Mutational Analysis of the *Caenorhabditis elegans* Cell-Death Gene *ced-3*

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Manuscript received June 16, 1999
Accepted for publication August 27, 1999

ABSTRACT

Mutations in the gene *ced-3*, which encodes a protease similar to interleukin-1β converting enzyme and related proteins termed caspases, prevent programmed cell death in the nematode *Caenorhabditis elegans*. We used site-directed mutagenesis to demonstrate that both the presumptive active-site cysteine of the CED-3 protease and the aspartate residues at sites of processing of the CED-3 proprotein are required for programmed cell death *in vivo*. We characterized the phenotypes caused by and the molecular lesions of 52 *ced-3* alleles. These alleles can be ordered in a graded phenotypic series. Of the 30 amino acid sites altered by *ced-3* missense mutations, 29 are conserved with at least one other caspase, suggesting that these residues define sites important for the functions of all caspases. Animals homozygous for the *ced-3*(*n2452*) allele, which is deleted for the region of the *ced-3* gene that encodes the protease domain, seemed to be incompletely blocked in programmed cell death, suggesting that some programmed cell death can occur independently of CED-3 protease activity.

THE gene *ced-3* functions cell-autonomously to promote programmed cell death in the nematode *Caenorhabditis elegans* (Ellis and Horvitz 1986; Yuan and Horvitz 1990; Shaham and Horvitz 1996a). *ced-3* appears to act downstream of both *ced-4*S, a positive regulator of cell death (Ellis and Horvitz 1986; Shaham and Horvitz 1996a,b) that encodes a protein similar to the human cell-death protein Apaf-1 (Zou *et al.* 1997), and *ced-9*, a negative regulator of cell death and a member of the *bcl-2* gene family (Hengartner *et al.* 1992; Shaham and Horvitz 1996a).

ced-3 encodes a member of the CED-3/ICE (interleukin-1β converting enzyme) family of cysteine proteases (Yuan *et al.* 1993; Xue *et al.* 1996). These proteases have been named caspases, for cysteine aspases, because they have active-site cysteines and cleave after aspartate residues (Alnemri *et al.* 1996). Caspase genes encode precursor proteins that are activated by cleavage at specific aspartate residues. Such cleavage generates C-terminal and central polypeptides that associate to form a heterodimeric protease and an N-terminal polypeptide not present in the active protease and called the prodomain (Thornberry *et al.* 1992; Faucheu *et al.* 1995; Xue *et al.* 1996). X-ray crystallographic studies of caspase-1 (ICE) suggest that the active protease consists of two interacting heterodimers (Walker *et al.* 1994; Wilson *et al.* 1994). A similar X-ray structure has been determined for caspase-3 (CPP32; Rotonda *et al.* 1996). All caspases examined cleave after particular aspartate residues (Thornberry *et al.* 1992; Xue *et al.* 1996), a substrate specificity shared with only one other known eukaryotic protease, granzyme B/fragmentin 2, which is thought to function in cell death mediated by cytotoxic T cells (Shi *et al.* 1992a,b; Heusel *et al.* 1994).

Several observations indicate roles for mammalian and fly caspases in apoptosis. First, some caspases are activated during apoptosis. For example, caspase-8 (MACH/FLICE) binds FADD, a Fas-associated protein, and is activated in cells undergoing apoptosis following Fas stimulation (Boldin *et al.* 1996; Muzio *et al.* 1996). Second, overexpression of mammalian caspases can result in cell death in culture (Miura *et al.* 1993; Fernandes-Alnemri *et al.* 1994, 1995a,b; Kumar *et al.* 1994; Wang *et al.* 1994; Faucheu *et al.* 1995; Munday *et al.* 1995; Tewari *et al.* 1995), and overexpression of two *Drosophila melanogaster* caspases, DCP-1 and drICE, can cause cell death both in flies and in cultured cells (Fraser and Evan 1997; Song *et al.* 1997). Third, inhibition of caspase activity can prevent apoptosis. The caspase inhibitor p35 can prevent programmed cell death in *C. elegans* (Xue and Horvitz 1995) and in *D. melanogaster* (Hay *et al.* 1994). Similar effects can be seen in mammalian cells. For example, expression in neurons of the viral caspase-1 inhibitor protein crmA (Ray *et al.* 1992) prevents the deaths of these neurons following trophic factor deprivation (Gagliardini *et al.* 1994). Caspase-1 inhibitors also prevent the *in vivo* deaths of chick embryo neurons that die when unable to innervate their target muscles (Milligan *et al.* 1995). In addition, disruption of the caspase-1 gene in mice results in a defect in Fas-mediated cell death (Kuida *et al.* 1995), suggesting that caspase-1 might normally have a role in

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programmed cell death. As another example, caspase-3 is likely to be a key component of cytoplasmic extracts that trigger morphological changes similar to those observed during physiological cell death in isolated nuclei (Nicholson *et al.* 1995), and inhibitors of caspase-3 prevent Fas-induced cell death (Enari *et al.* 1996). Disruption of the caspase-3 gene as well as disruption of the caspase-3 activator caspase-9 in mice results in a significant reduction in cell death in the brain, indicating that both genes are normally important for mediating cell death (Kuida *et al.* 1996, 1998; Hakem *et al.* 1998). These results suggest that caspases are involved in cell death not only in *C. elegans* but in vertebrates and insects as well.

To define those regions of caspase proproteins required for protease activation and/or enzymatic activity and to understand better the function of caspases in programmed cell death, we have analyzed the effects of mutations in *ced-3* on programmed cell death *in vivo.*

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°. 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 were described previously by Brenner (1974), Trent *et al.* (1983), Hedgecock *et al.* (1983), Ellis and Horvitz (1986), Clark *et al.* (1988), Ellis *et al.* (1991), Hengartner *et al.* (1992), and Yuan *et al.* (1993), or were isolated by us and members of our laboratory. These mutations are listed below:

Linkage Group (LG) I: *sem-4*(*n1378*), *ced-1*(*e1735*)
LGIII: *ced-9*(*n1950 n2077*, *n1950 n2161*), *ced-11*(*n2744*), *ced-5*(*n2098*)
LGIV: *ced-3*(*n717*, *n718*, *n1040*, *n1129*, *n1163*, *n1164*, *n1165*, *n1286*, *n1949*, *n2424*, *n2425*, *n2426*, *n2427*, *n2429*, *n2430*, *n2432*, *n2433*, *n2436*, *n2438*, *n2439*, *n2440*, *n2442*, *n2443*, *n2444*, *n2445*, *n2446*, *n2447*, *n2449*, *n2452*, *n2454*, *n2719*, *n2720*, *n2721*, *n2722*, *n2830*, *n2854*, *n2859*, *n2861*, *n2870*, *n2871*, *n2877*, *n2883*, *n2885*, *n2888*, *n2889*, *n2921*, *n2922*, *n2923*, *n2924*, *n2998*, *n3001*, *n3002*), *dpy-4*(*e1166*), *sDf21*
LGV: *egl-1*(*n487*).

Isolation and characterization of *ced-3* mutants: We isolated the *ced-3* alleles *n2859*, *n2861*, *n2870*, *n2877*, *n2883*, *n2885*, *n2888*, *n2889*, *n2921*, *n2922*, *n2923*, *n2924*, *n3001*, and *n3002* as suppressors of the maternal-effect lethality caused by the massive ectopic programmed cell death of embryos homozygous for the loss-of-function allele *ced-9*(*n1950 n2161*) (Hengartner *et al.* 1992). Specifically, *unc-69*(*e587*) *ced-9*(*n1950 n2161*)/*qC1* animals were mutagenized using ethyl methanesulfonate (EMS) and allowed to produce self-progeny (Brenner 1974). Unc-69 F_1 animals were then placed 10 to a plate, and F_2 animals 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 using the *ced-3*(*n717*) allele, followed by mapping to establish linkage to *unc-30*(*e191*) on chromosome IV. The *ced-3* alleles *n2719*, *n2720*, *n2721*, *n2722*, *n2830*, and *n2998* were isolated by Gillian Stanfield (personal communication) in our laboratory as suppressors of phenotypes of persistent corpses or abnormal corpse morphology of *ced-5* or *ced-11* mutants, respectively. *ced-3* mutations suppress the *ced-5* and *ced-11* defects by preventing programmed cell death and thus not allowing corpse formation. The *ced-3* alleles *n2424*, *n2429*, *n2432*, *n2436*, *n2439*, *n2440*, *n2442*, *n2443*, *n2444*, *n2445*, *n2446*, *n2447*, *n2449*, *n2452*, *n2454*, *n2854*, and *n2871* were isolated by Michael Hengartner (personal communication) in our laboratory as suppressors of the maternal-effect lethality of animals homozygous for *ced-9* loss-of-function mutations using a protocol similar to that described above. The *ced-3* alleles *n717*, *n718*, *n1040*, *n1129*, *n1163*, *n1164*, *n1165*, *n1286*, *n1949*, *n2426*, *n2430*, and *n2433* were described previously (Ellis and Horvitz 1986; Yuan *et al.* 1993) as were the *ced-3* alleles *n2425*, *n2427*, and *n2438* (Hengartner and Horvitz 1994b; see results). These alleles were isolated in four separate screens as suppressors of mutations that block the engulfment of dead cells (see above), as suppressors of the maternal-effect lethality conferred by *ced-9* loss-of-function mutations (see above), as mutations that prevent programmed cell deaths, or as mutations that fail to complement *ced-3*(*n717*) for suppression of the egg-laying defect of *egl-1*(*n487*) animals. The *egl-1*(*n487*) mutation causes the HSN neurons required for egg laying to undergo programmed cell death. These ectopic deaths are suppressed by mutations in *ced-3* (Trent *et al.* 1983; Ellis and Horvitz 1986). All 52 *ced-3* alleles described in this study were identified as *ced-3* alleles on the basis of complementation tests and linkage to chromosome IV.

