preventing multimerization of *ced-3* or by inhibiting the activity of another component of the cell

death machinery. Whether this mode of dominance is equivalent to the one described in the previous section is unclear.

Cells that normally die are more sensitive to the genetic state of *ced-3* than cells that die ectopically

We have previously suggested that *ced-3* is expressed in a large number of cells in C. elegans, being functional only in those that normally die (Shaham and Horvitz, manuscript in preparation). In *ced-9*(lf) animals *ced-3* gets inappropriately activated resulting in many ectopic cell deaths (Hengartner et al., 1992; Shaham and Horvitz, manuscript in preparation). Our results suggest that *ced-3* is less active in cells that die ectopically than in cells that normally die. In particular we demonstrated that weak mutations of *ced-3* can prevent the lethality associated with the ectopic cell deaths of *ced-9*(lf) animals, yet do not prevent normal programmed cell deaths. This observation implies that cells that normally die either produce more *ced-3* than ectopically dying cells, or are better capable of transducing a killing signal mediated by *ced-3*. The nature of this difference could lie in the levels of *ced-3* expression, or in the presence or absence of cell death components in cells that normally die versus those that die ectopically. We propose that this difference is, in part, responsible for determining which cells live and which cells die.

Materials and Methods

General Methods and Strains

The techniques used for culturing *C. elegans* were 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 mutations used have been previously described by Trent et al. (1983), Hedgecock et al. (1983), Ellis et al. (1991), Ellis and Horvitz (1986), Brenner (1974), Clark et al. (1988), Hengartner et al. (1992), or were isolated by us and members of our lab. These mutations are listed below:

LGI: *sem-4*(*n1378*), *ced-1*(*e1735*)

LGIII: *ced-9*(*n1950n2161*, *n1950n2077*), *ced-11*(*n2744*), *ced-5*(*n2098*)

LGIV: *ced-3*(*n2923*, *n2446*, *n2449*, *n2425*, *n2447*, *n2427*, *n2443*, *n2438*, *n2436*, *n2877*, *n2921*, *n1040*, *n2861*, *n1129*, *n2452*, *n1949*, *n2885*, *n2870*, *n1163*, *n2998*, *n2722*, *n2924*, *n2429*, *n2888*, *n2445*, *n2854*, *n2426*, *n1165*, *n1286*, *n2444*, *n2859*, *n2922*, *n2442*, *n2721*, *n2889*, *n717*, *n2439*, *n2441*, *n2720*, *n2719*, *n2454*, *n2440*, *n2432*, *n2830*, *n2871*, *n2430*, *n718*, *n3002*, *n2883*, *n2433*), *dpy-4*(*e1166*), *sDf21*

LGV: *egl-1*(*n487*)

Isolating *ced-3* alleles and characterizing their phenotypes

The alleles used in this work were isolated in several screens by several members of our lab. We isolated the alleles *n2859*, *n2861*, *n2870*, *n2877*, *n2883*, *n2885*, *n2888*, *n2889*, *n2921*, *n2922*, *n2923*, *n2924*, and *n3002* as suppressors of the maternal-effect lethality of the loss-of-function mutation *ced-9(n1950n2161)*. Specifically, *unc-69(e587) ced-9(n1950n2161)/qC1* animals were mutagenized using 30 mM ethylmethanesulfonate (EMS) and allowed to self. Unc-69 F1s were then placed at 10 to a plate and any F2s that grew to adulthood were picked and used to establish a suppressed strain. The presence of a *ced-3* mutation in the strain was confirmed by a complementation test, followed by mapping to show that the *ced-9* suppressor was linked to chromosome IV. The alleles we isolated as well as those isolated by others were generally removed from all other markers in the background except in the cases noted in table 1. To quantitate cell survival we scored for the presence of extra cells in the anterior region of the pharynx as has been previously described (Hengartner et al., 1992). Specifically, for each strain animals were mounted on a 5% agar pad on a slide containing 3-5 µl of 50 mM NaN_3 in M9 buffer (Sambrook et al.). A cover slip was applied, and animals were viewed using Nomarski optics at a magnification of 100X. The number of nuclei in the pharynx was counted and compared to that present in wild-type animals. The difference represented the number of extra cells. In wild-type animals an extra cell can be detected in approximately 5% of the animals.

Allele sequence determination

To isolate coding regions and exon/intron junctions from mutant strains we amplified the *ced-3* genomic coding region using PCR and a set of 4 primer pairs. Specifically, primers SHA2 and PCR2 were used to amplify exons 1-3, primers PCR3 and PCR4 were used to amplify exon 4, primers PCR5 and 650 were used to amplify exons 5-7, and primers BD1 and 1200 were used to amplify exon 8. The sequence and location of these primers is shown in table 6. DNA was amplified as follows. 1-10 worms were placed in 3 µl PCR lysis buffer (60 µg/ml proteinase K in 10 mM Tris (pH8.2), 50 mM KCl, 2.5 mM $MgCl_2$, 0.45% Tween 20 and 0.05% gelatin) and frozen at -70°C for 20-30 minutes. Samples were then placed in a PCR machine and allowed to incubate at 60°C for 1 hr followed by a 15 minute incubation at 95°C. Each sample was then fully used as the DNA source in a standard PCR reaction using one of the primer pairs described above. Samples were run on a 1.4% agarose gel, purified using β-agarase (New England Biolabs, Beverly, MA) and resuspended in 20 µl of TE buffer. Samples were sequenced using the fmol sequencing kit (Promega Corporation,

Madison, WI) following instructions of the manufacturer for ^{33}P labeling. Samples were run on a standard acrylamide sequencing gel (Life Technologies, Gaithersburg, MD). The gel was dried and exposed to X-ray film for 1-5 days. For each allele we determined the entire sequence of the *ced-3* open reading frame as well as all the exon/intron junctions. Sites where a potential mutation was identified were generally resequenced.

RT-PCR , Southern and Northern Hybridization

Southern analysis was performed on *n2452* and wild-type genomic DNA as described in Sambrook et al. (1989) using the restriction enzymes HindIII, XhoI, and XbaI (New England Biolabs, Beverly, MA) and using a *ced-3* cDNA as a probe. None of the wild-type bands downstream of exon 3 were detected in the *n2452* lanes.

RNA for northern analysis and Reverse Transcriptase PCR (RT-PCR) was prepared as follows. 1-2 9 cm plates of worms were added to a liquid culture containing S medium (Sulston and Hodgkin, 1988) and antibiotics. Frozen bacteria were added to the culture as a food source. Cultures were harvested after 5-7 days, and mRNA was prepared using the FastTrack mRNA preparation kit (Invitrogen, San Diego, CA). Northern blots were performed as described in Sambrook et al. (1989) using either a *ced-3* cDNA as a probe or a *lin-26* genomic fragment (M. Labouesse, personal communication) as a control. RT-PCR was performed using the RNA GeneAmp kit (Perkin Elmer Cetus, Norwalk, CT). The resulting bands were purified as described in the previous section. The sequences of the *n2440*, *n717*, and *n2854* products were determined using and ABI sequencer (Applied Biosystems, Foster City, CA).

Plasmid constructions

Construct A was made by digesting the heat-shock vectors pPD49.79 and pPD49.83 (A. Fire and P. Candido, personal communication) with the enzymes NheI and EcoRV, and ligating to a *ced-3* cDNA derived from plasmid pS126 (Shaham et al., manuscript in preparation) which had been in vitro mutagenized using an in vitro mutagenesis system (Amersham, Arlington Heights, IL) to alter the sequence TGT to GCT resulting in a cysteine to alanine change in the active site of *ced-3*, and cut with the enzymes SpeI and SmaI. Construct B was made by digesting the heat-shock vectors described above with NheI, digesting pS126 using SpeI and partially digesting using BglII, and digesting the GFP vector Tu#62 (M. Chalfie, personal communication) with BamHI and SpeI, followed by ligation of the mixture. Construct C was produced in the identical manner to construct B except that the *lacZ* vector pPD21.28 was used instead of the GFP vector. Construct D was made in the identical manner to construct C.

Construct E was made by digesting the *ced-3* genomic plasmid pJ40 (Yuan et al., 1993) with the enzymes Bgl II and ApaI and ligating to the GFP vector Tu#62 which had been cut using the enzymes BamHI and ApaI. Construct F was made in the identical manner to construct E except that the *lacZ* vector pPD21.28 was used instead of the GFP vector. Construct G was made by digesting pJ40 with the enzymes SalI and Apa, and ligating to the *lacZ* vector pPD21.28 which had been cut using the enzymes SalI and ApaI.

Germline transformation

Our procedure for microinjection and germline transformation followed that of Fire (1986) and Mello et al. (1991). DNA for injections was purified using the Qiagen system for DNA purification (Qiagen, Inc., Chatsworth, CA) and following the instructions of the manufacturer. The concentrations of all plasmids used for injections were between 50-100 μg/ml. All constructs were co-injected with the pRF4 plasmid containing the *rol-6(su1006)* gene as a dominant marker. Animals carrying the pRF4 plasmid exhibit a Rol phenotype. All transformation experiments were into wild-type animals. Approximately 30 animals were injected in each experiment, and approximately 50-100 F1 Rol animals were picked onto separate plates. F1 animals segregating Rol animals were established as lines containing extrachromosomal arrays (Way and Chalfie, 1988).

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Table 1. Phenotypes and sequence lesions of *ced-3* mutants.

Column 1 indicates the allele examined, *sDf21* is a deficiency uncovering *ced-3* (see materials and Methods). Column 2 indicates the number of extra cells in the anterior pharynx of animals homozygous for a given allele ± standard deviation, numbers in parentheses indicate the number of animals observed. Column 3 indicates the number of extra cells in the anterior pharynx of animals heterozygous for a given allele ± standard deviation, numbers in parentheses indicate the number of animals observed. Column 4 indicates the nucleotide alteration observed in each allele, undelined residues are altered, wild type sequence is to the left of each arrow, mutant sequence is to the right. Column 5 indicates the predicted change in the RNA or protein produced in a given mutant. Numbers indicate the amino acid residue. All of the *ced-3* alleles were separated from background mutations except for some which were scored in the following genetic backgrounds: *n1163*; *egl-1*(*n487*), *sem-4*(*n1378*) *ced-1*(*e1735*); *ced-11*(*n2744*); *n2998*, *sem-4*(*n1379*); *ced-5*(*n2098*) *ced-3*(*n2722*), *ced-3*(*n1165*); *egl-1*(*n487*), *sem-4*(*n1378*); *ced-5*(*n2098*) *ced-3*(*n2721*), *sem-4*(*n1378*); *ced-5*(*n2098*) *ced-3*(*n2719*), *sem-4*(*n1378*); *ced-5*(*n2098*) *ced-3*(*n2830*), *ced-3*(*n2871*) *dpy-4*(*e1166*), *ced-3*(*n3002*) *nIs50*.

