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The *ced-8* Gene Controls the Timing of Programmed Cell Deaths in *C. elegans*

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Summary

Loss-of-function mutations in the gene *ced-8* lead to the late appearance of cell corpses during embryonic development in *C. elegans*. *ced-8* functions downstream of or in parallel to the regulatory cell death gene *ced-9* and may function as a cell death effector downstream of the caspase encoded by the programmed cell death killer gene *ced-3*. In *ced-8* mutants, embryonic programmed cell death probably initiates normally but proceeds slowly. *ced-8* encodes a transmembrane protein that appears to be localized to the plasma membrane. The CED-8 protein is similar to human XK, a putative membrane transport protein implicated in McLeod Syndrome, a form of hereditary neuroacanthocytosis.

Introduction

It is critical for animals to be able to eliminate unwanted cells in a regulated, timely manner in response to developmental and environmental cues. This elimination is accomplished through a tightly controlled process of programmed cell death. The functions of specific cell death genes within cells that die are required for their demise, and a number of the key molecules that control this process have been identified and are conserved among animals (Horvitz, 1999; Vaux and Korsmeyer, 1999). Failure to kill appropriate cells can lead to severe developmental defects (Kuida et al., 1996, 1998; Hakem et al., 1998; Varfolomeev et al., 1998).

In the *Caenorhabditis elegans* hermaphrodite, 131 of the 1090 somatic cells generated during development are destined to die (Sulston and Horvitz, 1977; Sulston et al., 1983). The identities of the cells that undergo programmed cell death in *C. elegans* are essentially invariant from animal to animal. The majority of cell deaths occur early in development during embryonic stages coincident with a period of rapid cell division and the initiation of morphogenesis. In genetic screens for mutants abnormal in the pattern or appearance of cell corpses, *C. elegans* mutants variously defective in the specification, execution, engulfment, or degradation of programmed cell deaths have been identified. Analyses of these mutants have provided a framework for understanding how cells die by programmed cell death, not only in *C. elegans* but also in other animals (reviewed by Metzstein et al., 1998). The activities of the genes *ced-3* (*c*ell *d*eath abnormal), which encodes a caspase (Yuan et al., 1993; Xue et al., 1996), and *ced-4*, which encodes a protein similar to mammalian Apaf-1 (Yuan and Horvitz, 1992; Zou et al., 1997), are required for cells to undergo programmed cell death in *C. elegans* (Ellis and Horvitz, 1986). Cells destined to survive are protected by the activity of the gene *ced-9*, which encodes a member of the Bcl-2 family of proteins (Hengartner and Horvitz, 1994b); in dying cells, *ced-9* is negatively regulated by the gene *egl-1* (*egg-laying* defective), which encodes a BH3 (*B*cl-2 *h*omology 3) domain–containing protein (Conradt and Horvitz, 1998).

As a cell dies by programmed cell death, morphological changes in many aspects of its structure are visible (Kerr et al., 1972). These changes define the process of apoptosis. The plasma membrane blebs, and the cell becomes detached from its neighbors. The dying cell may fragment into smaller membrane-bound bodies. As observed using electron microscopy (EM), the cytoplasm condenses and becomes darkly staining (electron dense), while organelles remain grossly intact. The DNA becomes condensed at the nuclear periphery and is degraded by endonuclease activity; a cell corpse can be visualized using the TUNEL (*t*erminal transferase-mediated d*U*TP *n*ick *e*nd *l*abeling) technique, which labels DNA nicks in situ (Gavrieli et al., 1992). The cell corpse is eventually engulfed and degraded within the engulfing cell.

By most morphological criteria, cell corpses in *C. elegans* are similar to cells dying by apoptosis in mammals, although dying cells in *C. elegans* do not fragment into smaller bodies. As visualized by Nomarski microscopy, *C. elegans* cell corpses are highly refractile and easily distinguished from living cells (Sulston and Horvitz, 1977). These refractile corpses probably correspond to the electron-dense stage seen by EM (Robertson and Thomson, 1982). The DNA of *C. elegans* cell corpses is degraded (Sulston, 1976), and *C. elegans* cell corpses can be visualized using TUNEL (Wu et al., 2000).

Although genes important for controlling the killing step of programmed cell death have been well characterized, it is not clear how downstream events, such as the morphological changes that occur as cells die and the engulfment and degradation of cell corpses, are effected. Caspases are thought to be key mediators of these downstream events (e.g., Liu et al., 1997; Janicke et al., 1998), but while many in vitro targets of caspase proteolysis have been identified, the functions of these targets in cell death are in general unclear. DNA degradation may be triggered by the caspase-dependent cleavage of a protein that binds to and inhibits a cell death endonuclease (Enari et al., 1998; Sakahira et al., 1998). Several molecules implicated in the engulfment process have been characterized recently (Franc et al., 1996; Liu and Hengartner, 1998; Wu and Horvitz, 1998a, 1998b). Nonetheless, it is not known how the initiation of programmed cell death leads to the engulfment of a dying cell or whether the triggering of the engulfment process is a direct or indirect result of caspase activity.

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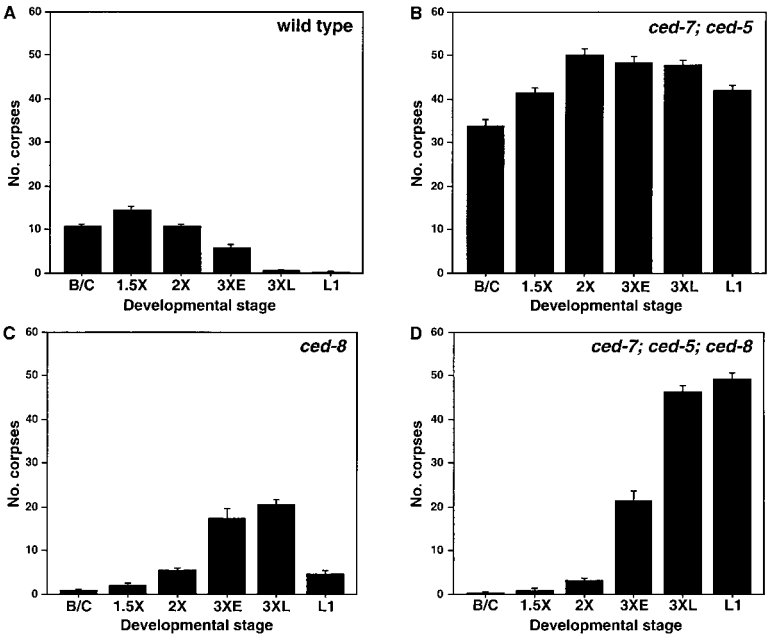

Figure 1. Delayed Appearance of Programmed Cell Deaths in *ced-8* Animals

(A) N2.
(B) *ced-7(n1892); ced-5(n1812)*.
(C) *ced-8(n1891)*.
(D) *ced-7(n1892); ced-5(n1812); ced-8(n1891)*.
The y axis represents the average number of corpses visible in the heads of embryos. Stages of embryos examined: bean and comma stage embryos (B/C); 1.5-fold and 2-fold embryos (1.5/2×); early 3-fold embryos, which are 3.5 times the length of the egg but do not yet have a well-developed pharynx (3×E); late 3-fold embryos, which have a well-developed pharynx and grinder (3×L); and early L1 larvae with four cells in the gonad (L1). Error bars indicate one standard error of the mean. At least 14 embryos of each stage were examined.