To quantitate cell survival in *ced-3* mutants we scored for the presence of extra cells in the anterior region of the pharynx (Hengartner *et al.* 1992). We observed one extra cell in ~5% of wild-type animals. Except for those alleles noted in Table 3, all *ced-3* mutants we analyzed were backcrossed at least twice to wild-type N2 animals to remove any non-*ced-3* mutations that might be present in the strain.

Determination of allele sequences: To characterize coding regions and exon/intron junctions from mutant strains, we amplified the *ced-3* genomic coding region using the polymerase chain reaction (PCR) and a set of four 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 sequences of these primers are shown in Table 1. DNA was amplified as follows. One to 10 worms were placed in 3 μl PCR lysis buffer (60 μg/ml proteinase K in 10 mm Tris pH 8.2, 50 mm KCl, 2.5 mm $MgCl_2$, 0.45% Tween 20, and 0.05% gelatin) and frozen at −70° for 20–30 min. Samples were allowed to incubate at 60° for 1 hr followed by a 15-min incubation at 95°. The entirety of each sample was then 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. Sample sequences were determined using the fmol sequencing kit (Promega, Madison, WI), following instructions of the manufacturer for ^{33}P-labeling and using the primers listed in Table 1, except for primers PWR.30, PWR.32, and PWR.40. Samples were run on standard polyacrylamide sequencing gels (Life Technologies, Gaithersburg, MD). Gels were 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 of all exon/intron junctions. Sequences of sites at which a potential mutation was identified were redetermined for both strands.

DNA flanking the *ced-3*(*n2452*) deletion site was isolated using the CLONTECH (Palo Alto, CA) Advantage cDNA PCR kit, using primers PWR.30 and PWR.32, and following the instructions of the manufacturer. Sequences of the resulting

TABLE 1

PCR and sequencing primer sequences

Primer	Position	Sequence
LOG4	2163, upstream of exon 1	5′ TCAGACTAAATCGAAAATC 3′
SHA2	2182, upstream of exon 1	5′ AAATCGTACTCTGACTACGGG 3′
PWR.30	2953, upstream of exon 3	5′ AGTTCACCGTGACAGCGTCTCTTC 3′
PCR2	3162, downstream of exon 3	5′ TTCGCTACGAGATATTTGCGCG 3′
PWR.40	3803, in intron 3	5′ GAAATGGGATCTCGGTCGATG 3′
PCR3	4225, upstream of exon 4	5′ GCGAAATTAAAATGTGCGAAACGTC 3′
PCR11	4397, in exon 4	5′ CATATTCCATGAAGAGGA 3′
PCR4	4700, downstream of exon 4	5′ ATTTAACACAAATTGTCGTGTCGAGA 3′
PCR5	5502, upstream of exon 5	5′ ATTTCCCAGCCTTGTTCCTAAT 3′
PCR13	5690, in exon 5	5′ TCTCAACGCGGCAAATGC 3′
650	6606, downstream of exon 7	5′ GGTGACGCGCGGCAGGCTT 3′
BD1	6885, upstream of exon 8	5′ GTTGTCCACGAGTATTACACGG 3′
1200	7123, downstream of exon 8	5′ GGGCGAAAGAGAACTGGGGG 3′
PCR6	7128, downstream of exon 8	5′ ACTAATTGGGCGAAAGAGAACT 3′
PWR.32	19.6 kb downstream of PWR.40	5′ CGCGGCGACTTGCTGATTGTGGG 3′

Primer positions are based on the *ced-3* genomic DNA sequence as published by Yuan *et al.* (1993) and indicate the position of the 5′ end of each primer.

DNA were determined using primer PWR.40 and an ABI sequencer (Applied Biosystems, Foster City, CA).

Southern hybridization and RT-PCR experiments: Southern analysis of *ced-3*(*n2452*) and wild-type genomic DNA was performed as described by Sambrook *et al.* (1989) using the restriction enzymes *Eco*RV, *Hin*dIII, *Xho*I, and *Xba*I (New England Biolabs, Beverly, MA) and a full-length *ced-3* cDNA as a probe. RNA for reverse transcriptase PCR (RT-PCR) was prepared as follows. Worms grown on one or two 9-cm plates were added to a liquid culture containing S medium and antibiotics as described by Sulston and Hodgkin (1988). 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). 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 *ced-3*(*n2440*), *ced-3*(*n717*), and *ced-3*(*n2854*) products were determined using an ABI sequencer (see below).

Plasmid constructions: Construct A was made by partially digesting the pJ40 plasmid containing *ced-3* genomic sequences (Yuan *et al.* 1993) with *Bgl*II (to delete sequences 3′ of the *Bgl*II site in the *ced-3* coding region) and self-ligating. Construct B was made by digesting pJ40 with *Mlu*I and *Apa*I, filling in overhangs with the Klenow enzyme, and ligating the vector-containing fragment to the *lacZ* moiety of pPD22.04 (Fire *et al.* 1990) digested with *Bam*HI and *Apa*I, and filled in with the Klenow enzyme. Construct C was made by digesting pJ40 with the enzymes *Bgl*II and *Apa*I and ligating the vector-containing fragment to the *lacZ* moiety of pPD22.04, which had been cut using the enzymes *Bam*HI and *Apa*I. Construct D was made by digesting pJ40 with the enzymes *Sal*I and *Apa*I, and ligating the vector-containing fragment to the *lacZ* moiety of vector pPD22.04, which had been cut using the enzymes *Sal*I and *Apa*I. Construct E was made by digesting the heat-shock vector pPD49.78 (Mello and Fire 1995) with *Nhe*I, digesting the *ced-3* cDNA plasmid pS126 (Shaham and Horvitz 1996a) using *Spe*I and then partially digesting it using *Bgl*II, and digesting pPD22.04 with *Bam*HI and *Spe*I, followed by ligation of the three components. Construct F was made in the identical manner as construct E, except that the smaller pS126 fragment was used. Construct G was made in the identical manner as construct C, except that the green fluorescent protein (GFP) vector Tu#62 (Chalfie *et al.* 1994; M. Chalfie, personal communication) was used instead of the *lacZ* vector. Construct H was produced in the identical manner as construct E, except that the the GFP vector Tu#62 was used instead of the *lacZ* vector. Constructs in Table 2 were made by mutating pS126 to introduce the appropriate changes using an *in vitro* mutagenesis kit (Amersham, Arlington Heights, IL) and following the instructions of the manufacturer. The mutant *ced-3* cDNAs were digested with *Spe*I and *Sma*I and ligated to the $P_{mec\text{-}7}$-containing vector pPD52.102 (Mello and Fire 1995) digested with *Nhe*I and *Eco*RV.

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) according to the instructions of the manufacturer. The concentrations of all plasmids used for injections were between 50 and 100 μg/ml. All constructs were coinjected 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 or *ced-9*(*n2812*); *ced-3*(*n717*) animals. Approximately 30 animals were injected in each experiment, and ~50–100 F_1 Rol animals were picked onto separate plates. F_1 animals segregating Rol animals were established as lines containing extrachromosomal arrays (Way and Chalfie 1988).

Splicing mutants of *ced-3*: We examined in more detail three of the four *ced-3* alleles (*n2854*, *n717*, *n2440*, and *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 3) instead of the AGGCG|gttcg present in the wild type. To characterize the *ced-3* transcripts made in animals carrying this *ced-3* mutation, we isolated RNA from mutant animals (see above), 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 the *ced-3*(*n2854*) mutant was 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 open reading

TABLE 2

Residues required for CED-3 protease activity and CED-3 precursor processing are essential for *ced-3* killing activity

Construct	Line no.	No. ALMs/ no. sides observed
$P_{mec\text{-}7}$*lacZ*[a]	1	40/40
$P_{mec\text{-}7}$*ced-3*(+)	1	9/15
	2	4/13
	3	5/12
$P_{mec\text{-}7}$*ced-3*(C358A)	1	18/18
	2	24/24
	3	17/17
$P_{mec\text{-}7}$*ced-3*(C358S)	1	22/25
	2	25/26
	3	14/14
$P_{mec\text{-}7}$*ced-3*(D374A)	1	22/23
	2	18/20
	3	15/18
$P_{mec\text{-}7}$*ced-3*(D371A)	1	10/20
	2	13/20
	3	13/20
$P_{mec\text{-}7}$*ced-3*(D371A, D374A)	1	25/26
	2	23/23
	3	25/25
$P_{mec\text{-}7}$*ced-3*(D131A)	1	7/20
	2	8/19
	3	12/20
$P_{mec\text{-}7}$*ced-3*(D221E)	1	21/22
	2	20/20
	3	20/20
No transgene		40/40

The amino acid alteration encoded by each transgene is in parentheses. Three transgenic lines were established independently for each transgene. All transgenes were injected into *ced-9*(*n2812*); *ced-3*(*n717*) animals to facilitate scoring of ALM survival (Shaham and Horvitz 1996a). +, wild type.