Table 1

Genotype	Mutant/ Mutant no. extra cells in anterior pharynx ± SD	Mutant/+ no. extra cells in anterior pharynx ± SD	Nucleotide change	Amino acid /splice change
Wild type	0.13 ± 0.4 (40)	–	–	–
n2923	0 (15)	0.13 ± 0.4 (15)	GCG->GTG	A347V
n2446	0.13 ± 0.4 (15)	0.13 ± 0.4 (15)	GTG->TTG	V311L
n2449	0.2 ± 0.4 (15)	0.13 ± 0.4 (15)	CGC->CAC	R51H
n2425	0.3 ± 0.6 (17)	0.13 ± 0.4 (15)	GGC->GAC	G277D
n2447	0.8 ± 0.6 (15)	0.07 ± 0.3 (15)	TCG->TTG	S446L
n2427	1.2 ± 0.9 (19)	0 (15)	GGA->AGA	G474R
n2443	1.8 ± 1.7 (15)	0.07 ± 0.3 (15)	CCG->TCG	P400S
n2438	2.1 ± 1.2 (10)	0.07 ± 0.3 (15)	GGA->AGA	G474R
n2436	6.2 ± 1.5 (10)	0.13 ± 0.4 (15)	CTT->TTT	L269F
n2877	7.0 ± 1.5 (10)	0.3 ± 0.6 (15)	CTT->TTT	L269F
n2921	7.9 ± 2.3 (10)	0 (15)	CCT->CTT	P241L
n1040	8.3 ± 1.7 (31)	0.13 ± 0.4 (15)	CTC->TTC	L27F
n2861	8.4 ± 1.6 (10)	0 (15)	GGT->AGT	G261S
n1129	8.7 ± 1.3 (10)	0.07 ± 0.3 (15)	GCA->GTA	A449V
n2452	9.5 ± 1.5 (15)	0 (15)	deletion in intron 3	–
n1949	9.5 ± 2.3 (10)	0.3 ± 0.5 (15)	CAA->TAA	Q412ochre
n2885	9.6 ± 1.3 (10)	0.07 ± 0.3 (15)	GAG->AAG	E456K
n2870	9.7 ± 1.6 (10)	0.2 ± 0.4 (15)	AGA->AAA	R429K
n1163	10.2 ± 1.7 (15)	0.13 ± 0.4 (15)	TCC->TTC	S486F
n2998	10.3 ± 1.7 (15)	0.08 ± 0.3 (12)	TGG->TAG	W436amber
n2722	10.6 ± 1.0 (15)	0.2 ± 0.4 (15)	GCA->GTA	A418V
n2924	10.6 ± 1.7 (14)	0.13 ± 0.4 (15)	GAG->AAG	E318K
n2429	10.6 ± 1.6 (15)	0.2 ± 0.4 (15)	TCA->TTA	S314L
n2888	10.6 ± 1.7 (10)	0 (15)	CGA->TGA	R154stop
n2445	10.7 ± 1.1 (13)	0.13 ± 0.4 (15)	TAA->AAA	ochre504K
n2854	10.7 ± 1.8 (10)	0.3 ± 0.5 (15)	G|gattt->G|gttgg	Exon 5 donor
n2426	10.7 ± 2.1 (10)	0.3 ± 0.6 (15)	GAG->AAG	E483K
n1165	10.9 ± 1.5 (15)	0.13 ± 0.4 (15)	CAG->TAG	Q404amber
n1286	10.9 ± 1.5 (15)	0.07 ± 0.3 (15)	TGG->TGA	W428opal
n2444	10.9 ± 1.9 (10)	0.2 ± 0.4 (15)	GCA->ACA	A420T
n2859	10.9 ± 1.9 (10)	0.3 ± 0.5 (15)	TGG->TAG	W406stop
n2922	10.9 ± 1.5 (10)	0.07 ± 0.3 (15)	GCA->ACA	A420T
n2442	11.0 ± 1.3 (15)	0.13 ± 0.4 (15)	GGA->GAA	G243E
n2721	11.1 ± 1.7 (15)	0.07 ± 0.3 (15)	CAC->TAC	H315Y
n2889	11.1 ± 1.2 (10)	0.2 ± 0.4 (15)	GAG->AAG	E318K
n717	11.2 ± 2.0 (10)	0 (15)	cag|CA->caa|CA	Exon 7 acceptor
n2439	11.3 ± 1.3 (15)	0 (15)	CTC->TTC	L30F
n2441	11.5 ± 1.7 (15)	0.14 ± 0.5 (14)	AGA->AAA	R429K
n2720	11.6 ± 1.5 (15)	0.2 ± 0.6 (15)	CAC->TAC	H315Y
n2719	11.6 ± 1.9 (15)	0.07 ± 0.3 (15)	GGA->AGA	G65R
n2454	11.6 ± 2.4 (15)	0.07 ± 0.3 (15)	GCT->ACT	A466T
n2440	11.7 ± 1.7 (15)	0.5 ± 0.6 (15)	CGCAA->CGTAA	Q401ochre/splice donor
n2432	11.7 ± 1.2 (10)	0.07 ± 0.3 (15)	TAC->AAC	Y235N
n2830	11.7 ± 1.5 (15)	0.07 ± 0.3 (14)	GGA->AGA,GGA->AGA	G65R and G474R
n2871	11.7 ± 1.7 (15)	1.0 ± 0.8 (15)	CGA->CAA	R359Q
n2430	11.8 ± 1.2 (10)	0.73 ± 0.6 (15)	GCT->GTT	A466V
n718	11.8 ± 1.1 (10)	0.07 ± 0.3 (15)	CTC->TTC	G65R
n3002	12.1 ± 1.5 (15)	0 (15)	GAG|gta->GAG|ata	Exon 7 donor
n2883	12.2 ± 1.1 (15)	0.13 ± 0.4 (15)	TCA->CCA	S314P
n2433	12.4 ± 1.0 (10)	0.5 ± 0.9 (15)	GGC->AGC	G360S
sDf21	–	0.07 ± 0.3 (15)	–	–

Table 2. Category I *ced-3* mutants do not prevent lethality of *ced-9*(*n1950n2077*) animals.

Column 1 indicates a given allele. Column 2 indicates the number of homozygous *ced-9* animals observed. Column 3 indicates the number of *ced-9* homozygotes producing live progeny. Each *ced-9* homozygote could contain zero, one, or two copies of a given *ced-3* allele since they were derived from the self progeny of *ced-9* /+; *ced-3* /+ animals. If suppression is occuring, 3/4 of the *ced-9* homozygotes scored should produce viable progeny. If no suppression occurs, none of the *ced-9* homozygotes should produce viable progeny. If intermediate numbers occur they might reflect either a bias in selection of the maternal population or reduced but not lack of suppression.

Table 2

Allele	No. *ced-9* homozygotes scored	No. *ced-9* homozygotes giving viable progeny
n2425	30	0
n2443	20	6
n717	20	10

Table 3. *ced-3*(*n2871*) is a dominant suppressor of *ced-9*(*n1950n2161*) lethality.

The progeny of five animals of an indicated genotype were observed. The *ced-9* chromosome was marked with the mutation *unc-69*(*e587*), and the *ced-3* chromosome was marked with *dpy-4*(*e1166*). The number of Dpy-4 progeny expected if the *ced-3* allele is a suppressor is indicated in the last column.

Table 3

Genotype	Animal	Total no. progeny	No. *ced-3*/+ progeny	Calculated no. *ced-3*/+ progeny (if dominant)
ced-9; ced-3(n2871)/+	1	20	13	15
ced-9; ced-3(n2871)/+	2	7	5	5
ced-9; ced-3(n2871)/+	3	49	26	37
ced-9; ced-3(n2871)/+	4	6	2	4
ced-9; ced-3(n2871)/+	5	2	2	1
ced-9; ced-3(n717)/+	1	22	0	17
ced-9; ced-3(n717)/+	2	9	0	7
ced-9; ced-3(n717)/+	3	7	0	5
ced-9; ced-3(n717)/+	4	20	0	15
ced-9; ced-3(n717)/+	5	21	0	16

Table 4. A *ced-3-lacZ* fusion construct can suppress the lethality of *ced-9*(lf) animals.

ced-9(lf) alleles were established containing an extrachromosomal array of construct F (table 5). The presence or absence of progeny that grew past the L4 stage of a given strain is indicated. +, progeny produced, -, no progeny produced.

Table 4

Genotype	Construct	Progeny
ced-9(n1950n2161)	none	-
ced-9(n1950n2161)	F	+
ced-9(n2812)	none	-
ced-9(n2812)	F	+

Table 5. *ced-3*-reporter fusion constructs can prevent programmed cell death.

Constructs are indicated graphically with names A-G indicated to the left of each construct. Black boxes indicate C. elegans heat shock promoters, slanted hatched boxes indicate GFP sequences, dotted boxes indicate *ced-3* cDNA sequences, checkered boxes indicate *lacZ* sequences, vertically hatched boxes indicate endogenous *ced-3* promoter sequences, white boxes indicate *ced-3* genomic coding sequences. The genotype into which each construct was introduced is indicated in column 2. The average number of extra cells in the anterior pharynx of animals of a given strain is indicated in column 3. The number of animals observed for each strain is indicated in column 4. The range of extra cells observed in each strain is indicated in column 5.

Table 5

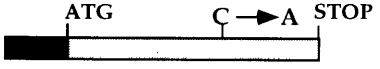
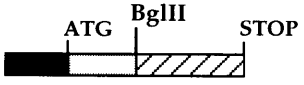
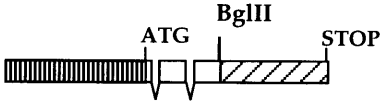
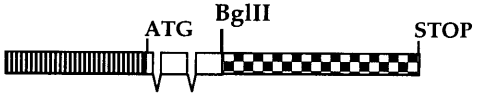
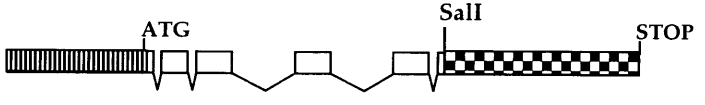
	Construct	Genotype	# extra cells	n	range
	None	Wild type	0.1	40	0-1
	None	*ced-3* (*n2433*)	12.4	10	11-14
A		Wild type	2.9 4.9 2.6	8 9 9	0-8 0-9 1-9
B		Wild type	1.0 1.2	9 11	0-4 0-3
C		Wild type	0.3 0.2 0.1	15 15 15	0-1 0-1 0-1
D		Wild type	1.3 0.7 0.4	15 15 15	0-3 0-3 0-1
E		Wild type	0.3 3.2	25 10	0-2 1-6
F		Wild type	1.5 2.4	23 31	0-4 0-9
G		Wild type	2.1 2.1	18 25	0-8 0-5

Table 6. PCR and sequencing primer sequences.