It is also unclear whether any downstream molecules in the cell death process are activated by caspase-independent mechanisms. The conservation among diverse organisms of both the morphology of dying cells and the molecules implicated in cell killing suggests that aspects of cell killing downstream of the key regulatory molecules are likely also to be conserved. For this reason, genetic analysis of programmed cell death in *C. elegans*, which has identified these regulatory molecules, should also permit the characterization of the events that occur downstream of cell death initiation and of how these downstream events contribute to the cell-killing process.

In this report, we demonstrate that the gene *ced-8* is involved in the execution step of programmed cell death in *C. elegans* and that the function of *ced-8* is important for the proper kinetics of programmed cell death during development. We cloned the *ced-8* gene and found that it is similar to XK, a putative membrane transport protein from humans (Ho et al., 1994). We suggest that *ced-8* functions in the downstream processes of cell killing.

Results

The Appearance of Cell Corpses Is Delayed in *ced-8* Embryos

During the approximately 14 hr of *C. elegans* embryogenesis, cell divisions and cell deaths together result in a total of 558 cells as the developing animal is transformed from a single cell into a vermiform larva capable of movement and feeding (Sulston et al., 1983). Most somatic cells destined to die in *C. elegans* do so soon after they are generated, and most cell corpses are visible during the developmental period between the bean and 2-fold stages when many cells are dividing and the embryo begins to elongate (Sulston and Horvitz, 1977; Sulston et al., 1983) (Figure 1A). As viewed by differential interference contrast (Nomarski) microscopy, a dying cell becomes highly refractile and distinct from its living neighbors. The refractile disk-like appearance of the dying cell disappears during the engulfment process, which occurs within an hour of the initiation of the cell's death. The total number of cell corpses visible at any time during development therefore represents the difference between the number of cells that have died up to that time and the number of cell corpses that have been engulfed and degraded.

ced-8 was identified in a screen for *C. elegans* mutants containing an increased number of cell corpses at the late embryonic and early larval stages (Ellis et al., 1991). Other mutants identified in this screen are defective in the engulfment of cell corpses by neighboring cells. In such engulfment-defective mutants, cell deaths occur at the appropriate time during early embryogenesis, but cell corpses persist in an unengulfed state (Hedgecock et al., 1983; Ellis et al., 1991) (Figure 1B). By contrast, we found that in *ced-8* mutants the majority of cell corpses did not appear until late in embryogenesis, and the greatest number of cell corpses was visible in late 3-fold embryos (Figure 1C; Table 1). Furthermore, nearly all of these cell corpses disappeared by the time of hatching, suggesting that they are engulfed rapidly, as usual. We observed a clear shift toward a later appearance of cell corpses than in the wild type for all *ced-8* alleles examined with the exception of *n3113*, which was identified as a suppressor of the lethality conferred by *ced-4(n2273) ced-9(n1653)* (Shaham and Horvitz, 1996a; E. K. Speliotes and H. R. H., unpublished data) and was nearly indistinguishable from the wild type in cell corpse assays (Table 1).

To confirm that the appearance of cell corpses is delayed in *ced-8* mutants, we examined the effect of a *ced-8* mutation on cell corpse number in a *ced-7; ced-5* background, in which most cell corpses remain unengulfed (Ellis et al., 1991). We found that most cell corpses in *ced-7; ced-5; ced-8* embryos were visible during the 3-fold stage of embryogenesis, just as in the *ced-8* single mutants (Figure 1D). We saw no more corpses in early

Table 1. *ced-8* Alleles

Genotype	Number of Corpses Early[a]	Number of Corpses Late[b]	Number of Extra Cells[c]	Range of Extra Cells[d]
Wild type	10.6 ± 0.6	2.3 ± 0.5	0.04 ± 0.03	0–1
ced-8(n1891)	0.9 ± 0.2	19.5 ± 1.1	0.7 ± 0.1	0–3
ced-8(n1999)	0.4 ± 0.1	12.0 ± 0.5	0.4 ± 0.09	0–2
ced-8(n2090)	1.8 ± 0.3	19.0 ± 1.2	0.8 ± 0.1	0–3
ced-8(n2093)	1.8 ± 0.3	19.5 ± 1.5	0.6 ± 0.1	0–3
ced-8(n3113)	9.7 ± 0.6	2.1 ± 0.4	0.1 ± 0.06	0–1
ced-8(n3115)	1.9 ± 0.2	16.7 ± 0.8	0.4 ± 0.1	0–2
ced-8(n3244)	0.7 ± 0.2	19.5 ± 1.1	0.7 ± 0.2	0–2
ced-8(n3245)	1.4 ± 0.2	17.1 ± 1.0	0.9 ± 0.2	0–4
ced-8(n3313)	0.9 ± 0.2	17.5 ± 0.7	0.8 ± 0.2	0–3
lon-2(e678) ced-8(n1891)/yDf2	ND	ND	0.8 ± 0.2	0–3
lon-2(e678)/yDf2	ND	ND	0.1 ± 0.07	0–1

[a] Average number of corpses in the head of bean and comma stage embryos ± SEM. ND, not determined.
[b] Average number of corpses in the head of 3-fold stage embryos ± SEM.
[c] Average number of extra cells in the anterior pharynx of L3 and L4 hermaphrodites ± SEM.
[d] Range in number of extra cells observed in the anterior pharynx. Number of animals examined: 50 for the wild type and 20–73 for other genotypes.

ced-7; ced-5; ced-8 embryos than in early *ced-8* embryos, consistent with the interpretation that cell corpses appear late, rather than persist, in animals mutant for *ced-8*. Furthermore, the total number of cell corpses that accumulated by the late 3-fold stage of embryogenesis in *ced-7; ced-5; ced-8* animals was similar to the maximum number observed in *ced-7; ced-5* animals, consistent with the idea that a delay rather than a reduction in cell killing is caused by mutations in *ced-8*.

ced-8 Mutations Weakly Diminish Cell Killing

Strong loss-of-function (lf) mutations in *ced-3* and *ced-4* result in the survival of nearly all cells that normally die in *C. elegans* (Ellis and Horvitz, 1986). Weak loss-of-function mutations in *ced-3* and *ced-4* result in the survival of a variable and apparently random subset of those cells that normally die (Hengartner and Horvitz, 1994a; Shaham et al., 1999; M. O. Hengartner and S. Shaham, personal communication) and in the delay of some cell deaths (M. O. Hengartner and H. R. H., unpublished data). Based on the number of corpses in embryos, *ced-8* mutations did not appear to cause many cells to survive ectopically. However, since weak mutations in *ced-3* and *ced-4* affect cell death kinetics and also weakly affect the cell-killing process, we assessed quantitatively whether *ced-8* mutations block cell death. Counts of extra cells in the anterior region of the pharynx, the nematode feeding organ, are a convenient measure of the degree to which cell death is blocked in a *ced* mutant (Hengartner et al., 1992). For instance, strong mutations in *ced-3* or *ced-4* result in approximately 13 extra cells in this region of the animal (Table 2). We found that very few, if any, extra cells were present in *ced-8* mutants as compared to strong *ced-3* and *ced-4* mutants (Tables 1 and 2).