[a] Data from Shaham and Horvitz (1996a).

frame of *ced-3*. Why this splicing pattern occurred is not understood. The *ced-3*(*n717*) mutation changes a conserved acceptor site G to an A in intron 7. To characterize the product(s) made in *n717* animals, we isolated RNA from mutants and used this RNA for a Northern blot probed with a *ced-3* cDNA. The size and level of the message were not discernibly different from those of the wild-type message (data not shown). We then prepared cDNAs from the *ced-3*(*n717*) RNA and amplified this DNA using PCR and *ced-3*-specific primers. Sequence determination of the resulting bands suggested that splicing occurred at positions −1, −2, −3, 0, +1, +2, and +3 (−, upstream; +, downstream) of the wild-type splice site (data not shown). The mutation in *ced-3*(*n2440*) changes the sequence CCGCAAGTT to CCGTAAGTT, altering codon 401 from a glutamine to an ochre stop codon. However, we noticed that this change also creates a potential splice-donor site (CC|gtaagtt), which might be used instead of the intron 6 splice donor immediately downstream of the mutation site. To determine if this splice-donor site is used, we determined the sequence of *ced-3* cDNAs prepared from *ced-3*(*n2440*) mutant RNAs (see above). Only one class of RNAs was discernible and used the predicted new 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, the mutation in *ced-3*(*n2440*) is likely not to be a nonsense mutation.

***ced-3* reporter constructs and expression patterns:** The construction of reporter transgenes is described above. All *lacZ* and GFP reporter transgenes we examined were expressed in many cells throughout the animal primarily during embryogenesis, starting at about the 200-cell stage, and during the first larval period (L1; data not shown). Very weak expression was seen in a small number of cells after the L1 stage (data not shown). Expression was detected both in cells that normally die and in those that normally live (data not shown), consistent with previous experiments suggesting that *ced-3* activity is present both in cells that do and in cells that do not die (Shaham and Horvitz 1996a). Because the expression patterns we observed were somewhat variable from strain to strain, perhaps because the expression constructs were present on unstable extrachromosomal arrays, we did not pursue a detailed characterization of these patterns.

RESULTS

Cys-358, asp-221, and asp-374 are important for CED-3-induced cell death: To determine whether CED-3 protease activity is required for programmed cell death, we used site-directed mutagenesis to generate mutant *ced-3* cDNAs that should lack either CED-3 protease activity or the CED-3 precursor cleavage sites. We expressed these cDNAs in the ALM neurons using the promoter of the gene *mec-7* ($P_{mec\text{-}7}$; Savage *et al.* 1989); such overexpression of a wild-type *ced-3* cDNA results in the programmed deaths of these cells (Shaham and Horvitz 1996a). In transgenic animals containing extrachromosomal arrays of wild-type $P_{mec\text{-}7}$*ced-3* constructs, ~50% of ALMs die (Shaham and Horvitz 1996a). By contrast, in animals containing mutant transgenes in which the presumptive active site cysteine 358 (Yuan *et al.* 1993; Shaham and Horvitz 1996a; Xue *et al.* 1996) was altered to either alanine (C358A) or serine (C358S), nearly all ALMs survived (Table 2; Figure 1). Similarly, in animals containing transgenes with D221E or D374A mutations, which alter sites of CED-3 proprotein processing (Xue *et al.* 1996), nearly all ALMs survived. On the other hand, ALM killing seemed normal in animals containing D131A or D371A mutated transgenes, which alter aspartate residues at which the CED-3 proprotein is not processed *in vitro*. These results suggest that both proprotein processing and protease activity of CED-3 are required for programmed cell death.

Most missense alleles of *ced-3* affect residues conserved with other caspases: To define additional residues important for *ced-3* function, we isolated 14 new *ced-3* alleles. We then characterized the phenotypes caused by and the molecular lesions of these alleles and of 38 previously existing *ced-3* alleles induced *in vivo*. One of the 52 alleles we examined, *n1949*, was isolated as an inhibitor of normal programmed cell death; 4 alleles (*n1163*, *n1164*, *n1165*, and *n1286*) were isolated in noncomplementation screens for suppression of the ectopic cell death of the HSN neurons in *egl-1* mutant

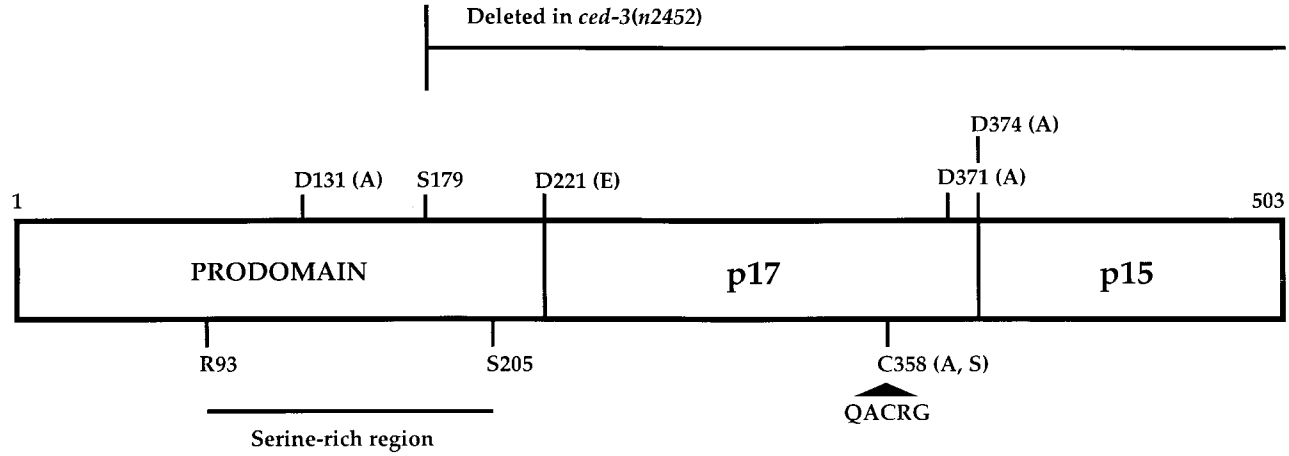

Figure 1.—Schematic diagram of the CED-3 protein. Boxes indicate regions of the CED-3 protein. Vertical lines separating boxes indicate sites at which the CED-3 proprotein is processed *in vitro*. Specific amino acid residues are indicated by a letter either above or below the boxes followed by a position number. The last residue present in *ced-3*(*n2452*) animals is indicated as S179. Mutations examined by *in vitro* mutagenesis are indicated in parentheses adjacent to the relevant residues. The serine-rich region (between amino acid residues R93 and S205), the region deleted in *ced-3*(*n2452*) animals, and the QACRG sequence surrounding the active-site cysteine 358 are indicated.

animals; 10 alleles (*n717*, *n718*, *n1040*, *n1129*, *n2719*, *n2720*, *n2721*, *n2722*, *n2830*, and *n2998*) were isolated as suppressors of mutations causing defects in either the engulfment or morphology of cell corpses; and 37 alleles (*n2424*, *n2425*, *n2426*, *n2427*, *n2429*, *n2430*, *n2432*, *n2433*, *n2436*, *n2438*, *n2439*, *n2440*, *n2442*, *n2443*, *n2444*, *n2445*, *n2446*, *n2447*, *n2449*, *n2452*, *n2454*, *n2854*, *n2859*, *n2861*, *n2870*, *n2871*, *n2877*, *n2883*, *n2885*, *n2888*, *n2889*, *n2921*, *n2922*, *n2923*, *n2924*, *n3001*, and *n3002*) were isolated as suppressors of the lethality conferred by the weak loss-of-function mutation *ced-9*(*n1950 n2161*) (see materials and methods).

To quantify the severity of the defects in programmed cell death of different *ced-3* mutants, we counted the number of extra surviving cells present in the anterior region of the pharynx, as has been previously described (Hengartner *et al.* 1992). To determine the molecular nature of the *ced-3* alleles studied, we used PCR to amplify coding regions and exon/intron boundaries from each mutant strain and determined the DNA sequences of these regions (see materials and methods). In the case of the allele *n2452* we failed to amplify sequences downstream of intron 3 of the *ced-3* gene, which suggests that a deletion might be present (see below; Figures 1–3).

All 52 alleles analyzed were isolated in mutant screens that used EMS as a mutagen. Of these alleles, 44 contained a single GC → AT transition, the mutation induced most often by EMS (Coulondre and Miller 1977; Anderson 1995). Two alleles (*n2432* and *n2445*) contained a single AT → TA transversion, one allele (*n2883*) contained a single AT → GC transition, one allele (*n2446*) contained a single GC → TA transversion, three alleles (*n2424*, *n2830*, and *n2854*) contained several altered nucleotides, and one allele (*n2452*) contained a deletion of 17,229 bp (see below). Of the alleles containing single point mutations, 38 had missense mutations, 6 had nonsense mutations, and 4, including the *n2440* allele, probably affected splicing. [Although *n2440* converts a glutamine codon to an ochre stop codon, our studies of this allele identified a single class of *ced-3*(*n2440*) RNA generated by a new splice-donor site located just upstream of the *n2440* mutation; this RNA presumably encodes an altered protein unaffected by the stop codon described above; see materials and methods.]