Table 6

Primer	Sequence
SHA2	5' AAATCGTACTCTGACTACGGG 3'
PCR2	5' TTCGCTACGAGATATTTGCGCG 3'
PCR3	5' GCGAAATTAAAATGTGCGAAACGTC 3'
PCR4	5' ATTTAACACAAATTGTCGTGTCGAGA 3'
PCR5	5' ATTTCCCAGCCTTGTTCCTAAT 3'
PCR6	5' ACTAATTGGGCGAAAGAGAACT 3'
PCR11	5' CATATTCCATGAAGAGGA 3'
PCR13	5' TCTCAACGCGGCAAATGC 3'
LOG4	5' TCAGACTAAATCGAAAATC 3'
650	5' GGTGACGCGCGGCAGGCTT 3'
BD1	5' GTTGTCCACGAGTATTACACGG 3'
1200	5' GGGCGAAAGAGAACTGGGGG 3'

Figure 1. Positions of *ced-3* mutations.

An alignment of sequences of CED-3 proteins from the nematodes C. elegans, C. briggsae, and C. vulgaris (Yuan et al., 1993) with sequences of mouse and human ICE (Thornberry et al., 1992), mouse Nedd-2 (Kumar et al., 1994), human ICH-1L and S (Wang et al., 1994), and CPP32 (Fernandez-Alnemri et al., 1994). Shaded regions represent sequences that are conserved between the nematode species and any one of the mammalian proteins. Missense mutations are indicated with the allele name and the altered residue above the wild-type residue. Nonsense mutations are indicated with the allele name and a stop sign above the altered residue. Introns are indicated by arrow heads. Mutations in splice donor or acceptor sites are indicated as Do or Ac respectively followed by the allele name.

Figure 1

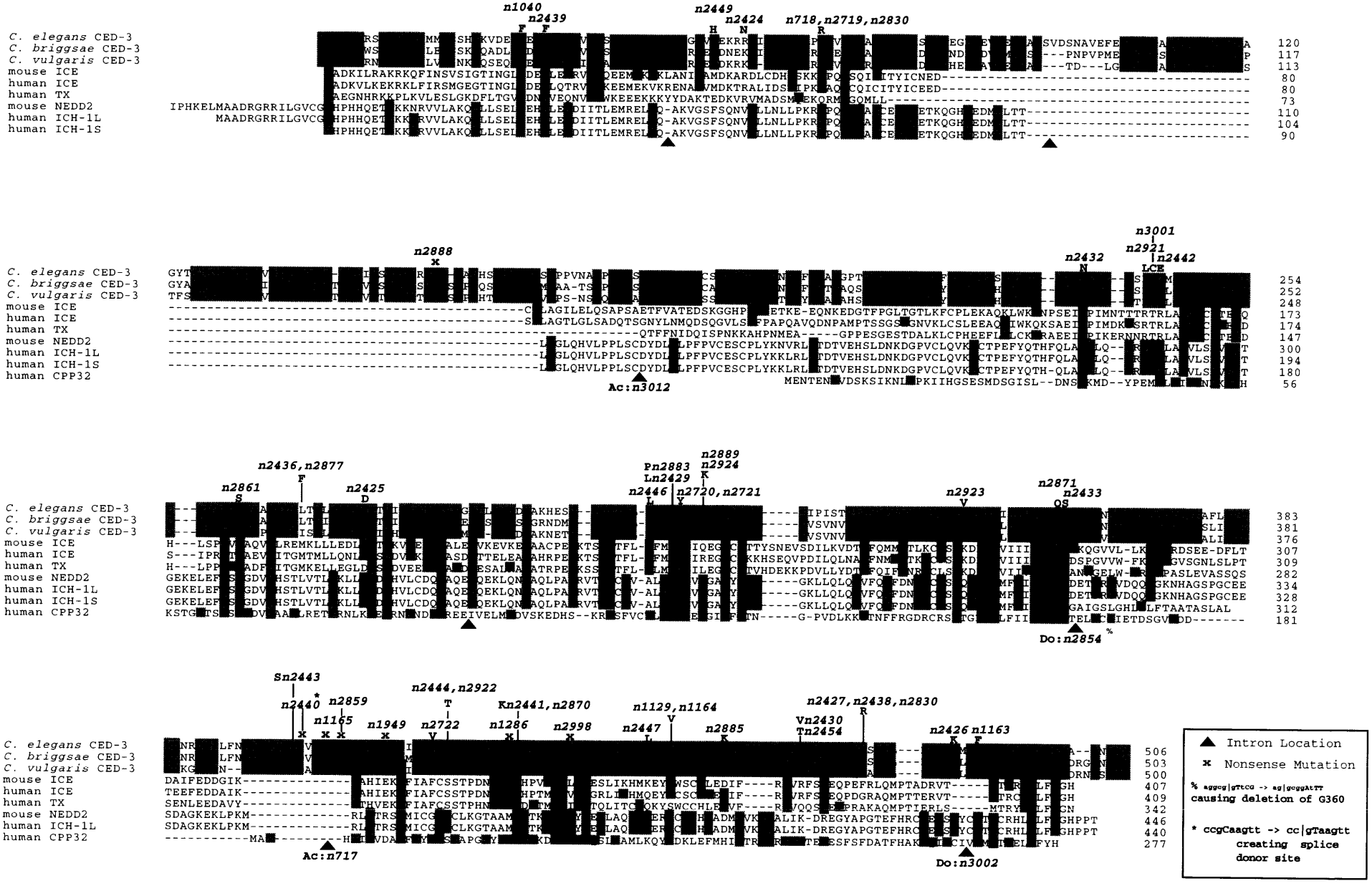

n1040
n2439
n2449
n2424
n718,n2719,n2830
C. elegans CED-3
C. briggsae CED-3
C. vulgaris CED-3
mouse ICE
human ICE
human TX
mouse NEDD2
human ICH-1L
human ICH-1S
n2888
n3001
n2921
n2442
n2432
LCE
human CPP32
Ac:n3012
n2436,n2877
n2861
n2425
n2889
n2924
Pn2883
Ln2429
n2446
n2720,n2721
n2923
n2871
n2433
Do:n2854
Sn2443
n2440
n2859
n1165
n1949
n2444,n2922
n2722
Kn2441,n2870
n1286
n2998
n1129,n1164
n2447
n2885
n2427,n2438,n2830
Vn2430
Tn2454
n2426
n1163
Ac:n717
Do:n3002
Intron Location
Nonsense Mutation
causing deletion of G360
creating splice donor site

Figure 2. Northern blot of 11 *ced-3* mutants.

RNA from 11 *ced-3* mutants was blotted and probed (see Materials and Methods) with either a *ced-3* cDNA probe (top panel) or with a *lin-26* cDNA probe as a loading control (bottom pannel).

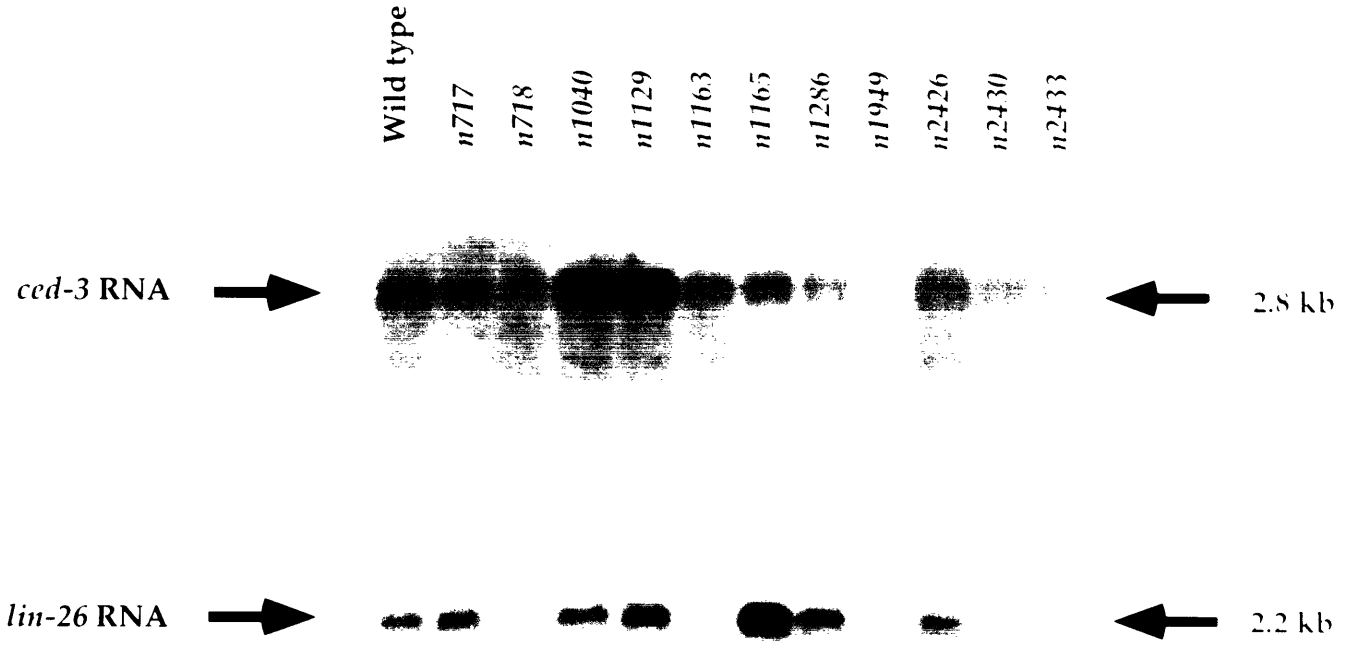

Wild type
n717
n718
n1040
n1129
n1163
n1165
n1286
n1949
n2426
n2430
n2433
ced-3 RNA
2.8 kb
lin-26 RNA
2.2 kb

Figure 3. *ced-3*(*n2452*) deletes regions necessary for CED-3 catalytic activity.