One possible explanation for a weak *ced-8* cell survival phenotype was that the known *ced-8* mutations might cause only a partial loss of function of this gene. To test whether stronger *ced-8*(lf) mutations might cause a more pronounced cell survival phenotype, we scored for extra cells in the pharynges of *ced-8(n1891)/yDf2* animals (*yDf2* spans the *ced-8* locus; L. Miller and B. Meyer, personal communication). We found that there was no significant difference in the number of extra cells in *ced-8(n1891)/yDf2* animals as compared to the number in *ced-8(n1891)* homozygotes (Table 1). These results are consistent with *n1891*s being a strong loss-of-function or null allele of *ced-8*.

Although mutations in *ced-8* alone did not result in the survival of a significant number of cells that normally die, it was possible that *ced-8* might enhance the weak cell survival phenotype that results from partial loss of *ced-3* or *ced-4* function. We scored for the presence of extra cells in the anterior pharynx of animals mutant both for *ced-8* and for one of the weak *ced-3* mutations *n2427* or *n2438*. We found that a *ced-8* mutation and a weak *ced-3* mutation had a synergistic effect on the number of extra cells in the anterior pharynx (Table 2). We also examined animals mutant for both *ced-8* and *ced-4(n2273)*, a weak *ced-4* mutation that results in the survival of only a few cells that normally die. *ced-4(n2273); ced-8(n1891)* animals also contained more extra cells than did either of the single mutants (Tables 1 and 2). Thus, mutations in *ced-8* enhance the cell survival phenotype of all weak *ced-3* and *ced-4* mutants tested. Double mutants between *ced-8* and the strong alleles *ced-3(n1040)*, *ced-3(n717)*, or *ced-4(n1162)* had no more extra cells than did the *ced-3* or *ced-4* single mutant animals (Table 2).

These data are consistent with a modulatory function for *ced-8* in cell killing. *ced-8* appears to play a role in cell death that is distinct from those of *ced-3* and *ced-4*: *ced-8* is not absolutely required for cell killing, but it can enhance the probability of dying for a cell poised near the threshold of programmed cell death.

ced-8 Acts Downstream of or in Parallel to *ced-9*

The gene *ced-9* is required for cell survival during development and for fertility: *ced-9*(lf) mutations result in ectopic cell deaths leading to maternal-effect lethality and in zygotic sterility (Hengartner et al., 1992). Mutations in either *ced-3* or *ced-4* suppress the *ced-9*(lf) phenotype, indicating that the lethality results from ectopic programmed cell deaths and that *ced-9* normally functions to negatively regulate *ced-3* and *ced-4* activity. We

Table 2. *ced-8* Enhances Weak *ced-3* and *ced-4* Alleles

Genotype	Number of Extra Cells[a]	Range[b]	n[c]
ced-3(n2427)	1.0 ± 0.1	0–4	42
ced-8(n1891)	0.7 ± 0.1	0–3	73
ced-8(n1999)	0.4 ± 0.1	0–2	50
ced-3(n2427); ced-8(n1891)[d]	5.2 ± 0.3	3–10	30
ced-3(n2427); ced-8(n1999)	4.8 ± 0.3	2–8	29
ced-3(n2438)	2.0 ± 0.3	0–7	28
ced-3(n2438); ced-8(n1891)	6.5 ± 0.3	3–11	32
ced-3(n2438); ced-8(n1999)	5.0 ± 0.3	3–8	26
ced-3(n1040)	11.5 ± 0.3	8–14	33
ced-3(n1040); ced-8(n1891)	10.3 ± 0.5	6–16	23
ced-3(n1040); ced-8(n1999)	11.1 ± 0.4	8–15	24
ced-3(n717)	12.8 ± 0.4	8–18[e]	30
ced-3(n717); ced-8(n1891)[d]	12.3 ± 0.3	9–15	30
ced-4(n2273)	3.9 ± 0.3	3–6	10
ced-4(n2273); ced-8(n1891)	7.1 ± 0.4	5–9	10
ced-4(n2273); ced-8(n1999)	7.0 ± 0.4	5–8	10
ced-4(n1162)	13.1 ± 0.3	10–16	31
ced-4(n1162); ced-8(n1891)[d]	13.3 ± 0.4	9–16	28

[a] Average number of cells in the anterior pharynx of L3 and L4 hermaphrodites ± SEM.
[b] Range in number of extra cells observed in the anterior pharynx.
[c] n, number of animals examined.
[d] These strains contained the mutation *lon-2(e678)*, which does not affect cell survival.
[e] Although 16 cells die in the anterior region of the pharynx, 18 extra cells were observed in one animal of this genotype. These cells likely originated in the posterior bulb of the pharynx, since the pharynx is separated from other cells by a basement membrane. Extra cells are often slightly misplaced in strong *ced* mutants.

sought to determine whether *ced-8* mutations similarly could suppress the *ced-9*(lf) phenotype. We found that the *ced-8(n1891)* and *ced-8(n1999)* mutations partially suppressed the loss of fertility and developmental defects of *ced-9(n1950 n2161)* mutants. For example, *ced-9(n1950 n2161) unc-69(e587); ced-8* homozygous mothers laid more eggs than did *ced-9(n1950 n2161) unc-69(e587)* mothers, and *ced-9(n1950 n2161) unc-69(e587); ced-8* embryos developed to later embryonic stages than did *ced-9(n1950 n2161) unc-69(e587)* embryos (data not shown). However, although *ced-9(n1950 n2161) unc-69(e587); ced-8* animals occasionally survived until hatching, none progressed past the L1 larval stage, so mutations in *ced-8* improved the viability of *ced-9*(lf) animals but did not completely suppress their defects. To test whether *ced-8* acts genetically upstream of *ced-9*, we determined whether *ced-9* function is required for the enhanced cell survival phenotype of a *ced-3*(weak); *ced-8* double mutant. We found that more extra cells were present in *ced-9(n2812); ced-3(n2427); ced-8(n1891)* mutant animals than were present in *ced-9(n2812); ced-3(n2427)* animals ($p < 0.0001$, paired t test) (Table 3). *ced-9(n2812)* is likely to be null for *ced-9* function (Hengartner and Horvitz, 1994a). Thus, mutations in *ced-8* probably can enhance cell survival even in the absence of *ced-9* activity, indicating that *ced-8* acts downstream of or in parallel to *ced-9*.