Of the 30 distinct sites affected by these missense mutations, 29 are conserved with at least one other nonnematode caspase, even though CED-3 is no more than 34% identical to any of these caspases (Figure 2). The nonconserved serine-rich region of the CED-3 protein (amino acids 93–205; Yuan *et al.* 1993) is not affected by any of these missense mutations, and its functional importance remains unknown. Interestingly, seven of the alleles we studied (*n1040*, *n2439*, *n2449*, *n2424*, *n718*, *n2719*, and *n2830*) alter residues within the CED-3 prodomain, suggesting that the prodomain is essential for CED-3 function during programmed cell death.

Some of the missense mutations we studied could be assigned to residues that have been implicated in a specific caspase function based on the caspase-1 and caspase-3 X-ray structures. These residues seem likely to have a similar role in CED-3 function. Specifically, the alleles *n2427*, *n2438*, and *n2830* (G474R) alter a glycine residue of CED-3 that, on the basis of its corresponding site in caspase-1, is probably located on the heterodimer-heterodimer interface (Wilson *et al.* 1994). CED-3 multimerization might be defective in these mutants. The alleles *n2429* and *n2883* (S314L and

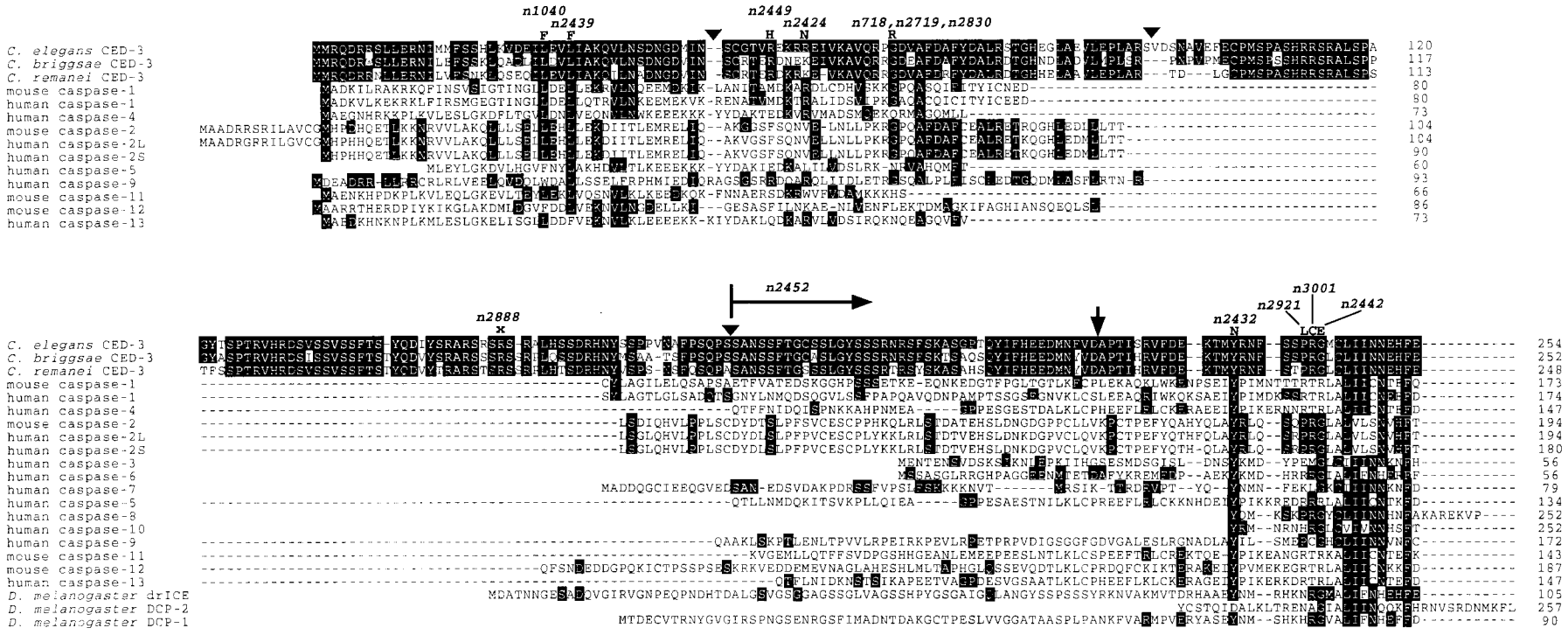

Figure 2.—Positions of *ced-3* mutations. An alignment of sequences of CED-3 proteins from the nematodes *C. elegans*, *C. briggsae*, and *C. remanei* (Yuan *et al.* 1993) with sequences of mouse and human caspase-1 (Cerretti *et al.* 1992; Thornberry *et al.* 1992; Molineaux *et al.* 1993), human caspase-4 (Faucheu *et al.* 1995), mouse caspase-2 (Kumar *et al.* 1994), human caspase-2L and caspase-2S (Wang *et al.* 1994), human caspase-3 (Fernandes-Alnemri *et al.* 1994; Tewari *et al.* 1995), human caspase-6 (Fernandes-Alnemri *et al.* 1995a), human caspase-7 (Fernandes-Alnemri *et al.* 1995b), human caspase-5 (Munday *et al.* 1995), amino acids 226–479 of human caspase-8 (Boldin *et al.* 1996; Muzio *et al.* 1996), amino acids 243–479 of human caspase-10 (Fernandes-Alnemri *et al.* 1996), human caspase-9 (Srinivasula *et al.* 1996), human caspase-11 (Wang *et al.* 1996), mouse caspase-12 (Van de Craen *et al.* 1997), human caspase-13 (Humke *et al.* 1998), *D. melanogaster* drICE (Fraser and Evan 1997), *D. melanogaster* DCP-2 (Inohara *et al.* 1997), and *D. melanogaster* DCP-1 (Song *et al.* 1997). Shaded regions represent residues conserved between *C. elegans* CED-3 and any of the aligned proteins. CED-3 missense mutations are indicated with the allele name and the altered residue above the wild-type residue or by the allele name followed by a colon and the altered residue with the mutation site identified by a vertical line. Nonsense mutations are indicated with the allele name and an x above the altered residue. The sites of exon borders in the *C. elegans* sequence are indicated by arrowheads above the alignment. The N-terminal end of the deletion in *ced-3*(*n2452*) animals, which is located in intron 3, is indicated as an arrow; all sequences to the right of the vertical bar are absent. Mutations in splice donor or acceptor sites are indicated as Do or Ac, respectively, followed by the allele name. CED-3 cleavage sites are indicated with arrows above the *C. elegans* CED-3 sequence. *, animals carrying the *n2445* allele probably contain a protein extended 26 amino acids beyond the wild-type protein.

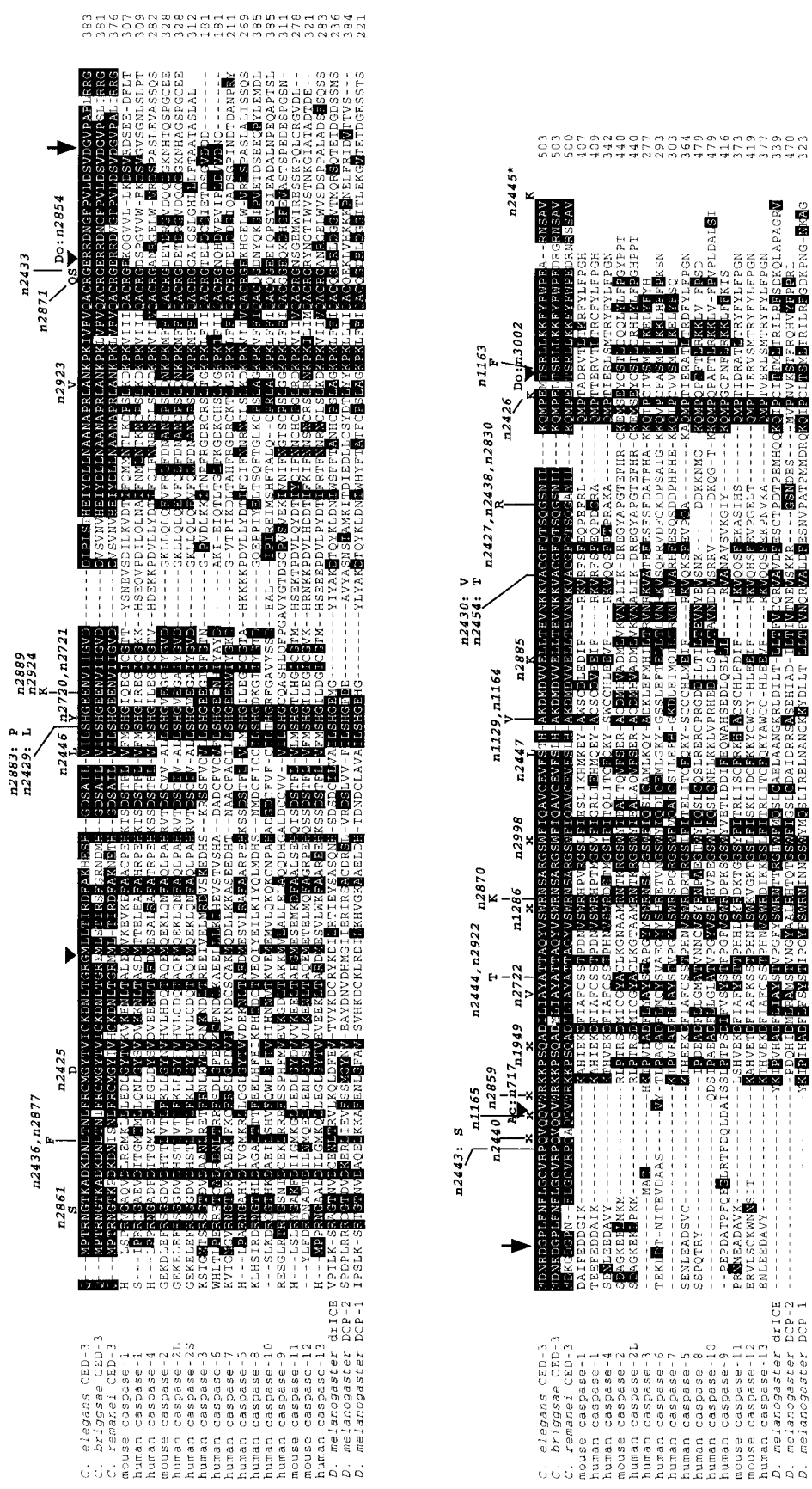

Figure 2.—*Continued.*

S314P, respectively), *n2870* (R429K), *n2721* and *n2720* (H315Y), *n2871* (R359Q), and *n2433* (G360S) all alter residues with equivalents in caspase-1 located within 4 Å of the bound substrate, suggesting that these residues might be important for the CED-3 active site.