Left panel, southern blot of genomic DNA from *ced-3*(*n2452*) and wild-type animals. DNA was digested with XhoI. Size of bands is shown to the left. Right panel, southern blot of genomic DNA from *ced-3*(*n2452*) and wild-type animals. DNA was digested with HindIII. Size of bands is shown to the right. Both blots were probed with a *ced-3* cDNA probe.

ced-3(n2452)
Wild type
15 kb
5 kb
2.4 kb
1.7 kb
XhoI digest
ced-3(n2452)
Wild type
5 kb
3.5 kb
HindIII digest

Appendix

The C. elegans cell death genes *ced-3* and *ced-4* might be expressed in both cells that die and cells that do not die

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This chapter represents a preliminary characterization of the expression patterns of *ced-3* and *ced-4*.

Abstract

Mutations in the genes *ced-3* and *ced-4* prevent programmed cell death in the nematode Caenorhabditis elegans. We used *lacZ* and GFP fusion constructs as well as RNA in situ hybridization to analyze the expression pattern of the *ced-3* gene during development. Our results suggest that the expression of *ced-3* is wide spread and that the gene can be expressed both in cells that die and cells that do not die. We also examined expression of the *ced-4* gene using both epitpoe tagging experiments and anti-*ced-4* Abs. Our preliminary data suggest that this protein is excluded from the nuclei of cells overexpressing the gene, and that the gene is expressed in many cells during embryogenesis.

Introduction

Programmed cell death is a molecularly conserved process that occurs in all metazoans examined (Glücksman, 1950; Ellis et al., 1991). This process plays many roles during the development of organisms such as shaping tissues, eliminating cells whose function is no longer needed, and eliminating cells that might be harmful. In the nematode Caenorhabditis elegans 131 of the 1090 cells born during the development of the hermaphrodite, undergo programmed cell death (Sulston and Horvitz, 1977; Kimble and Hirsh, 1979; Sulston et al., 1983). This process usually occurs within an hour after the cell is born and is morphologically and ultrastructurally similar to the mammalian process of cell death termed apoptosis. Mutations in the genes *ced-3* and *ced-4* prevent programmed cell death in C. elegans (Ellis and Horvitz, 1986) suggesting that the activity of these genes is necessary for cells to die. Recent work (Shaham and Horvitz, manuscript in preparation) has demonstrated that overexpression of these genes is sufficient to cause cells that normally live to undergo programmed cell death under certain conditions, suggesting that the activity of these genes can be sufficient to induce programmed cell death. However, it has been suggested, based on genetic criteria (Shaham and Horvitz, manuscript in preparation) that both *ced-3* and *ced-4* are expressed in many cells in the animal and that a network of regulatory interactions among the products of these two genes and the product of the cell survival gene *ced-9* (which is similar to the mammalian *bcl-2* gene which also functions in preventing programmed cell death, Hengartner et al., 1992; Hengartner and Horvitz, 1994; Vaux et al., 1988) post-translationally mediate activation or suppression of the cell death program. The *ced-3* gene encodes a protein with similarity to the class of mammalian proteases represented by the protease Interleukin-1β converting enzyme (ICE) (Yuan et al., 1993; Thornberry et al., 1992). Overexpression of members of this family in culture results in programmed cell death (Miura et al., 1993, Kumar et al., 1994, Wang et al., 1994; Fernandes-Alnemri et al., 1994). In addition, mice harboring a knockout mutation in ICE reveal defects in Fas-mediated cell death, suggesting that ICE plays an important in vivo role in regulating cel death (Kuida et al., 1995). The *ced-4* gene encodes two novel proteins designated CED-4L and CED-4S which have opposite functions (Shaham and Horvitz, manuscript in preparation; Yuan and Horvitz , 1992). CED-4S can cause cells to undergo programmed cell death (Shaham and Horvitz, manuscript in preparation), whereas CED-4L can protect cells from dying.

To better understand the functions of the CED-3 and CED-4 proteins we have attempted to characterize their expression patterns. Our results suggest that both CED-3 and CED-4 are likely to be widely expressed in C. elegans. Both proteins are likely to

be present in both cells that die and cells that do not die. Our results also suggest that the CED-4 protein is probably excluded from the nuclei of expressing cells.

Results

ced-3-lacZ fusion constructs are expressed in embryos and early larvae as well as in the tail of L4 and adult males

To determine the expression pattern of *ced-3* we constructed translational fusion constructs between the *ced-3* gene and E. coli *lacZ* gene (see Materials and Methods). Transgenic lines containing these constructs were stained using X-gal to visualize expression of the *lacZ* protein. We examined separate lines containing one of each of three different translational fusions shown in figure 1. Transgenic animals containing constructs 1 and 2 showed a similar staining pattern to animals containing construct 3 which did not contain a nuclear localization signal. As figure 2 shows we examined hermaphrodites of various stages and observed staining in 200 minute-old embryos, later staged embryos, and first larval stage (L1) larvae. Early embryos, older larvae, and adults either did not stain at all or contained only a small number of staining cells. This general staining pattern is consistent with our previous results (Yuan et al., 1993) suggesting that expression of *ced-3* RNA in hermaphrodites is primarily embryonic, and is greatly reduced in older larvae. Our results are also consistent with the observations that most programmed cell deaths in hermaphrodites occur in embryos and L1s, and are consistent with the notion that *ced-3* expression is required for cell death to occur. Interestingly, many of the cells we observed to stain do not die during normal development, suggesting that the mere expression of *ced-3* might not be sufficient to kill cells in vivo (see Discussion), however, some of the staining cells were cells destined to die (see below). Many cells in the male tail undergo programmed cell death during the L4 stage (Sulston and Horvitz, 1977). We examined the expression of construct 1 during this stage in males. As figure 2d shows several cells stained in the male tail during this period and in adult males that did not stain in the hermaphrodites. This result supports the notion that *ced-3* expression is necessary for programmed cell death to occur. Because these studies used transgenes which are overexpressed we can not rule out the possibility that aspects of the staining pattern we observed are artefactual.

ced-3-lacZ fusion constructs that prevent cell death are expressed in some cells destined to die

The *lacZ* constructs used in the experiments described above can prevent programmed cell death in wild-type animals (Shaham et al., manuscript in preparation).

Interestingly, some of the undead cells stain with X-gal, suggesting that cells that normally undergo programmed cell death actively transcribe the *ced-3* gene. Figure 3 shows two surviving tail spike cells which should have died during embryogenesis (Sulston et al., 1983), but survive in an L1 animal and express the *ced-3-lacZ* transgene. This observation is consistent with *ced-3*-GFP results (see below), and with the notion that *ced-3* activity is necessary within a cell that dies for that cell to die (Yuan and Horvitz, 1990; Shaham and Horvitz, manuscript in preparation) (see Discussion). Why expression of *ced-3* in some cells is sufficient to drive the death program, and in others is not is not clear (see Discussion).

A *ced-3*-GFP fusion construct is expressed in both cells that die and do not die

To further examine the expression pattern of the *ced-3* gene we examined expression of two different trangenes containing a fusion to the GFP reporter gene (figure 4). Transgene 4 (figure 1) containing a translational fusion to *ced-3* at the identical position as transgene 1 containing *lacZ* as the reporter gene did not show any levels of expression as assayed by observation of live animals under a fluoresence micoscope. Interestingly, this trangene was capable of preventing programmed cell death (Shaham et al., manuscript in preparation), suggesting that protein was made from the construct. These observations suggests that the GFP moiety might not be properly folded, or that its chromophore group is not accessible to the impinging light and suggests that a transcriptional fusion to GFP might be more appropriate for examining *ced-3* expression. As figure 4 shows, transgene 5 containing a transcriptional fusion to *ced-3* does show expression, yet does not prevent programmed cell death (data not shown). Using this GFP transgene we observed a similar expression pattern to that using the *lacZ* transgenes described above. 200-minute embryos stained strongly with the construct, and staining persisted into the first larval stage and greatly diminished in older animals. Expression was detected in many cells that do not die as shown in figure 4. Because we were able to observe GFP expression in live animals we could directly assay if cells undergoing programmed cell death were expressing our GFP constructs. As shown in figure 4 we could readily identify cell corpses in embryos that expressed GFP. This result suggests that some cells that die actively transcribe *ced-3*, and supports the indirect observations made in the previous section.

***ced-3* RNA is expressed in many cells during embryogenesis**

To examine the expression of the *ced-3* gene more directly we used RNA in situ hybridization to follow expression of endogenous *ced-3* RNA in embryos (see Materials and Methods). We were unable to assess the presence of *ced-3* RNA in late embryos,

larvae and adults. However, we were able to observe staining in earlier embryos. As shown in figure 5 we could identify *ced-3* RNA in many cells during mid embryogenesis. Interestingly, the RNA was mostly localized to the nuclei of expressing cells in a manner similar to that described by Seydoux and Fire, 1994. Staining was clearly detected in many cells that do not die, consistent with our observations described in the previous sections. This result supports our previous hypothesis (Shaham et al., manuscript in preparation) suggesting that *ced-3* is likely to be expressed in many and perhaps all cells in the animal, however we have not yet performed adequate controls to rule out staining artefacts in our in situ protocols.

ced-4 encodes a 67 kd protein which is present in _ced-3_ mutants

To examine the CED-4 protein we generated anti-CED-4 antibodies in rabbits (see Materials and Methods). After affinity purifying our antibodies we examined their staining pattern on a Western blot. As figure 6 shows our antibodies recognize a band of approximately 67 kd, the expected size for the CED-4 protein, which is not present in animals carrying the mutation *ced-4(n1162)* which does not produce any detectable RNA (Yuan and Horvitz, 1992). Introduction of a transgene containing wild-type *ced-4* genomic sequences into *n1162* animals restores the 67 kd band (data not shown). This observation suggests that *ced-4* encodes a 67 kd protein as has previously been previously described (Yuan and Horvitz, 1992). Interestingly, *ced-4* encodes two alternatively spliced messages, *ced-4*L and *ced-4*S (Shaham et al., manuscript in preparation). The *ced-4*L transcript is at least 10 fold less abundant than *ced-4*S. We did not detect two *ced-4*-specific bands in our western blots, suggesting that CED-4L is probably less abundant than CED-4S. To test whether mutations in the *ced-3* gene might affect the size or level of CED-4 protein we examined the expression of the protein in animals containing different *ced-3* mutations. As shown in figure 6, none of the *ced-3* mutants showed significant alterations in size or amount of CED-4 protein produced, suggesting that *ced-3* does not grossly affect the nature of the CED-4 protein.