DNA Degradation Is Somewhat Delayed in *ced-8* Mutants

How might we distinguish whether *ced-8* mutants initiate programmed cell death late or execute the process of programmed cell death slowly? If programmed cell deaths were initiated late, then the appearance of all markers of cell death should be delayed, but once killing is triggered, the morphological changes that result in a refractile corpse would presumably occur with normal kinetics. However, if programmed cell deaths were initiated at the normal time in *ced-8* mutants, then some cell death markers could appear at the appropriate time even though the progression of the cell to refractility is slowed.

Therefore, we sought additional biochemical markers of cell corpses besides the refractility visible by Nomarski microscopy. Degradation of the DNA of dying cells is a hallmark of apoptosis and can be visualized in situ using TUNEL (Gavrieli et al., 1992; Wu et al., 2000). In wild-type *C. elegans* embryos, a small fraction of dying cells are TUNEL reactive at any one time; by contrast, embryos mutant for the gene *nuc-1*, which is important for DNA degradation (Sulston, 1976; Hevelone and Hartman, 1988), contain many condensed TUNEL-reactive nuclei that appear when cells die and persist (Figures 2A and 2B; Wu et al., 2000). Thus, DNA degradation is apparently very rapid during programmed cell death in

Table 3. *ced-8* Functions Downstream of or in Parallel to *ced-9*

Genotype	Number of Extra Cells[a]	n[b]
ced-9(n2812); ced-3(n2427)	7.1 ± 0.4	18
ced-9(n2812); ced-3(n2427); unc-10(e102) ced-8(n1891)	10.0 ± 0.3	21

[a] Average number of extra cells in the anterior pharynx in L3 and L4 hermaphrodites ± SEM.
[b] n, number of animals examined.

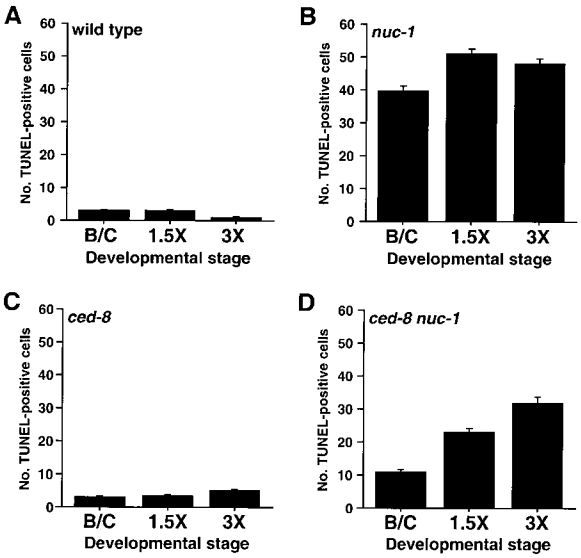

Figure 2. Delayed Onset of DNA Degradation in *ced-8* Embryos

The genotypes indicated are for the *ced-8* and *nuc-1* loci. All four strains also contained the mutation *egl-15(484)*, which does not affect TUNEL staining (data not shown), as indicated: (A) *egl-15(n484)*; (B) *nuc-1(e1392) egl-15(n484)*; (C) *ced-8(n1891) egl-15(n484)*; (D) *ced-8(n1891) nuc-1(e1392) egl-15(n484)*. The y axis represents the number of TUNEL-positive cells present in embryos. Stages examined: bean and comma stage embryos (B/C), 1.5-fold embryos (1.5×), and 3-fold embryos including both early and late stages (3×). Error bars indicate one standard error of the mean. At least 15 embryos of each stage were examined.

wild-type *C. elegans*, and DNA degradation is slowed in *nuc-1* mutants relative to in the wild type. Furthermore, engulfment per se is not necessary for the generation of TUNEL-reactive DNA in cell corpses (Wu et al., 2000), suggesting that TUNEL provides a cell-intrinsic marker for cell deaths. We therefore examined whether mutations in *ced-8* affect the kinetics of DNA degradation as visualized by TUNEL.

In *ced-8* embryos, as in wild-type embryos, few TUNEL-reactive corpses were present at any given developmental stage (Figures 2A and 2C). However, in a *ced-8 nuc-1* double mutant strain, many TUNEL-reactive corpses were present late in embryogenesis (Figure 2D), suggesting that the low number of TUNEL-reactive corpses in the *ced-8* single mutant was not caused by a block in DNA degradation but rather was caused by rapid progression through the DNA degradation process (Figure 2D).

To compare the time course of the appearance of TUNEL-reactive corpses with the time course of the appearance of refractile cell corpses, we examined TUNEL-stained *ced-8 nuc-1* double mutant animals at various developmental time points during embryogenesis and compared the number of TUNEL-positive cells to the number of refractile corpses visible in a *ced-7; ced-5; ced-8* mutant strain at the same developmental stage. The *nuc-1* DNA degradation-defective and the *ced-7; ced-5* engulfment-defective backgrounds allow a similar number of accumulated cell corpses to be visualized at each embryonic stage (Figure 2B versus Figure 1B), suggesting that these two methods of visualizing corpses have similar sensitivities. We found that the greatest number of TUNEL-reactive corpses was present in late-stage *ced-8 nuc-1* embryos. However, in early embryos, there were many more corpses in *ced-8 nuc-1* embryos as visualized by TUNEL staining than there were refractile corpses in *ced-7; ced-5; ced-8* mutant embryos as visualized by Nomarski optics (Figure 2D versus Figure 1D). That so many TUNEL-positive cells were present at this early stage suggests that although morphological changes causing refractility are delayed in most cell corpses until the 3-fold stage of embryogenesis, DNA degradation is less delayed. This result is consistent with the hypothesis that cell death is triggered at the appropriate developmental time in *ced-8* mutants but proceeds to the refractile corpse stage more slowly than in the wild type and that the biochemical and cytological changes of dying cells that occur nearly synchronously in the wild type are uncoupled from one another in *ced-8* mutants.

Cloning of *ced-8*

ced-8 previously had been mapped to LG*X* to the right of *unc-10*, near *dpy-6* (Ellis et al., 1991). We more precisely mapped *ced-8* to the left of *dpy-6*, which lies to the left of the polymorphism *nP81* (Kornfeld et al., 1995). We injected cosmids from the region to the left of *nP81* (Figure 3A) into *ced-8(n1999)* hermaphrodites and found that arrays containing either of the cosmids F54F4 or F37F1 restored cell corpses to early embryos. We localized the region required for rescue to a 4.2 kilobase (kb) fragment from the region of overlap between F54F4 and F37F1 (Figure 3B). We determined the sequence of this DNA fragment and found that it contains a single gene predicted by the program GENEFINDER (*C. elegans* Genome Sequencing Consortium, 1998).