The allele *n2871*(R359Q) encodes a protein with a QACQG pentapeptide containing the active site cysteine. This sequence is present in at least five other caspases that possess proteolytic activity. Interestingly, we found that the CED-3(*n2871*) protein expressed in *Escherichia coli* lacked proteolytic activity (data not shown), suggesting that the QACQG sequence is functional only in specific sequence contexts. Similarly, a number of other mutations also introduce into the CED-3 protein amino acids normally found in other caspases: *n1040*(L27F), *n2439*(L30F), *n3001*(R242C), *n2425*-(G277D), *n2889*(E318K), *n2924*(E318K), *n2923*(A347V), *n2870*(R429K), and *n1163*(S486F).

The phenotypic characterizations of and the sequence alterations caused by the 52 *ced-3* alleles are presented in Table 3 and described below.

***ced-3* alleles define a graded series of function:** As shown in Table 3, the *ced-3* alleles we analyzed define a graded series based on the number of extra cells present in the anterior pharynx. To determine if this assay was consistent with other measurements of *ced-3* killing activity, we compared our results from Table 3 to results from two other tests of *ced-3* activity.

First, we examined the ability of eight different *ced-3* alleles to suppress the maternal-effect lethality of animals homozygous for the strong *ced-9*(*n1950 n2077*) loss-of-function allele, which contains a nonsense mutation at codon 160 of the 280-codon *ced-9* open reading frame (Hengartner and Horvitz 1994a). Although all eight *ced-3* alleles suppressed the lethality caused by the weak *ced-9*(*n1950 n2161*) mutation (data not shown), not all suppressed the stronger *ced-9*(*n1950 n2077*) mutation. Specifically, as shown in Table 4, none of the *ced-9*(*n1950 n2077*) progeny of *ced-9*(*n1950 n2077*)/+; *ced-3*(*n2424*, *n2923*, *n2446*, *n2449*, *n2425*)/+ animals was fertile. By contrast, *ced-9*(*n1950 n2077*) progeny of *ced-9*(*n1950 n2077*)/+; *ced-3*(*n2447*, *n2443*, *n717*)/+ animals were increasingly fertile (~10, 30, and 50%, respectively). Fertility correlated with the extra cell counts shown in Table 3: animals homozygous for the alleles *n2424*, *n2923*, *n2446*, *n2449*, and *n2425* had no extra cells in the anterior pharynx, and *n2447*, *n2443*, and *n717* animals had increasing numbers of extra cells (0.8, 1.8, and 11.2 extra cells, respectively).

Using a second assay of *ced-3* activity, we observed that *ced-9*(*n1950 n2077*); *ced-3*(*n2447* or *n2443*) animals were severely egg-laying defective, presumably as a consequence of the deaths of the HSN neurons required for egg laying (data not shown; Hengartner *et al.* 1992), whereas *ced-9*(*n1950 n2077*); *ced-3*(*n717*) animals were egg-laying competent. None of these *ced-3* mutants is egg-laying defective in the absence of the *ced-9* mutation. These results suggest that the *ced-3* alleles *n2447* and *n2443* are less defective in *ced-3* function (*i.e.*, allow more cell death to occur) than is the allele *n717*, consistent with the results presented in Tables 3 and 4.

Taken together, our observations support the hypothesis that the 52 *ced-3* alleles we examined define a graded series of *ced-3* activities as listed in Table 3.

***ced-3*(*n2452*) animals lack the protease region of CED-3:** The *n2452* allele is deleted for the region of the *ced-3* gene that encodes the p17 and p15 subunits, which form the CED-3 protease (Figure 1). As shown in Figure 3, Southern blots of *ced-3*(*n2452*) genomic DNA digested with *Xho*I or *Hin*dIII and probed with a full-length *ced-3* cDNA revealed the absence of fragments present in wild-type genomic DNA. Specifically, as shown in Figure 3A, both a 1.7-kb *Xho*I fragment internal to the *ced-3* gene and an adjacent 20.2-kb *Xho*I fragment present in wild-type animals were absent in *ced-3*(*n2452*) animals, whereas a 2.6-kb *Xho*I fragment containing the *ced-3* promoter and first three exons remained intact in the mutant. Furthermore, a novel 4.7-kb *Xho*I fragment appeared in *ced-3*(*n2452*) animals. Similarly, as shown in Figure 3B, both a 4.8-kb and a 3.7-kb *Hin*dIII fragment present in wild-type animals were missing in *ced-3* (*n2452*) animals, and a novel 4.9-kb band appeared in the mutant. Similar results were observed using other enzymes (data not shown). On the basis of these results we propose that *ced-3*(*n2452*) is a deletion that removes all coding sequences downstream of intron 3 of *ced-3* (see Figures 1–3).

In support of this interpretation, we were able to amplify a wild-type-sized DNA fragment from *ced-3*(*n2452*) animals using PCR and primers located upstream of exon 1 and at the 5′ end of intron 3 (primers SHA2 and PCR2, Table 1; data not shown). However, we could not amplify any DNA fragments from *ced-3*(*n2452*) animals using PCR and primer pairs located at the 3′ end of intron 3 and in intron 4 (primers PCR3 and PCR4, Table 1), in introns 4 and 7 (primers PCR5 and 650, Table 1), or in intron 7 and downstream of the *ced-3* stop codon (primers BD1 and 1200, Table 1; data not shown). Furthermore, we were able to amplify a 3.2-kb genomic fragment of DNA from *ced-3*(*n2452*) animals using primers PWR.30 and PWR.32 located 59 nucleotides upstream of the *ced-3* intron 3 splice-donor site and 20.3-kb downstream of the *ced-3* intron 3 splice-donor site, respectively. Partial sequence of this 3.2-kb DNA fragment was determined using the primer PWR.40. The resulting sequence was consistent with a deletion of 17,229 bp downstream of position 4008 in the *ced-3* genomic sequence (Yuan *et al.* 1993; Figure 3). This deletion lacks the *ced-3* region encoding amino acids 180 to the end of the protein—the region necessary for CED-3 protease activity (Figures 1–3). This deletion also removes two other putative genes (C48D1.1 and F58D2.2) and disrupts a third putative gene (F58D2.1).

TABLE 3

Phenotypes and sequence alterations of *ced-3* mutants

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)	—	—	—
sDf21	—	0.07 ± 0.3 (15)	Large deletion	—
Recessive alleles[a]				
n2424	0 (15)	0.07 ± 0.3 (15)	AGA → AAT	R55N
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
n3001	0.8 ± 1.0 (15)	0.07 ± 0.3 (15)	CGT → TGT	R242C
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
n1164	8.6 ± 1.4 (15)	0.2 ± 0.4 (15)	GCA → GTA	A449V
n1129	8.7 ± 1.3 (10)	0.07 ± 0.3 (15)	GCA → GTA	A449V
n2452	9.5 ± 1.5 (15)	0 (15)	deletion	—
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	R154opal
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|gatt → G|gttcg	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	W406amber
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
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
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
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

(*continued*)

TABLE 3

(Continued)

Genotype	Mutant/mutant: no. extra cells in anterior pharynx ± SD	Mutant/+: no. extra cells in anterior pharynx ± SD	Nucleotide change	Amino acid/ splice change
Weakly semidominant alleles[b]				
n2440	11.7 ± 1.7 (15)	0.5 ± 0.6 (15)	CG<u>C</u>AA → CG<u>T</u>AA	Q401ochre/splice donor
n2871	11.7 ± 1.7 (15)	1.0 ± 0.8 (15)	C<u>G</u>A → C<u>A</u>A	R359Q
n2430	11.8 ± 1.2 (10)	0.73 ± 0.6 (15)	G<u>C</u>T → G<u>T</u>T	A466V
n2433	12.4 ± 1.0 (10)	0.5 ± 0.9 (15)	<u>G</u>GC → <u>A</u>GC	G360S

sDf21 is a deficiency spanning *ced-3* (see materials and methods). Numbers in parentheses indicate the number of animals observed. Nucleotide change: altered residues are underlined, wild-type sequence is to the left of each arrow, and mutant sequence is to the right. Amino acid/splice change: numbers indicate the amino acid residue. For scoring *mutant/mutant* animals, the *ced-3* alleles were separated from background mutations, except for some that were scored in the following genetic backgrounds: *ced-3*(*n1163*); *egl-1*(*n487*), *ced-3*(*n1164*); *egl-1*(*n487*), *sem-4*(*n1378*) *ced-1*(*e1735*); *ced-11*(*n2744*); *ced-3*(*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*), and *ced-3*(*n3002*) *nIs50*. For scoring *mutant*/+ animals, all the strains described above were crossed to wild-type animals and resulting heterozygous males were scored. The altered sequences in the *ced-3* alleles *n717*, *n718*, *n1040*, *n1129*, *n1163*, *n1164*, *n1165*, *n1286*, *n1949*, *n2426*, *n2430*, and *n2433* have been described previously (Yuan *et al.* 1993).