CED-4 protein is likely to be excluded from nuclei and is expressed in many cells

To examine the intracellular localization of the CED-4 protein as well as to characterize its expression pattern we attempted to observe CED-4 reactivity in situ with our antibodies. We did not observe any staining in wild-type animals, suggesting that CED-4 might be present in only a small amount, might be diffuse, or might be hidden from our antibodies. To check that our antibodies were capable of detecting CED-4 protein in situ we examined staining in lines presumably overexpressing the CED-4 protein. Figure 7a shows staining in an embryo transgenic for several copies of

the *ced-4* genomic region. Staining is clearly excluded from the nucleus and seems to be punctate. The staining pattern also seems to be membrane-associated. We observed an identical localization in animals carrying a *ced-4* transgene containing an epitope tag which is capable of rescuing the Ced-4 mutant phenotype, suggesting that this construct is expressed at least in part in the correct location (see Materials and Methods) and stained with an anti-epitope antibody (figure 7a). We also examined the presence of CED-4 reactivity in the ALM neurons of animals containing a transgene in which a *ced-4* cDNA is transcribed under the control of the *mec-7* promoter which is expressed in the ALM neurons (Savage et al., 1989). Interestingly, although immunoreactivity was excluded from the ALM nuclei, the punctate staining pattern was not observed, and staining was more cytoplasmic (figure 7b). Because the *mec-7* promoter is highly expressed it is possible that the difference between the intracellular localizations in the two experiments described above is due to levels of expression. These observations suggest that our antibodies are capable of detecting *ced-4* expression in situ, and that the CED-4 protein is excluded from the nuclei of expressing cells.

Discussion

The determination of the intracellular localization and tissue distributions of proteins involved in cell death is important to understanding their mechanisms of action. In this appendix we have presented preliminary data concerning the expression of the C. elegans cell death genes *ced-3* and *ced-4*. Our results suggest that both proteins are likely to be expressed throughout the animal in both cells that die and cells that do not die. For *ced-3* we demonstrated using transgenes containing *lacZ* and GFP fusions to *ced-3* genomic sequences that these fusions are expressed in many cells, and results of RNA in situ hybridization suggest a widespread expression pattern at least during mid-embryogenesis. For *ced-4*, epitope tagging experiments as well as antibody stainings suggest that the protein is present in many cells. These results are consistent with our previous hypothesis based on genetic evidence that *ced-3* and *ced-4* are likely to be expressed in many cells that do not die as well as in cells that do (Shaham and Horvitz, manuscript in preparation). Our results also suggest that in vivo it is probably not always the case that expression of either *ced-3* or *ced-4* is sufficient to kill cells, since many cells that do not normally die seem to express both proteins. We have previously described a set of potential post-translational regulatory interactions among the products of the *ced-3* and *ced-4* genes and the product of the *ced-9* gene (Shaham and Horvitz, manuscript in preparation; Shaham and Horvitz, manuscript in preparation; Hengartner et al., 1992). It is possible that these interactions directly determine the cell death fate of a cell and that expression of *ced-3* and *ced-4* is not the unique determining

factor of that fate. The fact that not all the cells we observed stained using the techniques outlined above could be interpreted in a number of ways. One possibility is that we have not optimized staining conditions in the experiments described above. It is also possible that protein at the required levels is normally present in all cells, but is only transcribed in a smaller subset of cells. This perdurance would result in staining patterns in animals transgenic for either transcriptional fusions to reporter genes or in animals containing many copies of a given gene that would not be expressed in all cells. It is also possible that the animals we observed were mosaic for the transgenes used, and were thus not expressing in all cells. Finally, it is possible that the staining patterns we observed are similar to the endogenous distributions of the CED-3 and CED-4 proteins. The latter possibility seems less likely since cells which have been suggested to contain CED-3 and CED-4 protein (such as the PVM neuron, Shaham and Horvitz, manuscript in preparation) have not stained using the techniques described above.

It is likely that the CED-4 protein is excluded from nuclei of expressing cells as shown in figure 7. However, we can not reliably indicate a localization outside the nucleus. CED-4 staining patterns in animals transgenic for multiple copies of the *ced-4* gene seem to contain immunoreactivity which is punctate and is plasma membrane associated. Interestingly, the CED-4 protein terminates in a dicysteine sequence which has been shown to be a substrate for geranyl geranylation in some instances. Addition of this hydrophobic moiety would serve to anchor the protein in the membrane. Two observations shed some doubt about the membrane localization and its mode. First, sequences of CED-4 genes from related nematodes do not terminate in dicysteines (S. Shaham, data not shown) suggesting that geranyl geranylation might not be important for CED-4 function. Second, CED-4 immunoreactivity in ALM neurons expressing a *ced-4* cDNA under the control of the *mec-7* promoter is not confined to the membrane and is equally distributed in the cytoplasm (figure 7b).

Our results suggest preliminary possibilities for the localization and cell distribution of CED-3 and CED-4. More refined analyses using reagents that can detect expression of the endogenous products is necessary to more precisely define these parameters.

Materials and Methods

Plasmid constructions

All the plasmids described in this work have been prviously described (Shaham et al., manuscript in preparation) except for the ones described below. Plasmid 3 was

constructed in the identical manner to plasmid 1 except that a *lacZ* gene not containing a nuclear localization signal was used. Plasmid 5 containing a transcriptional *ced-3*-GFP fusion was constructed as follows: plasmid pJ40 (Yuan et al., 1993) was amplified using the M13 reverse primer (New England Biolabs, Beverly, MA) and a 21 nucleotide primer complementary to sequences immediately upstream to the ATG and containing a SalI site at its 5' end. The resulting product, as well as pBluescript (Stratagene, La Jolla, CA), was digested with the enzymes BamHI and SalI and ligated. The resulting plasmid was then digested with SalI and NotI and ligated to the GFP insert of plasmid Tu#62 (M. Chalfie, personal communication) cut with SalI and EagI. The epitope tagged *ced-4* genomic fragment was constructed as follows. In vitro mutagenesis on the plasmid C10D8-5 (Yuan and Horvitz, 1992) was performed using the primer CED-4-OLIGO-1 (5' CCGATGCCTGTTGGATACCCATACGACGTCCCAGACTACGCTGAAAAAGAAGA AGAT 3') and following the instructions of the manufacturer. This resulted in the insertion of a nine amino acid flu epitope (Kolodej and Young, 1991) having the corresponding 12CA5 antibody epitope at the carboxy terminous of the CED-4 protein.

RNA in situ hybridization and antibody techniques

Techniques for RNA in situ hybridizations were as described in Mitani et al., 1993 using the oligo CED-3 ANTI2 (5'CACGAGTGAATTTTAAGCG TAGTCTGGGACGTCGTATGGGTAGACGGCAGAGTTTCG 3'). Antiserum against the CED-4 protein was generated as follows: gel purified CED-4 protein generated from the plasmid pJ76 (Yuan and Horvitz, 1992) was electroeluted from the gel, dialyzed against PBS (Sambrook et al., 1989), emulsified with complete Freund's adjuvant and injected into New Zeland White rabbits. The rabbits were boosted twice with a protein produced in E. coli from the pMal-c plasmid (New England Biolabs, Beverly, MA) containing the E. coli *malE* gene fused to the BamHI-EcoRI fragment of the *ced-4* cDNA containing the terminal 340 amino acids (Yuan and Horvitz, 1992). This protein was purified on an amylose resin, dialyzed against PBS and injected with incomplete Freund's adjuvant into rabbits. Whole serum or affinity purified serum was used to detect CED-4 protein on methanol/paraformaldehyde fixed animals. Fixation of animals was done as described in Finney and Ruvkun (1990) or using a modified method (M. Finney and G. Ruvkun, personal communication) except that we used a 30 minute 2-mercaptoethanol treatment. Fixed animals of mixed stages were incubated at room temperature with 1% serum diluted in PBS and 1% BSA (Bovine Serum Albumin) overnight. Animals were then washed with PBS three times for 20 minutes each, incubated at 37°C with 5% goat anti-rabbit antibodies conjugated to FITC (Cappel,

Durham, NC) for two hours, washed again three times for 20 minutes each, and resuspended in 30 µl of PBS. Five microliters of the final suspension were mixed with five microliters of phenylenediamine (Sigma, St. Louis, MO; 1mg/ml in 90% glycerol in PBS) and mounted on a slide for observation using a fluorescent microscope with a 100X objective. Only lines containing extra copies of the *ced-4* gene showed any staining. Western blots were performed as described in Harlow et al., 1988 using an alkaline phosphatase conjugated secodary antibody (Cappel, Durham, NC).

lacZ and GFP staining procedures

lacZ staining procedures were as described by Fire et al., 1992. GFP detection procedures were as described by Chalfie et al., 1994.

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Figure 1. Reporter constructs used to assay *ced-3* expression.

Constructs are described in Materials and Methods. White boxes represent *ced-3* genomic coding sequences, black boxes represent a nuclear localization signal, stippled boxes represent *ced-3* sequence 5' of the ATG, hatched slanted boxes represent GFP, hatched vertical boxes represent *lacZ*. Construct are named 1-5 as indicated to the left of each construct.