We identified a cDNA (Figure 3C; see Experimental Procedures) that rescued the Ced-8 phenotype when expressed in *ced-8(n1891)* embryos under the control of *C. elegans* heat-shock promoters (data not shown). This transcript presumably corresponds to *ced-8*. To determine whether we had identified the 5′ end of this rescuing transcript, we analyzed 5′ RACE products from *C. elegans* embryonic RNA and performed Northern analysis. Putative full-length *ced-8* transcripts identified by RACE were 1576 bases in length and had 41 additional nucleotides of contiguous genomic sequence as compared to the longest cDNAs isolated from phage libraries. A Northern blot identified a single band migrating at 1.4–1.6 kb, the approximate size of the putative full-length transcript, in RNA from embryos but not from mixed stage animals (data not shown). Since the *ced-8* transcript has two in-frame stop codons 5′ to the putative transcriptional start, it is likely that we have identified the complete *ced-8* coding sequence.

To confirm that we had identified the *ced-8* gene, we determined the sequences of the coding region from *ced-8* mutants and identified mutations for all nine alleles (Figure 3C). The nature of the lesions in the *ced-8* alleles are consistent with their causing loss of *ced-8* function. Eight of the *ced-8* alleles cause an identical phenotype that is consistent with their being strong or complete loss of function. The *n3113* mutation, which eliminates the splice acceptor for intron 3 and is predicted to result in an in-frame insertion of 16 amino

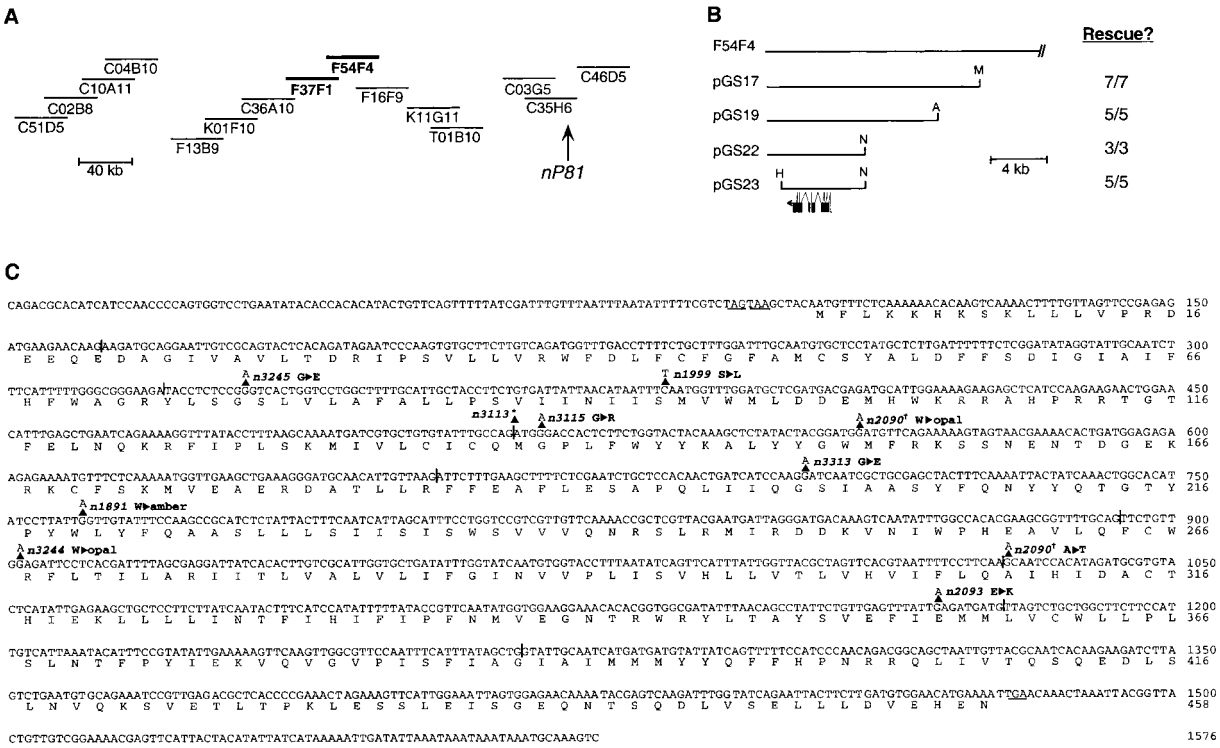

Figure 3. *ced-8* Cloning and cDNA Sequence

(A) Cosmids assayed for transformation rescue. Rescuing cosmids are in bold.

(B) Subclones assayed for transformation rescue; the fraction of assayed transgenic lines showing rescue of the Ced-8 phenotype is indicated on the right. A, AgeI; H, HpaI; M, MluI; N, NheI.

(C) A composite of the *C. elegans ced-8* sequence derived from cDNA and 5′ RACE analysis. Intron positions are indicated by vertical bars. The predicted CED-8 protein sequence is shown beneath. Arrowheads designate base changes in the indicated *ced-8* alleles. The *ced-8(n3113)* allele (*) is predicted to eliminate the splice acceptor for intron 3, resulting in an in-frame insertion of the sequence VTHFLGSVRKTKSDLQ into the CED-8 protein between amino acids 137 and 138. The *ced-8(n2090)* allele (†) contains two nucleotide changes relative to the wild-type sequence. Two in-frame stop codons upstream of the predicted initiation codon are underlined; the termination codon is also underlined.

acids, results in a weaker phenotype: very few cell deaths are delayed until late stages of embryogenesis in *n3113* embryos (Table 1).

CED-8 Is Similar to XK

The *ced-8* cDNA encodes a 458 amino acid protein that is weakly similar to the product of the human *XK* gene (Figure 4A) (Ho et al., 1994). CED-8 and XK share 19% identity and 37% similarity overall and have similar hydropathy plots (Figure 4B). Both proteins contain 10 hydrophobic predicted transmembrane-spanning segments, and most sequence similarity between the two proteins lies within these regions. Consistent with this structural prediction for CED-8, a *ced-8:gfp* (*g*reen *f*luorescent *p*rotein) translational fusion, which includes most of the *ced-8* coding region and rescued the delayed death phenotype of *ced-8(n1999)* embryos (data not shown), was localized to the plasma membrane (Figure 5). Two regions of similarity between CED-8 and XK are shared with a predicted protein from the ascidian *Ciona intestinalis*, and one of these regions is also shared with a *Drosophila* EST (Figure 4C).

Discussion

ced-8 was discovered in a screen for animals containing cell corpses in L1 larvae, a stage at which few programmed cell deaths occur and embryonic cell corpses have been cleared from the animal by engulfment (Ellis et al., 1991). That screen primarily identified mutants with defects in cell corpse engulfment. *ced-8* mutants, like the engulfment-defective mutants identified in that screen, contain visible cell corpses at these late stages, and *ced-8* was assumed initially to be defective in the engulfment step of programmed cell death. However, by examining earlier stages of development, we discovered that in *ced-8* embryos corpses were not visible at the stages at which they normally appear both in the wild type and in engulfment mutants; rather, corpses were present at later stages than in the wild type. This observation revealed that the presence of cell corpses in *ced-8* L1 larvae reflects a delay in cell killing rather than a defect in engulfment.