[a] Less than 15% of heterozygous animals contained one extra cell, and none contained more than one extra cell in the anterior pharynx.

[b] More than 50% of heterozygous animals contained at least one extra cell in the anterior pharynx.

TABLE 4

Weak *ced-3* alleles did not prevent the sterility caused by the *ced-9*(*n1950 n2077*) allele

Parental genotype: *ced-9*(*n1950 n2077*)/+; *ced-3*/+	*ced-9* homozygotes: Fertile/total
ced-3 allele (avg. no. extra cells)	
n2424 (0)	0/30
n2923 (0)	2/30*
n2446 (0.13)	0/30
n2449 (0.2)	0/30
n2425 (0.3)	0/30
n2447 (0.8)	3/30
n2443 (1.8)	6/20
n717 (11.2)	10/20

The *ced-9*(*n1950 n2077*) chromosome was marked with the *unc-69*(*e587*) mutation. Unc-69 animals were inferred to be homozygous for *ced-9*(*n1950 n2077*). *ced-9* mutations result in sterility, which is dominantly rescued by strong *ced-3* mutations (Hengartner *et al.* 1992). Thus, only *ced-9*; *ced-3*/+ or *ced-9*; *ced-3* animals are fertile. Each *ced-9* homozygote assayed contained zero, one, or two copies of a given *ced-3* allele, because these animals were derived from the self-progeny of *ced-9*/+; *ced-3*/+ animals. For *ced-3* mutations resulting in little *ced-3* activity, 75% of *ced-9* homozygotes scored should be fertile (one-third of these are *ced-9*; *ced-3* and two-thirds are *ced-9*; *ced-3*/+). For weaker *ced-3* mutations that retain significant *ced-3* activity, <75% of *ced-9* homozygotes scored should be fertile. Fertility was scored as the ability to lay >20 eggs with at least one embryo developing into a fertile adult. *, each of the two animals gave rise to one viable, sick, and infertile progeny.

Programmed cell death may occur in the absence of CED-3 protease function: *ced-3*(*n2452*) animals had 9.5 ± 1.5 extra cells in the anterior pharynx (Table 3). By contrast, numerous other *ced-3* mutants contained significantly more extra cells in the anterior pharynx than did *ced-3*(*n2452*) animals. For example, *ced-3* (*n2433*) animals contained 12.4 ± 1.0 extra cells in the anterior pharynx ($P < 0.001$ by the unpaired Student's *t*-test). Mutant animals carrying a number of *ced-4* mutations or the gain-of-function *ced-9*(*n1950*) allele similarly contained significantly more extra cells in the anterior pharynx than did *ced-3*(*n2452*) animals. *ced-4*(*n1162*) animals, for example, had 11.9 ± 1.1 extra cells and *ced-9*(*1950*) animals had 12.5 ± 0.8 extra cells. These observations suggest that some cells die by programmed cell death in *ced-3*(*n2452*) animals. If so, the protease activity of CED-3 might not be necessary to cause all programmed cell deaths in *C. elegans*.

To confirm that cells can undergo programmed death in *ced-3*(*n2452*) animals we examined the number of cell corpses present in the heads of *ced-3*(*n2452*) L1 animals. To facilitate our analysis, animals were scored in a *ced-1*(*e1735*) background, which results in the persistence of cell corpses. As shown in Table 5, half of the *ced-1*(*e1735*); *ced-3*(*n2452*) animals we examined contained at least one cell corpse. These results support the notion that programmed cell death can still occur in the absence of CED-3 protease activity. Interestingly, even animals harboring stronger *ced-3* alleles such as *ced-3*(*n717*) or *ced-3*(*n718*) contained some corpses.

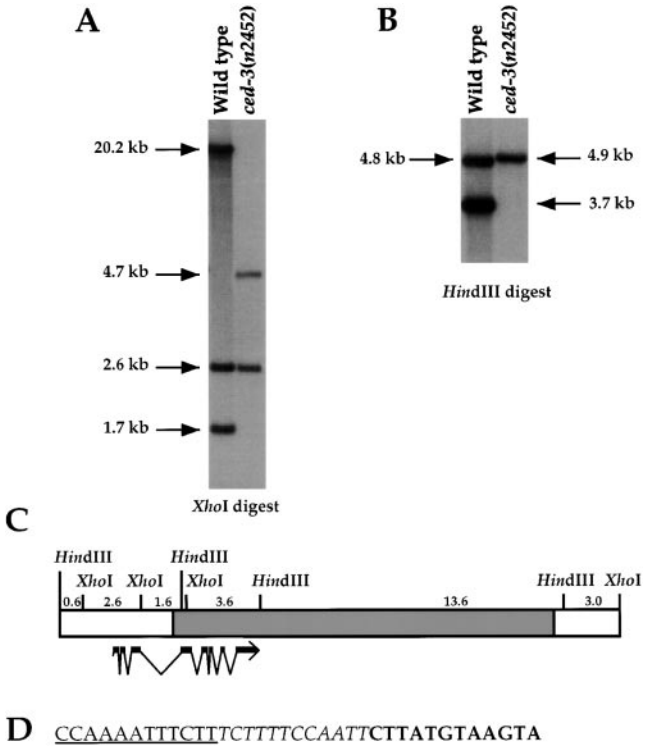

Figure 3.—*ced-3*(*n2452*) is a deletion. (A) Southern blot of *ced-3*(*n2452*) and wild-type genomic DNA digested with *Xho*I and probed with a full-length *ced-3* cDNA. Note the absence of the 1.7-kb and 20.2-kb bands and the appearance of a new band of 4.7 kb in the *ced-3*(*n2452*) lane. (B) Southern blot of *ced-3*(*n2452*) and wild-type genomic DNA digested with *Hin*dIII and probed with a full-length *ced-3* cDNA. Note the absence of the 3.7-kb and 4.8-kb bands and the appearance of a 4.9-kb band in the *ced-3*(*n2452*) lane. (C) A schematic diagram of the *ced-3* genomic region in wild-type and *ced-3*(*n2452*) animals. Shaded box corresponds to sequences that are deleted in *ced-3*(*n2452*) animals. The *ced-3* mRNA is indicated below the horizontal bar. Numbers correspond to the sizes in kilobases of intervals flanked by restriction sites. (D) Sequences surrounding the *ced-3*(*n2452*) deletion breakpoints. Underlined residues are *ced-3* intron 3 residues. Bold residues lie 17.3 kb downstream of the *ced-3* intron 3 breakpoint. Italicized residues cannot be accounted for by the known sequence of the region and indicate an insertion of 12 bp.

It is conceivable that the *ced-3*(*n2452*) strain we studied contains a second mutation that bypasses a need for CED-3 protease activity to induce programmed cell death. To explore this possibility, we backcrossed the *ced-3*(*n2452*) strain to the wild-type N2 strain four times. Also, we generated strains in which the backcrossed *ced-3*(*n2452*) chromosome IV had undergone recombination with a non-*ced-3*(*n2452*) chromosome IV on either side of the *ced-3* gene (see materials and methods). Four-times backcrossed *ced-3*(*n2452*) animals had 9.5 ± 1.5 (n = 15) extra cells; *ced-3*(*n2452*) *dpy-4*(*e1166*) animals in which the right arm of the original *ced-3*(*n2452*) chromosome IV was replaced with an arm from a *dpy-4*(*e1166*) strain by recombination had 9.7 ± 1.4 (n = 18) extra cells; and *unc-32*(*e189*) *ced-3*(*n2452*) in which the left arm of the original *ced-3*(*n2452*) chromosome IV was replaced with an arm from an *unc-32*(*e189*) strain by recombination had 9.3 ± 2.0 (n = 15) extra cells. These results indicate that if a modifier mutation exists in the original *ced-3*(*n2452*) strain, it is likely to be located within four map units to the right of *ced-3* and two map units to the left of *ced-3*. It remains possible that the disruption of C48D1.1, F58D2.2, or F58D2.1 in the *ced-3*(*n2452*) strain bypasses a need for CED-3 protease activity, although none of these genes has a sequence suggesting a direct or indirect role in programmed cell death (our unpublished observations).

The N-terminal nonprotease prodomain of CED-3 might be important for programmed cell death: Whether the phenotype of *ced-3*(*n2452*) animals represents the phenotype caused by a true *ced-3* null allele is unclear (see discussion). For example, it is possible that the prodomain of the CED-3 protein, which might be functional in *ced-3*(*n2452*) animals, could affect programmed cell death.