Figure 1

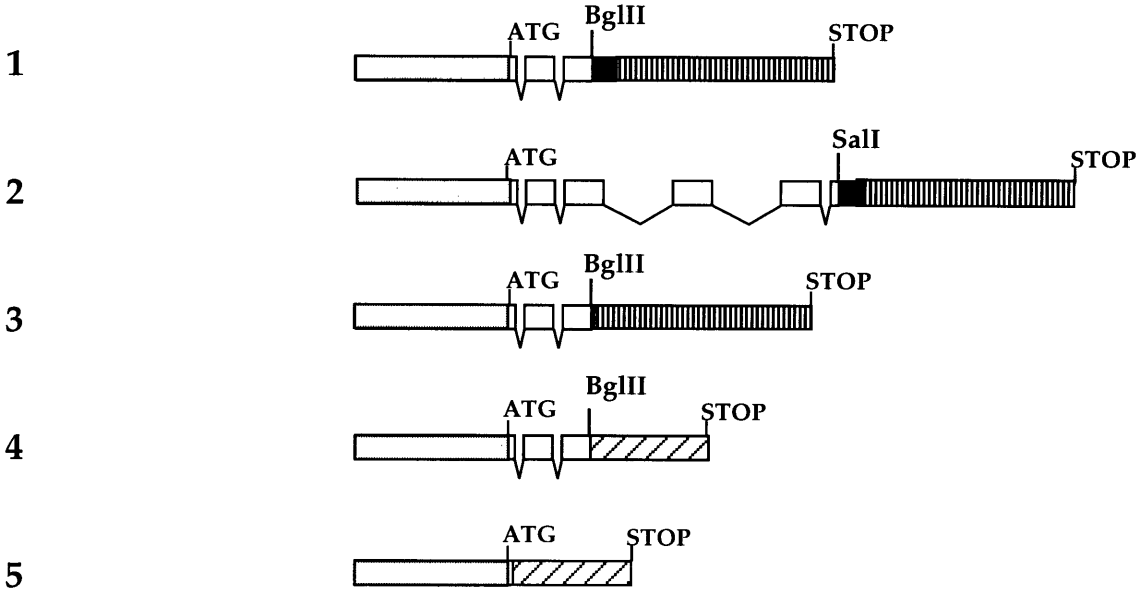
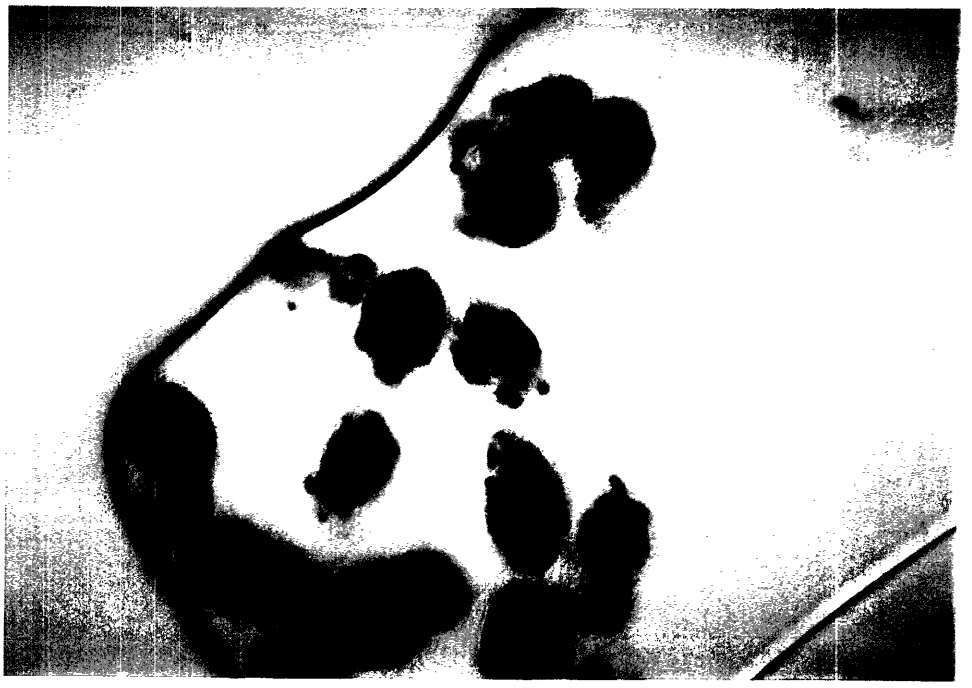
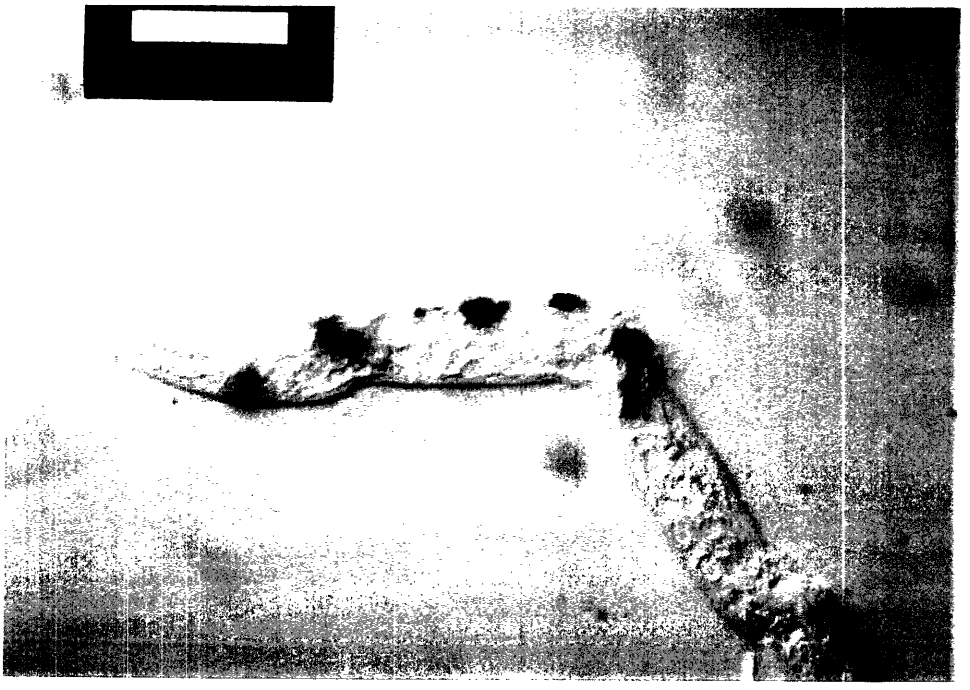

Construct
Structure
1
ATG
BglII
STOP
2
ATG
SalI
STOP
3
ATG
BglII
STOP
4
ATG
BglII
STOP
5
ATG
STOP

Figure 2. *ced-3-lacZ* expression pattern.

Transgenic animals carrying construct 2 (figure 1) stained for *lacZ* expression (see Materials and Methods). a, embryos, b, L1 larva, c, older larvae, d, Adult male tail.





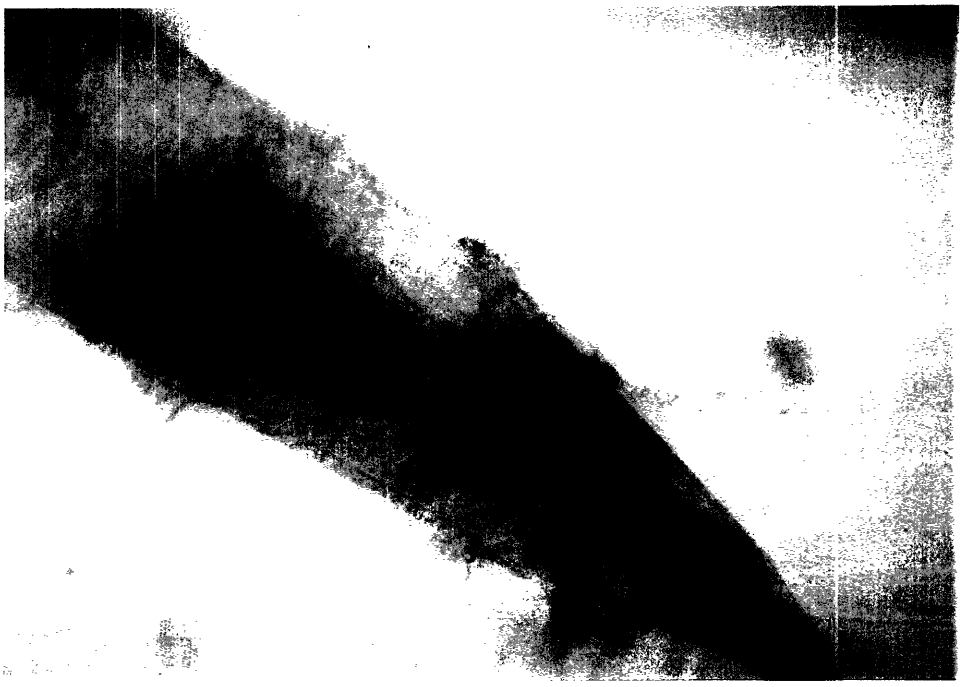


Figure 3. Undead tail spike cells express a *ced-3-lacZ* transgene.

Transgenic L1 animal carrying construct 2 (figure 1) stained for *lacZ* expression (see Materials and Methods). Arrows are pointing at the two tail spike cells

Figure 4. *ced-3*-GFP expression pattern.

Transgenic embryos carrying construct 5 were visualized under a fluoresence microscope. a, embryo, b, Nomarski phtograph of an embryo, arrow indicates a cell corpse, c, fluorescent image of the same embryo shown in b, arrow pointing at the same cell corpse shown in b.

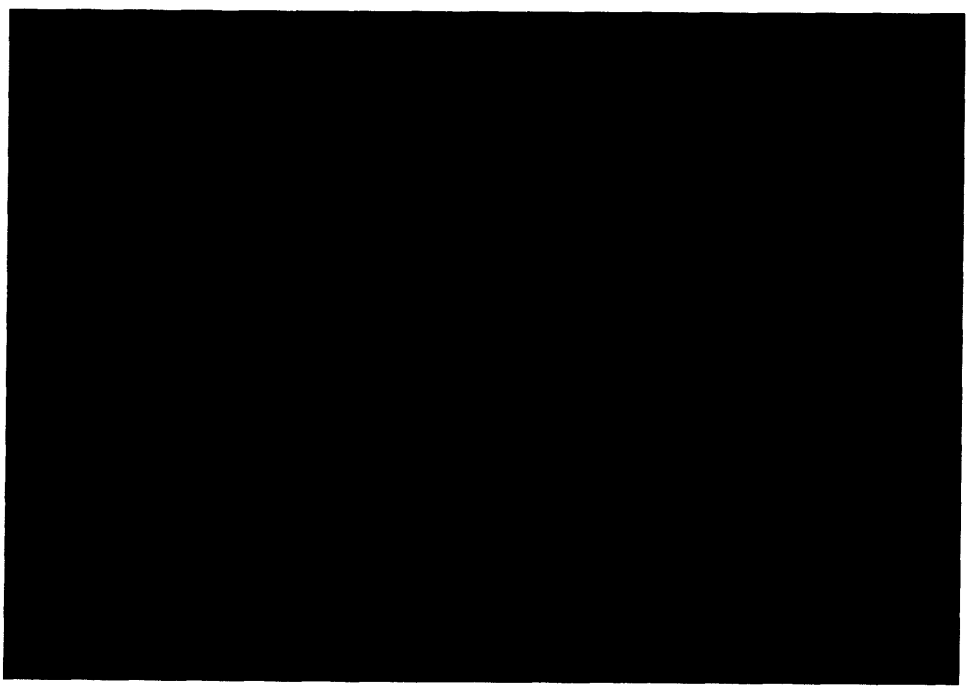


Figure 5. *ced-3* RNA in situ hybridization.

Wild-type embryos stained for *ced-3* RNA expression using an in situ hybridization technique (see Materials and Methods). Arrows indicate some staining cells.