Our previous ultrastructural studies indicated that cell corpses generated postembryonically in the ventral cord of *ced-8* mutants are engulfed (Ellis et al., 1991), consistent with our current conclusion that the defect of *ced-8* mutants is in cell killing rather than cell corpse engulfment. However, we also reported previously that cell corpses that are normally generated during early to mid embryogenesis are not engulfed in recently hatched *ced-8* L1 larvae. We now understand that these corpses are present at this stage simply because they have just been generated. Specifically, in *ced-8* mutants, embryonic deaths are delayed until very late embryogenesis

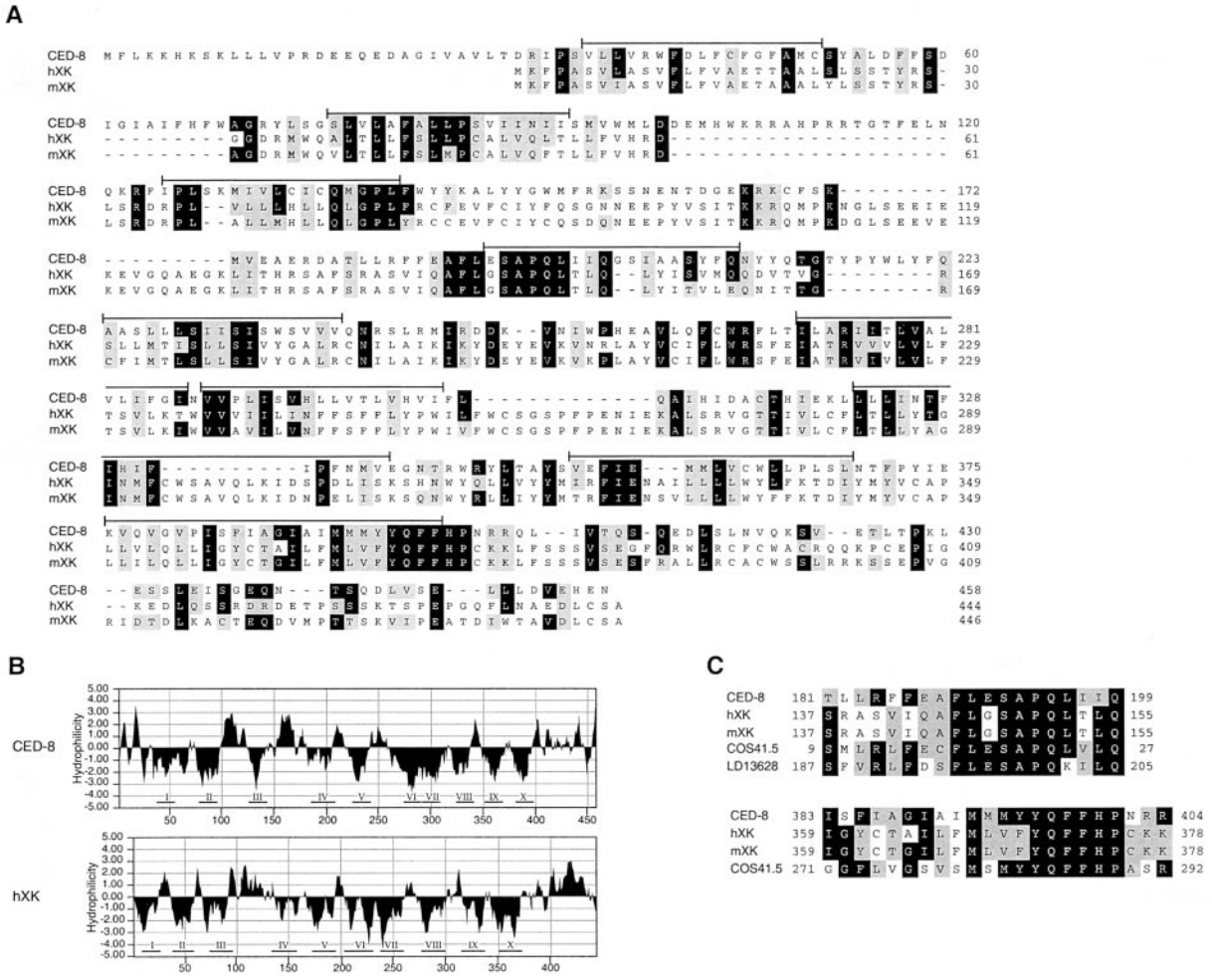

Figure 4. CED-8 Protein Sequence Comparisons

(A) Alignment of the full-length proteins *C. elegans* CED-8, human XK (hXK) and mouse XK (mXK; Collec et al., 1999). Identities are shaded black; similarities are shaded grey. Predicted transmembrane-spanning domains in CED-8 are indicated with brackets.

(B) Kyte-Doolittle hydropathy plots (Kyte and Doolittle, 1982) (scanning window, seven amino acids) of CED-8 and human XK. Bars indicate predicted transmembrane-spanning domains.

(C) Alignment of similar sequences of CED-8, human XK, mouse XK, *Ciona intestinalis* COS41.5, and *Drosophila* EST LD13628.

or even the early L1 larvae stage (Figures 1A and 1C); furthermore, unlike in engulfment *ced* mutants, in *ced-8* mutants cell corpses do not accumulate (Figures 1B and 1C), indicating that in *ced-8* mutants engulfment is not defective and probably proceeds at a normal rate once corpses have been generated.

We have shown that *ced-8* is important to ensure that cell corpses appear on time and that in sensitized genetic backgrounds (with weak *ced-3* and *ced-4* mutants) *ced-8* is important for the cell-killing process. While expression of *ced-8* under the control of heat-shock promoters was able to rescue the mutant phenotype of *ced-8(n1891)* animals, no additional abnormalities were observed: e.g., there was no increase in the number of cell deaths or any loss of viability associated with overexpression of *ced-8* (data not shown). By contrast, overexpression of *ced-3* or *ced-4* either under the control of heat-shock promoters or under cell type–specific promoters is able to induce programmed cell death (Shaham and Horvitz, 1996b; S. Shaham and H. R. H., unpublished data). Thus, while *ced-8* is neither required for programmed cell death nor sufficient to induce cell killing, *ced-8* enhances the efficiency of the cell death program during development.

Function of *ced-8* in Programmed Cell Death

Programmed cell death in *C. elegans* is believed to be initiated molecularly by the activation of a core cell death pathway, consisting of *egl-1*, *ced-9*, *ced-4*, and *ced-3* (reviewed by Metzstein et al., 1998). Morphological changes and other downstream events are thought to be dependent on cleavage by CED-3 of cell death substrates, although it is not known precisely how the cell-killing process results in the refractility or other morphological characteristics of a cell corpse. We have found that *ced-8*, like *ced-3* and *ced-4*, functions downstream of or in parallel to *ced-9*. Thus, *ced-8* could function either to activate *ced-3* and/or *ced-4*, that is, in the initiation of cell killing, or to effect cell killing after cell death has been initiated by *ced-3* and *ced-4*.