We have obtained data that suggest that the prodomain of CED-3 can prevent programmed cell death when fused to a heterologous protein. Specifically, while examining the expression patterns of translational fusions of *ced-3* to either *lacZ* or GFP reporter genes containing nuclear localization signals (NLS), we observed that wild-type animals expressing transgenes encoding the prodomain of CED-3 displayed extra cells in the anterior pharynx. (We have not characterized the expression patterns of these transgenes in detail; see materials and methods.) First, we expressed genomic regions of *ced-3* containing sequences 2.5 kb upstream of the start codon ($P_{ced\text{-}3}$) and terminating at different locations within the *ced-3* coding region, fused to *lacZ* (Figure 4, transgenes B–D). Second, we expressed a *C. elegans* heat-shock promoter fused to a truncated *ced-3* cDNA fused, in turn, to *lacZ* (Figure 4, transgenes E and F).

Transgenes C and D partially inhibited programmed cell death (Figure 4). For example, two lines of animals transgenic for transgene C had on average 1.5 or 2.4 extra cells in the anterior pharynx. Extra cells were also seen in animals carrying transgene F, indicating that *ced-3* promoter sequences are not required to generate extra cells. The presence of extra cells also was not dependent on the *lacZ* moiety, because transgenes containing fusions to GFP also caused extra cells to be present (Figure 4, transgenes G and H). Finally, we demonstrated that reporter transgenes equivalent to transgenes C and D but lacking a NLS could also promote the presence of extra cells (data not shown), suggesting that the NLS is not required for this phenotype. In short, the expression of CED-3 residues 1–151 resulted in extra cells.

To test the hypothesis that the extra cells we observed resulted from an inhibition of programmed cell death,

TABLE 5

Some cell deaths occur in *ced-3* mutants

Genotype	Average no. of head corpses[b]	No. animals[a] with:				
		0 corpses	1 corpse	2 corpses	3 corpses	>3 corpses
ced-1(*e1735*)	27.1 ± 3.8	0	0	0	0	50
ced-1(*e1735*); *ced-3*(*n2427*)[c]	18.1 ± 3.7	0	0	0	0	50
ced-1(*e1735*); *ced-3*(*n1040*)	7.0 ± 2.7	0	1	1	4	44
ced-1(*e1735*); *ced-3*(*n1129*)	3.9 ± 1.5	0	2	7	13	28
ced-1(*e1735*); *ced-3*(*n717*)[d]	1.0 ± 0.8	14	24	10	2	0
ced-1(*e1735*); *ced-3*(*n2452*)	0.7 ± 0.9	25	18	4	3	0
ced-1(*e1735*); *ced-3*(*n718*)	0.5 ± 0.7	33	12	4	1	0

[a] Fifty animals were scored for each genotype.
[b] Corpses were counted in the heads of L1 larvae with four cells in the gonad. Numbers indicate average number of head corpses ±SD.
[c] Strain also contained the *unc-30*(*e191*) allele.
[d] Strain also contained the *unc-31*(*e928*) allele.

we introduced transgene D into animals homozygous for the *ced-9*(lf) alleles *n1950*, *n2161*, or *n2812*. Transgene D suppressed the lethality of both strains, further showing that this transgene can inhibit programmed cell death. These observations indicate that the expression of CED-3 prodomain fusion constructs can interfere with programmed cell death and raise the possibility that this region of the CED-3 proprotein might normally interact with components of the cell-death machinery. It has been suggested that CED-3 activation is mediated by binding of CED-4 to the CED-3 prodomain (Chinnaiyan *et al.* 1997). If so, our CED-3 prodomain fusion constructs might directly compete with wild-type CED-3 for binding to CED-4, resulting in inhibition of programmed cell death.

Transgenes B and E, which encode residues 1–71 and 1–94 of CED-3, respectively, did not inhibit programmed cell death (Figure 4). This observation suggests that the region between residues 94–151 is important for protection. Interestingly, transgenes B and E both interrupt a region (caspase recruitment domain, amino acids 1–86) postulated to be required for interaction with the CED-4 protein, and it is possible that our CED-3 prodomain fusions interact with CED-4. That transgene A, containing identical N-terminal-encoding *ced-3* sequences as transgene C but without a reporter fusion, was unable to protect against programmed cell death suggests that this region on its own might produce an unstable protein or require fusion to a heterologous protein to prevent cell death. Alternatively, this transgene might not have been expressed at adequate levels to prevent cell death.

Interestingly, *ced-3*(*n2452*) animals harboring transgene D (Figure 4) contained significantly more surviving cells in the anterior pharynx (11.7 ± 1.2, $P < 0.001$) than *ced-3*(*n2452*) animals alone (9.5 ± 1.5), suggesting that transgene D might affect a CED-3 protease-independent mode of programmed cell death.

Mutations in the conserved QACRG active-site pentapeptide of CED-3 are weakly dominant-negative: While examining cell survival in animals heterozygous for the 52 EMS-induced *ced-3* alleles, we noticed that 4 of these alleles (*n2871*, *n2433*, *n2440*, and *n2430*) were weakly semidominant (Table 3). Animals heterozygous for these alleles showed weak cell survival (0–2 extra cells per animal with more than half of the animals having at least one extra cell). These values are statistically different from those of wild-type animals (unpaired Student's *t*-test: $P < 0.004$ for *n2440* and *n2433*, $P < 0.001$ for *n2430* and *n2871*). To test for another semidominant effect of the *ced-3*(*n2871*) allele, we examined the viability of animals homozygous for the weak *ced-9* allele *n1950 n2161* and heterozygous for *ced-3*(*n2871*). *unc-69*(*e587*) *ced-9*(*n1950 n2161*) animals do not produce viable progeny (Hengartner and Horvitz 1994a). However, *unc-69*(*e587*) *ced-9*(*1950 n2161*); *ced-3*(*n2871*) *dpy-4*(*e1166*)/+ + produce viable animals. Of 84 such progeny examined, 48 were non-Dpy. Only 3 or 4 of the non-Dpy progeny would be expected to be homozygous for *ced-3*(*n2871*) as a result of recombination between *ced-3* and *dpy-4*. Thus, the majority of the non-Dpy progeny observed were likely heterozygous for *ced-3*. Furthermore, 10 of 10 non-Dpy progeny scored were not homozygous for *ced-3*(*n2871*) as assessed by examination of their Ced phenotype. These results confirm that the *ced-3*(*n2871*) allele has semidominant effects.

To test whether the semidominant phenotype conferred by these alleles was caused by a haplo-insufficiency of the *ced-3* locus, we examined animals either heterozygous for the deficiency *sDf21*, which spans *ced-3*, or heterozygous for the *ced-3*(*n2452*) deletion allele. As shown in Table 3, neither *sDf21*/+ animals nor *ced-3*(*n2452*)/+ animals showed significant extra cell survival in the anterior pharynx, suggesting that the semidominant phenotype conferred by the *ced-3*(*n2871*, *n2433*, *n2440*, *n2430*) alleles was caused not by haplo-