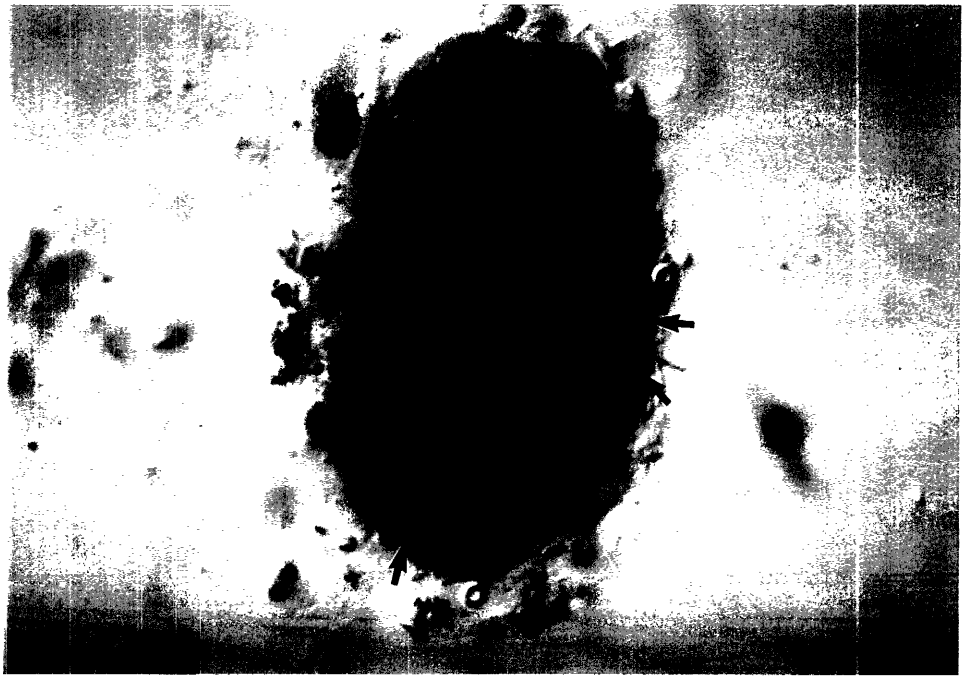


Figure 6. CED-4 is expressed at normal size and levels in *ced-3* mutant backgrounds.
Western blot showing reactivity of anti-CED-4 antibody (see Materials and Methods). Lanes are labeled with the relevant genotype. Arrow indicates the location of the CED-4 band.

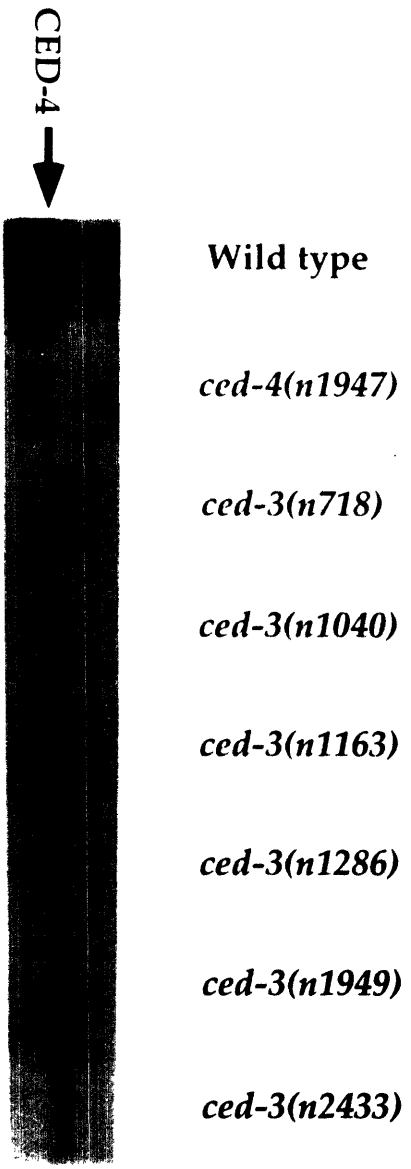
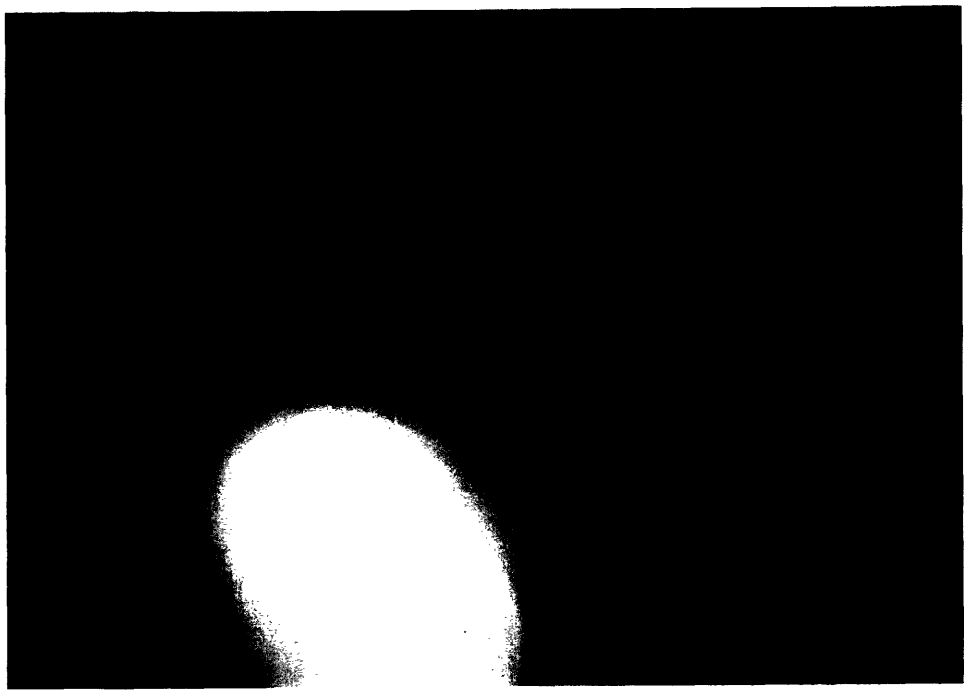
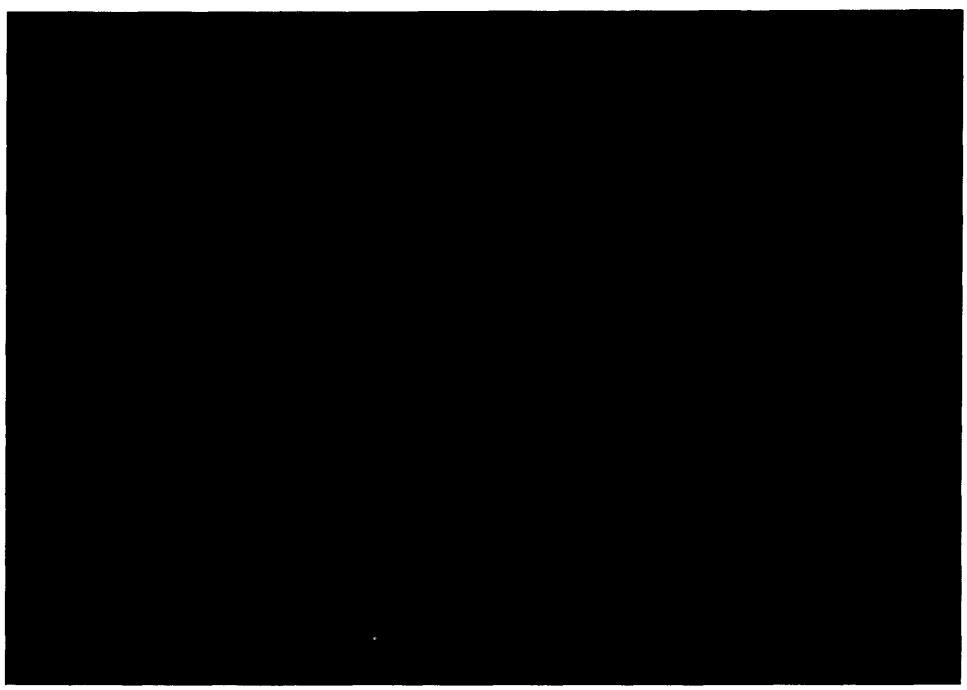

CED-4
Wild type
ced-4(n1947)
ced-3(n718)
ced-3(n1040)
ced-3(n1163)
ced-3(n1286)
ced-3(n1949)
ced-3(n2433)

Figure 7. CED-4 protein is excluded from nuclei.

a, *ced-4* mutant animal transgenic for several copies of a flu epitope tagged *ced-4* genomic fragment stained using an anti-epitope antibody. Arrows indicate some staining cells. b, An ALM neuron overexpressing CED-4 in an animal transgenic for a $P_{mec\text{-}7}$*ced-4* fusion construct stained with anti-CED-4 antibodies. Arrow indicates the staining cell.





Perspectives

Speculations on a few issues

Shai Shaham

In this section I would like to discuss a number of issues relating the work described in the previous chapters to cell death in general. Key questions remain concerning the applicability of the findings in worms to mammals, and even in *C. elegans* it is not clear how the various cell death genes interact on a molecular level. Below are described a number of these issues peppered with rampant speculations about what the answers might be.

What determines if a cell lives or dies?

The work described in previous chapters has alluded to the fact that cells that normally die in *C. elegans* have a greater propensity to die than cells that normally survive. Two observations are the following. 1) Cells that normally die die, and cells that normally live live. This observation clearly shows a difference between living cells and dying cells, and since in most cases in *C. elegans* cell death is thought to have a cell-autonomous component it is reasonable to assume that the differences between dying cells and living cells might, in part, reside within each cell. 2) Weak mutations in *ced-3* block ectopic cell deaths induced by loss-of-function mutations in *ced-9* but will not block the deaths of cells that normally die. This observation suggests that there is an inherent difference between cells that normally live and normally die, and that this difference is in some way related to the function of *ced-3*. Cells that normally die are either more sensitive to the activity of *ced-3* or are more sensitive to some aspect of the regulation of *ced-3* activity. Thus, it seems plausible that some aspect of *ced-3* function is responsible for the differences between cells that live and those that die. This difference could occur upstream or downstream of *ced-3*. A number of hypotheses are presented below.