In the former case, *ced-8* might act directly on either *ced-3* or *ced-4* to potentiate their activities and thereby

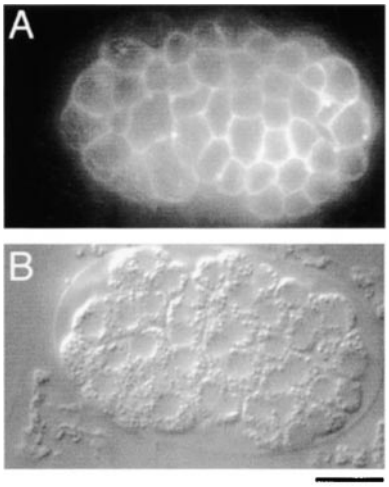

Figure 5. *ced-8:gfp* Is Expressed in the Plasma Membrane

(A) Embryo in which a *ced-8:gfp* fusion was expressed in most, if not all, cells of early embryos and was localized to the plasma membrane.

(B) Nomarski image of the same embryo.

Scale bar, 10 μm.

initiate the cell death process. For instance, CED-8 might function as an adaptor protein between CED-3 and a protein required for the activation of CED-3 enzymatic activity. *ced-4* appears to function genetically upstream of *ced-3* (Shaham and Horvitz, 1996b), and biochemical studies suggest that CED-4 helps induce the conversion of pro-CED-3 to active CED-3 enzyme (Chinnaiyan et al., 1997; Seshagiri and Miller, 1997; Wu et al., 1997a). When coexpressed with CED-9 in mammalian cells, CED-4 is localized to intracellular membranes and the perinuclear region (Wu et al., 1997b). However, we observed that a *ced-8:gfp* fusion is localized to the plasma membrane, suggesting that CED-8 does not function in the CED-4-dependent activation of CED-3. Alternatively, CED-8 might function to localize and thereby activate other proteins that promote cell killing or to sequester and thereby inactivate proteins that inhibit cell killing. A localization role for CED-8 is plausible given the Ced-8 phenotype of delayed death. If *ced-8* functions in a rate-enhancing role in the formation of a cell-killing complex, it is reasonable that cell death occurs eventually, albeit more slowly, in its absence.

Alternatively, if *ced-8* functions in some aspect of cell death execution downstream of *ced-3* and *ced-4*, CED-8 might function as a substrate of CED-3 or by interacting with such a substrate(s). We have found that the timing of certain downstream cell death events, the onset of refractility and DNA degradation, are uncoupled in *ced-8* cell corpses. Defects in the timing and/or morphology of cell killing may be a common result of compromising events downstream of the central cell-killing pathway, since the Ced-8 phenotype is reminiscent of the effect of blocking the cleavage of lamins during apoptosis in mammalian cells (Rao et al., 1996). When lamins with a mutated caspase cleavage site were introduced into mammalian cells in culture, a delay in death associated with changes in the morphology of dying cells was observed.

How does mutation of *ced-8* in a sensitized *ced-3* or *ced-4* genetic background result in the survival of cells that normally die? It is possible that if a cell death is delayed sufficiently then that cell can lose its competence to die, either because cell-killing protein(s) are no longer present or because cell-protective function(s) are activated. While such weak defects in cell death have a very minor effect on *C. elegans*, which is viable even if cell death is essentially completely blocked, it is possible that inefficient cell killing would cause more serious defects in other organisms.

Evolutionary Conservation of CED-8

CED-8 shares similarity with human XK, the loss of which is reported to cause McLeod Syndrome, a hereditary disease defined by abnormalities in erythrocyte Kell antigens (Ho et al., 1994). McLeod erythrocytes also have an acanthocytic (spiculated) appearance, and the McLeod phenotype comprises progressive myopathy and neurodegeneration. The cellular basis of these defects is not understood.

The XK protein is associated with the Kell protein in the erythrocyte plasma membrane, and loss of XK leads to the absence of Kell antigens at the cell surface (Khamlichi et al., 1995; Daniels et al., 1996; Russo et al., 1998). The Kell protein is similar to neprilysin-like membrane metallopeptidases, although its substrate is not known (Turner and Tanzawa, 1997). XK has been postulated to function as a transporter (Ho et al., 1994), so one possibility is that XK transports either a substrate or a cleavage product of Kell out of or into the cell. By analogy, it is possible that CED-8 functions in transporting some substrate important for effecting the downstream events of programmed cell death. However, although the two proteins share some sequence similarity and have similar hydropathy profiles, and both are apparently localized to the plasma membrane, the similarity between CED-8 and XK is not extensive. Functional analysis of CED-8, XK, and the other related proteins is needed to determine the extent of their functional similarity.

It is possible that both CED-8 and XK act as transporters that act to define the lipid content of the plasma membrane. One proposed basis for acanthocytic morphology, such as seen in McLeod erythrocytes, is asymmetry between the inner and outer leaflets of the plasma membrane (Zwaal et al., 1993; Handin et al., 1995); such asymmetry could be a consequence of decreased phospholipid transport by XK. CED-8 might similarly function to modulate the distribution of lipids in the plasma membrane; unlike living cells, dying cells display phosphatidylserine on the outer surface of their plasma membranes (Fadok et al., 1992; Martin et al., 1995; Vermes et al., 1995). Perhaps an abnormality in plasma membrane lipid distribution leads to the slow progression of membrane changes in dying cells, altering the kinetics of programmed cell death in *ced-8* mutants. We propose, by analogy with the role of *ced-8* in *C. elegans*, that XK may function in apoptosis in humans and that the late onset neurodegeneration associated with McLeod Syndrome is a consequence of a disruption in apoptosis. Such an effect could be direct, that is, degenerating cells may represent late occurring programmed cell

deaths, or indirect, that is, degenerating cells may be a subsequent consequence of earlier defects in programmed cell death.

Experimental Procedures

Nematodes

C. elegans was cultured at 20°C on NGM agar seeded with *E. coli* strain OP50 as described by Brenner (1974). Worms used for RNA preparations were grown in liquid culture essentially as described in Wood et al. (1988). All mutant strains were derived from the wild-type Bristol strain N2. Mutations are described by Riddle et al. (1997) unless otherwise noted. A list of the mutations used follows. LG III: *ced-7(n1892); ced-9(n2812)* (Hengartner and Horvitz, 1994a); *qC1*[*dpy-19(e1259*ts*) glp-1(q339)*] (Epstein and Shakes, 1995); *ced-4(n1162, n2273)*. LG IV: *ced-3(n717)*; *ced-3(n2427, n2438)* (Hengartner and Horvitz, 1994a); *ced-3(n2452)* (Shaham et al., 1999); *ced-5(n1812)*. LGX: *lon-2(e678)*; *unc-10(e102)*; *xol-1(y9)*; *dpy-6(e14)*; *nuc-1(e1392)*; *egl-15(n484)*; *yDf2* (L. Miller and B. Meyer, personal communication); *szT1*[*lon-2(e678)*]. The *ced-8* alleles *n1891*, *n1999*, *n2090*, and *n2093* are described by Ellis et al. (1991). *ced-8(n3113)* and *ced-8(n3115)* were isolated as suppressors of the lethality and sterility caused by *ced-4(n2273) ced-9(n1653)* (Shaham and Horvitz, 1996a; E. K. Spelliotes and H. R. H., unpublished data), *ced-8(n3244)* and *ced-8(n3245)* were isolated as containing late cell corpses (Z. Zhou and H. R. H., unpublished results), and *ced-8(n3313)* was isolated as an enhancer of the extra cell phenotype of *ced-3(n2427)* animals (P. W. Reddien and H. R. H., unpublished data).