One intriguing observation presented in chapter 3 is that of the effect of endogenous *ced-4* activity on the ability of overexpression of *ced-3* to cause cell death. It turns out that it is irrelevant whether the endogenous *ced-4* gene is wild-type or mutant when the ALM neurons are killed by overexpression of *ced-3* in an otherwise wild-type background. However, if a loss-of-function mutation in *ced-9* is introduced into the background the *ced-4* mutation does make a difference. In fact, in the absence of *ced-9*, a *ced-4* mutation will inhibit some of the killing activity of the overexpressed *ced-3*. Based on the model proposed in chapter 3, one possible interpretation of these results is as follows. The ALM neurons are cells that normally do not die, and thus their endogenous cell death machinery should exist in an inactive form. If we assume that *ced-9* normally acts to inhibit the activity of *ced-4*S in cells that live, then it would make sense that the state of endogenous *ced-4* is irrelevant to killing by *ced-3* overexpression

in a wild-type background. When a wild-type *ced-4* product is present it should be fully inhibited by the wild-type *ced-9*, and hence will be inactive. This is the same as not having any *ced-4* around at all as in the case of the mutated *ced-4*. However, in the absence of wild-type *ced-9* *ced-4* is now not subject to negative regulation. Thus, if *ced-4* is wild-type it will help activate *ced-3*, but if *ced-4* is mutated it will not activate *ced-3*, leading to reduced killing. This interpretation suggests that one way in which cells that live and cells that die are different is in the state of their *ced-9* product. Active *ced-9* results in survival, and inactive *ced-9* results in death. This conclusion is, in fact, similar to that described by Hengartner et al. (1992)[1]. In their paper the authors concluded that since the absence of *ced-9* results in ectopic cell death, then it is possible that the state of *ced-9* regulates the survival decision.

However, it seems that control of the difference between cells that die and those that survive is likely to be regulated on other levels as well. We demonstrated in chapter 4 that the *ced-4*L product is likely to protect cells from cell death. Mutations in this transcript are likely to make cells that normally live more sensitive to death and overexpression of this transcript will prevent the deaths of cells that normally die. These characteristics of *ced-4*L are very similar to those of *ced-9*. Does *ced-4*L act similarly to *ced-9*? We do not have a mutation in *ced-4*L which does not also affect the *ced-4*S product. However, it is tempting to speculate that selective inhibition of *ced-4*L will result in lethality and ectopic cell death, just as does elimination of *ced-9*. Thus, according to this model, neither *ced-9* nor *ced-4*L are sufficient on their own to protect from cell death. Both are needed for protection to occur. This model is completely consistent with that outlined in the previous paragraph. Thus, it is also possible that the state of *ced-4*L is crucial to the decision of whether a cell lives or dies. In the case of *ced-4*L we have an inkling into how this product might be regulated. Since *ced-4*L results from alternative splicing of the *ced-4* locus it is possible that splicing plays a key role in regulating the decision of a cell to live or die.

This last point brings up the final major difference that is likely to exist between cells that live and cells that die. Expression of all the components of the cell death pathway involved in killing is a prerequisite for any cell to die. It is possible that expression of key components such as *ced-3* or *ced-4*S is restricted only to a certain set of cells, and thus, cells not expressing these products will never die. This possibility, although quite attractive, is made less plausible by two observations. First, experiments in chapter 3 have demonstrated that it is likely that *ced-3*, *ced-4*S, and *ced-9* are all expressed in at least a number of cells that do not die, including all *mec-7-* and *unc-30*-expressing cells. Second, experiments described in the appendix of this thesis suggest that *ced-4* and *ced-3* are likely to be expressed in many cells, including cells that

normally do not die. Thus, although it is certainly possible that some cells in *C. elegans* survive because they do not express *ced-3* or *ced-4*, it is unlikely that this is the main mechanism for differentiating between cells that live and cells that die.

How is *ced-3* regulated by *ced-9* and *ced-4*?

The issues raised above beg the question of how *ced-9* and *ced-4* act to inhibit or activate *ced-3* respectively. Although no biochemical experiments have been performed to address this question a number of hypothesis exist and can be tested biochemically.

The results of genetic analysis presented in chapter 3 suggest that *ced-9* does not act to directly inhibit the activity of *ced-3*. Rather, it seems likely that a major component of negative regulation in the cell death pathway in *C. elegans* involves the negative regulation of *ced-4*S by *ced-9*. It is, of course, possible that *ced-9* does have some direct regulatory role on *ced-3* but this is likely to be minor. Thus, the original question posed above can be split into at least four questions. How does *ced-4*S activate *ced-3*, how does *ced-9* inhibit *ced-4*S, how does *ced-9* inhibit *ced-4*L, and how does *ced-4*L inhibit cell death? To address these question biochemical interaction studies among the various components of the cell death pathway must be performed. Currently the answer to the last question posed above is more difficult to assess because it is not clear if *ced-4*L prevents cell death by acting upstream, downstream or in parallel to *ced-3*.

The first question posed above might be answered by considering an interesting observation. Within the genetic pathway presented in chapter 4 *ced-9* can either inhibit or cause cell death. Thus, *ced-9* acts in a permissive way to decide if *ced-3* gets activated or not. One possible model for the regulation of *ced-3* is thus that *ced-9* is actually irrelevant in some respect, and that there is a competition between *ced-4*L and *ced-4*S to block or activate *ced-3*, respectively. This suggests that *ced-4*S might bind *ced-3* and be involved in regulating autocleavage of *ced-3*, and that *ced-4*L could bind *ced-3* but not be able to regulate autocleavage. Other models are also clearly possible.

The *n1950* mutation of *ced-9* is likely to be an interesting mutation to study in the context of the second and third questions posed above concerning interactions between *ced-9* and *ced-4*L and *ced-9* and *ced-4*S. Experiments in chapter 4 suggest that it is possible that *n1950* results in survival of cells that normally die by being unable to negatively regulate *ced-4*L yet still capable of negatively regulating *ced-4*S. This intriguing possibility suggests that perhaps *ced-9* normally interacts with both *ced-4*L and *ced-4*S and that in *n1950* this interaction is selectively perturbed. This possibility is readily amenable to biochemical analysis. Binding studies should reveal if this idea is correct. Again, many other models are plausible and it is certainly possible that *ced-9*, *ced-3* and *ced-4* do not interact at all.

The role of CED-3/ICE-like proteases in mammalian programmed cell death

The results described in this thesis about the regulation of programmed cell death suggest that perhaps a similar set of genes and interactions exist in mammalian systems. Two issues are critical to address when making these comparisons. First, are the molecular players similar between worms and man? Second, are interactions among cell death genes in mammals similar to those observed in *C. elegans*?

It has become clear that mammals contain a number of proteins similar to both CED-3[2-7] and CED-9[8-10]. In most cases all of these components have been shown to exhibit some sort of death-related activity. However, only in three cases has this assertion been rigorously tested by knockout mutations. Elimination of ICE in mice does not seem to have a devastating effect on the animals, at least from a cell death point of view. The only cell death defect detectable in these animals is a debatable inability for some cells to undergo Fas-induced cell death[11]. Thus, it is unlikely that ICE on its own is the only cysteine protease required for cell death in mammals. Because a large family of these proteases exists and because they have relatively different tissue distributions one possibility is that within each mammalian cell there exist at least two CED-3-like proteases that become induced during programmed cell death. Thus, one model suggests that there is a redundancy in the cysteine-protease killing activity. This model makes sense particularly in the immune system where it is often very important to eliminate cells that are potentially harmful to the animal, and thus it is advantageous to ensure that the death program is properly executed. However, this redundancy also suggests that it would be more difficult to regulate the activity of two genes as opposed to a single gene. Thus, diversity of negative regulators of cysteine-proteases involved in cell death is important (see below). Of course, it is also possible that there is normally only one protease involved in mammalian programmed cell death and that it is not ICE.

Elimination of the bcl-2 gene in mice results in generally few cell death defects, although some defects are eventually observed in the immune system where massive cell death can be demonstrated[12]. Similar observations exist for mice deficient in the bcl-x [8]gene although in this case the mice die embryonically from what seem to be cell death related defects. Thus, both bcl-2 and bcl-x are likely to have genuine roles in the negative regulation of cell death in mammals. Because the expression patterns of these genes are different it is possible that they each act in a different set of tissues. However, the redundancy model might also apply here. Thus, both bcl-2 and bcl-x might be important in keeping a given cell alive, although either alone might not always be sufficient.

Thus, genetic evidence suggests that at least some of the molecular components of cell death that have sequence similarity to the *C. elegans* genes have similar functions as well. Yet, clearly the mammalian situation is more complex.

It seems that some of the ICE-like genes and some of the BCL2-like genes in mammals interact in a way consistent with the way in which *ced-9* negatively regulates *ced-3*. Overexpression of *bcl-2* will prevent death induced by overexpression of ICE[2] or ICH-1[3], suggesting that in mammals *bcl-2* acts to negatively regulate the activity of cysteine proteases.

An intriguing issue which is currently unresolved is whether a mammalian equivalent of the *ced-4* gene exists. Currently, all efforts to isolate *ced-4*-like genes in mammals have failed, suggesting that if a *ced-4*-like gene exists it does not share more than 30-40% identity (the limit of detection by hybridization) with the *C. elegans* gene. It is, of course, possible that *ced-4* does not exist in mammals, however, it seems likely that proteins sharing a similar function to that of *ced-4* in *C. elegans* must exist. If one assumes the pathway in *C. elegans* to be applicable to mammals then since *ced-9*-like proteins can negatively regulate *ced-3*-like proteins in mammals, *ced-4*-like function must also exist. Thus, one possibility is that a functional homolog of *ced-4* exists in mammals which shares little if any sequence similarity to *ced-4*. In fact, it is possible that two classes of proteins exist in mammals, those with functions similar to *ced-4*L, and those with functions similar to *ced-4*S. These might not share any sequence similarities to the *C. elegans* proteins. Further work on the mammalian cell death systems will reveal how parallel these systems are to *C. elegans*.

Isolating more cell death genes in *C. elegans*

Finally, the work described in this thesis has suggested a number of genetic ways to try and identify new components of the cell death pathway in *C. elegans*. Because many screens have been performed looking for mutations resulting in the prevention of normally occurring cell death it is likely that any remaining components fall into one of two classes. Mutations in these killing components could result in lethality for some reason. Or there might exist as sets of redundant genes ordered in redundant pathways, each of which is sufficient to activate cell death. Screens for mutations in genes involved in protection from cell death have not been extensively explored and some possibilities are presented below.

Since overexpression of *ced-3* or *ced-4* can induce cell death in the appropriate background then suppressors of these deaths can reveal new cell death components. Weak mutations in genes that are essential might be revealed, as well as dominant mutations in genes that prevent cell death.

Enhancer screens to enhance the weak *ced-3* alleles should be tried to obtain mutations in genes that are involved in killing. This screen might isolate weak mutations in essential genes or mutations in redundant pathways (assuming there isn't complete redundancy).

Finally, looking for cell survival genes by screening for lethals balanced by a *ced-3* dominant negative extrachromosomal array should reveal additional *ced-9*-like genes. I used this strategy to isolate the *ced-9* allele *n2812*.

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