Genetic Mapping

From the progeny of *unc-10(e102) xol-1(y9) dpy-6(e14)/ced-8(n1891)* heterozygotes, we selected 56 Unc non-Dpy recombinants and found that 53 segregated Ced-8 progeny, indicating that *ced-8* lies between *unc-10* and *dpy-6*, very close to *dpy-6* and therefore left of the physical marker *nP81* (Kornfeld et al., 1995).

Cell Death Assays

The number of extra cells in the anterior pharynx of L4 animals was scored using Nomarski microscopy as described by Hengartner et al. (1992). TUNEL experiments were performed as described by Wu et al. (2000). DAPI (4′-6-diamidino-2-phenylindole dihydrochloride) was included at 1 μg/ml in the final wash, and animals were staged using the pattern of DAPI-stained nuclei to identify developmental landmarks.

ced-8 Dosage Experiments

We crossed *lon-2* or *lon-2 ced-8* males with *szT1/+; unc-32(e189); yDf2/szT1*[*lon-2(e678)*] hermaphrodites. Non-Lon cross progeny were scored for extra cells in the pharynx. To assay viability, several of these non-Lon hermaphrodites were picked to a plate and allowed to lay eggs for 2 to 3 hr. The number of eggs was counted immediately after removal of the adult worms and then counted again after at least 16 hr, and the number of worms reaching at least the L3 stage was counted 2 days later. There was no decrease in viability of embryos from *lon-2(e678) ced-8(n1891)/yDf2* mothers as compared to embryos from *lon-2(e678)/yDf2* mothers; in both cases, approximately a quarter of embryos failed to hatch (data not shown), as expected if only the *yDf2* homozygous class of progeny was inviable.

Rescue Experiments

We performed injections as described by Mello et al. (1991) using the coinjection markers pRF4 at 50–75 ng/μl and *egl-5:gfp* (A. Chisholm and H. R. H., unpublished data) at 50–75 ng/μl, and we selected transgenic lines using the Roller phenotype.

We injected cosmids at 20 ng/μl each into *ced-8(n1999)* hermaphrodites. We examined 3-fold embryos that expressed the *egl-5:gfp* marker for the presence of cell corpses in the head region; embryos with fewer than three corpses in the head were scored as rescued. We confirmed that the absence of cell corpses was a consequence of rescue of the Ced-8 phenotype rather than a block in cell death by examining bean and comma stage embryos from candidate lines for cell corpses; embryos with more than six corpses in the head at this stage were scored as rescued.

We injected heat-shock constructs at 50 ng/μl each into *ced-8(n1891)* hermaphrodites. For heat-shock experiments, gravid Rol hermaphrodites were allowed to lay eggs for 1.5–2 hr, the adults were removed from the plates, and the plates were incubated at 33°C for 1 hr. The number of corpses was counted when embryos reached either the bean and comma stages or the 3-fold stage of development. Non-heat-shocked control animals were studied in parallel and treated identically, except that the plates were placed at 20°C rather than 33°C for 1 hr.

Molecular Biology

Standard molecular biological procedures were followed (Sambrook et al., 1989). The sequences of primers used for determining DNA sequences, for PCR amplification from *ced-8* mutants, and for 5′-RACE are available upon request. We determined sequence from the minimal rescuing fragment pGS23 by a shotgun protocol using an ABI 373A sequencer. CED-8 corresponds to the predicted gene F08F1.5 (*C. elegans* Genome Sequencing Consortium, 1998), except that the splice donor predicted by GENEFINDER for exon five of F08F1.5 is incorrect. We isolated RNA from nematodes by standard methods and performed poly(A) selection using Fast Track (Invitrogen). 5′-RACE reactions were performed on embryonic RNA using the 5′ RACE system (GIBCO).

cDNA Analysis

To identify *ced-8* cDNAs, a 3.5 kb HpaI-SphI fragment of pGS23 was used to probe an embryonic stage cDNA library in λgt11 (Okkema and Fire, 1994) and a mixed stage cDNA library in λZAP (Barstead and Waterston, 1989). We found two classes of alternatively spliced cDNA clones in addition to a clone containing introns 1 and 3, which were spliced out of all other isolated cDNAs. We refer to these two classes of alternatively spliced cDNA clones as type I and type II, and they differed in two ways: the splice donor for the second intron differed between the two clones, and the type II cDNA was shorter at the 5′ end. The type I splicing pattern was present in five independent clones, while the type II splicing pattern was present in only a single independent clone. The type I cDNA is predicted to encode a 458 amino acid protein. The longest ORF from the type II cDNA can encode a protein corresponding to the 363 C-terminal amino acids of the type I ORF. Plasmids pGS35 (type I) and pGS33 (type II) were constructed by digesting phage DNA with BsiWI and ligating the insert fragment into pSL1190 (Brosius, 1989) digested with BsiWI. To determine whether either or both of the two alternatively spliced transcripts encode(s) functional CED-8 protein, we placed the longest ORF from each of the two transcripts under the control of *C. elegans* heat-shock promoters (Fire et al., 1990) and assayed the ability of these heat shock–cDNA constructs to rescue the Ced-8 phenotype of *ced-8(n1891)* embryos. Only the type I ORF rescued, suggesting that the type II transcript does not encode a functional *ced-8* gene.

To assess further the possibility that the type II transcript might be relevant for either the function or regulation of *ced-8*, we cloned and determined the sequence of the *ced-8* region from the closely related nematode *C. briggsae*. We obtained clones containing genomic *C. briggsae ced-8* by probing a λCharon4 library (T. Snutch and D. Baillie, personal communication) and a fosmid library (Genome Systems; fosmid clones were provided by the *C. elegans* Genome Sequencing Consortium). We found that the type I splice donor sequence is conserved from *C. elegans* to *C. briggsae*, but the type II splice donor site is absent in *C. briggsae*. We also found that the majority of the predicted amino acid sequence of *C. briggsae* CED-8 is well conserved as compared to that of *C. elegans* type I, and in particular, the coding potential in the region spliced out of the *ced-8* message in the event of a type II splice donor choice is identical between *C. elegans* and *C. briggsae*. Our findings that the type II long ORF does not appear to encode a functional protein and that the splice site necessary to generate a type II transcript are not conserved between *C. elegans* and *C. briggsae* are consistent with the idea that the type II cDNA identified in the library is not functional. This cDNA may represent an aberrant transcript that is not normally

produced. We consider the type I cDNA, referred to in the text as the *ced-8* cDNA, to be the functional *ced-8* product.

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