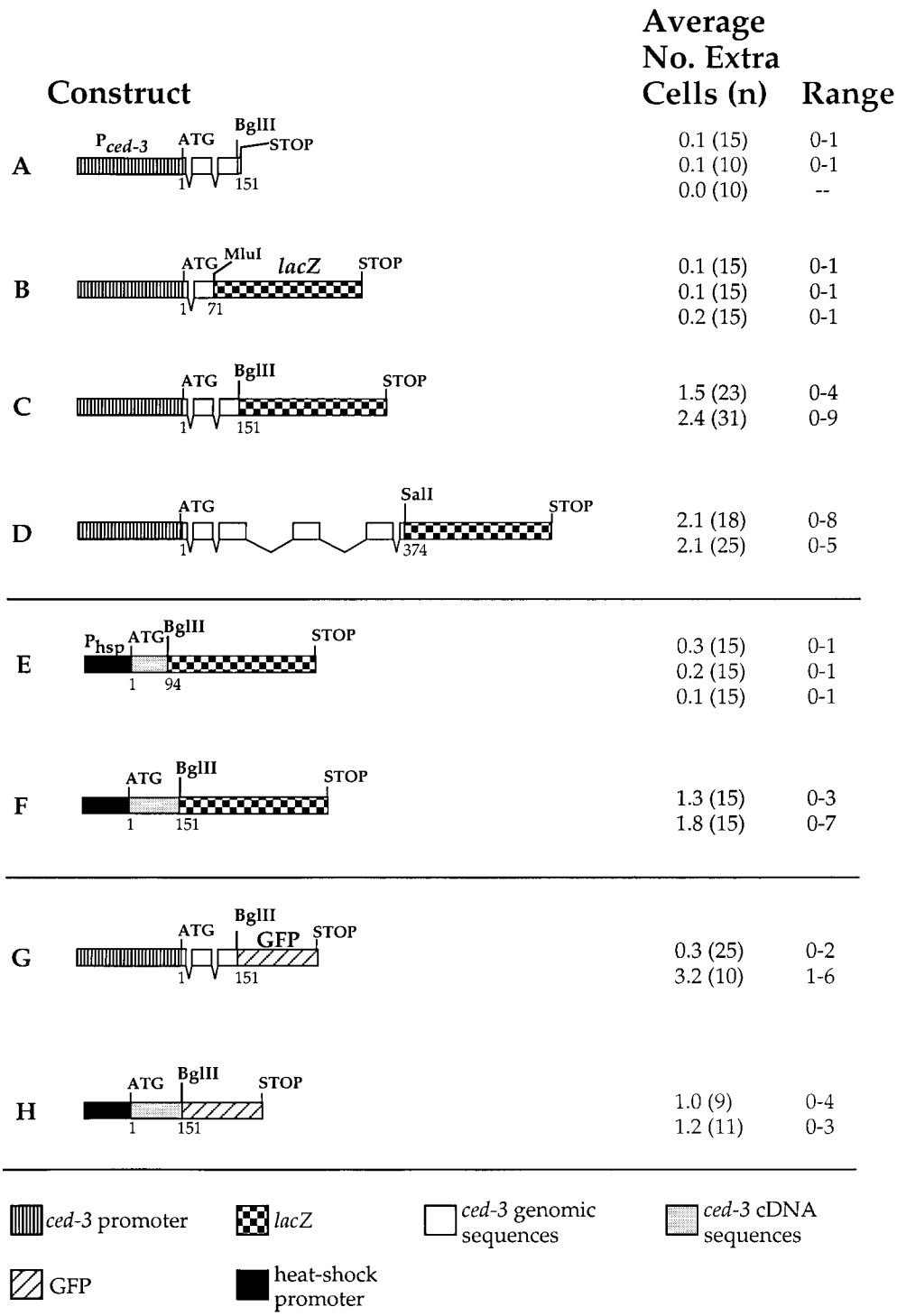

Figure 4.—*ced-3*-reporter fusion transgenes can inhibit programmed cell death. The structure of each construct is indicated graphically, with the names A–H indicated to the left. CED-3 amino acid positions are indicated under the *ced-3* portion of each construct. *lacZ* and GFP fragments indicated contain an SV40 T antigen-derived nuclear localization signal (Fire *et al.* 1990). The average number of extra cells in the anterior pharynx of animals of a given transgenic line is indicated. For heat-shock promoter transgenes the number of extra cells was scored in L3 larvae heat-shocked at 33° for 1 hr at the 200-cell embryo stage. No significant extra cell survival was detected in nonheat-shocked animals (data not shown). GFP expression in line 1 of construct G was significantly reduced compared with line 2, which presumably accounts for the observed lower number of extra cells. Each number represents an independent transgenic line. n, the number of animals observed for each strain. The range of extra cells observed in the anterior pharynx of each strain is indicated.

insufficiency but rather by a dominant-negative interaction.

Two of the four *ced-3* alleles with semidominant effects, *ced-3*(*n2871*) and *ced-3*(*n2433*), alter the arginine (R359) and glycine (G360) residues, respectively, in the highly conserved pentapeptide QACRG, which surrounds the active site of CED-3 and is characteristic of most caspases (Table 3, Figure 2; Yuan *et al.* 1993). To determine if other active-site CED-3 mutants might have dominant-negative effects, we expressed in wild-type animals a *ced-3* transgene containing a heat-shock promoter fused to a *ced-3* cDNA encoding an active-site cysteine-358-to-alanine mutant protein (see Figure 4 legend for heat-shock protocol). We found that in three separate lines containing this mutant transgene, animals contained on average 2.9 ($n = 8$), 4.9 ($n = 9$), and 2.6 ($n = 9$) extra cells in the anterior pharynx. This result further indicates that *ced-3* alleles containing mutations

affecting the conserved QACRG motif can prevent programmed cell death in a dominant-negative fashion.

DISCUSSION

The *C. elegans* CED-3 proprotein consists of an N-terminal prodomain with no known catalytic function and central and C-terminal regions that are cleaved from the proprotein and associate to form an active protease (Yuan *et al.* 1993; Xue *et al.* 1996). In this study we have characterized the effects of site-directed mutations introduced *in vitro* into the *ced-3* gene, as well as *ced-3* mutations induced *in vivo* by the chemical mutagen EMS and identified in genetic screens, to address the roles of the CED-3 protease and N-terminal prodomain in programmed cell death. This study has allowed a detailed analysis of the effects of specific amino-acid substitutions on CED-3 caspase function *in vivo*. Specifically, key residues in the prodomain and in the protease subunits of CED-3 have been identified as important for CED-3 function.

The protease activity of CED-3 and the processing of the CED-3 proprotein are important for programmed cell death in *C. elegans*: In this article we demonstrate that a mutation in the presumptive active-site cysteine 358 of CED-3, previously shown to perturb CED-3 protease activity *in vitro* (Xue *et al.* 1996), also perturbed the *in vivo* killing activity of *ced-3* (Table 2). In addition, we describe seven EMS-induced *ced-3* mutant strains (*n2429*, *n2883*, *n2870*, *n2721*, and *n2720*, *n2871*, and *n2433*), each of which is defective in programmed cell death, and each of which contains an alteration in a residue (serine 314, histidine 315, arginine 359, glycine 360, or arginine 429) that, on the basis of comparison to the caspase-1 crystal structure, seems likely to lie within 4 Å of the bound substrate and be an integral element of the CED-3 active site. Together, these findings indicate that CED-3 protease activity is important for programmed cell death.

Our data also indicate that processing of the CED-3 proprotein is important for programmed cell death, because site-directed mutations in two residues at which the CED-3 proprotein is processed *in vitro* (aspartate 221 and aspartate 374; Xue *et al.* 1996) abolished the killing activity of CED-3 *in vivo*. The processing of the CED-3 proprotein could be important only for generating active CED-3 protease. Alternatively, such processing could be important for releasing the N-terminal prodomain of CED-3, which might have a role in programmed cell death (see results).

CED-3 protease activity might not be essential for all programmed cell deaths in *C. elegans*: The *ced-3* allele *n2452* eliminates CED-3 protease function, because *ced-3*(*n2452*) animals contain a deletion that removes all sequences present in the mature protease (Figures 1–3; Xue *et al.* 1996). Nonetheless, some programmed cell deaths still occur in *ced-3*(*n2452*) animals. Specifically, we showed that animals carrying the *ced-3*(*n2452*) mutation contain an average of 9.5 extra surviving cells in the anterior pharynx, whereas at least 17 strains homozygous for other *ced-3* alleles (*n2442*, *n2721*, *n2889*, *n717*, *n2439*, *n2720*, *n2719*, *n2454*, *n2432*, *n2830*, *n718*, *n3002*, *n2883*, *n2440*, *n2871*, *n2430*, and *n2433*) as well as strains homozygous for strong *ced-4* alleles or for the *ced-9*(*n1950*) gain-of-function allele (Hengartner *et al.* 1992; Shaham and Horvitz 1996b; S. Shaham and H. R. Horvitz, unpublished results) contain more extra surviving cells (up to an average of 12.5; unpaired Student's *t*-test: $P < 0.01$ for the number of extra surviving cells in the least cell-death defective strains listed). These results suggest that on average ~3 cells undergo programmed cell death in the anterior pharynx of *ced-3*(*n2452*) animals. Furthermore, in *ced-3*(*n2452*) animals containing a *ced-1*(*e1735*) mutation, which allows the visualization of cell corpses, corpses were observed in the head, providing additional evidence that programmed cell death occurs in this *ced-3* mutant strain. We also have shown that *ced-3*(*n2452*) animals harboring an additional mutation affecting programmed cell death, *ced-8*(*n1891*), contain an average of 11.9 extra cells in the anterior pharynx (S. Shaham, G. Stanfield and H. R. Horvitz, unpublished observations), supporting the hypothesis that cell deaths do occur in *ced-3*(*n2452*) animals, because it appears that these deaths can be prevented by the *ced-8*(*n1891*) mutation.

We cannot preclude the possibility that simply eliminating CED-3 protease activity would result in the absence of all programmed cell deaths and that in *ced-3*(*n2452*) animals there is loss of a death-protective function in addition to the loss of CED-3 protease activity. Such a death-protective function could be provided by the CED-3 protein, by an alternative product of the *ced-3* gene, or by the product of a gene closely linked to *ced-3* and also disrupted in the *ced-3*(*n2452*) strain (see results). In each of these cases, cell death would occur in the absence of CED-3 protease activity, again suggesting that this activity is not absolutely essential for all programmed cell deaths.

We previously showed that the ectopic overexpression of *ced-4* can induce programmed cell death to a limited extent in animals homozygous for the strong *ced-3* (*n2433*) mutation, which substitutes a serine for glycine at codon 360 (Shaham and Horvitz 1996a). That cells can still die in *ced-3*(*n2433*) animals suggests three possibilities: (1) the *ced-3*(*n2433*) mutation does not fully eliminate CED-3 protease activity, (2) the CED-3 protein contains a nonprotease killing activity, or (3) a CED-3-independent activity is capable of killing cells in the absence of CED-3 activity. The latter two possibilities are also suggested by our finding that in the *ced-3*(*n2452*) deletion mutant cell death can occur in the absence of CED-3 protease activity.

How might some *ced-3* alleles prevent programmed cell death more than the protease deletion mutant *ced-*

***3*(*n2452*)?** First, as noted above, it is possible that the CED-3 protein contains a nonprotease killing activity that is not disrupted in the *ced-3*(*n2452*) mutant. In this case, the *ced-3*(*n2452*) mutation would not be a *ced-3* null allele, and stronger, null, *ced-3* alleles would disrupt both the protease and the nonprotease CED-3 killing activities. Second, if *ced-3*(*n2452*) is a null allele, two possibilities seem plausible. On the one hand, *ced-3*(*n2452*) might eliminate not only a *ced-3* killing function but also a *ced-3* protective function; both *ced-4* (Shaham and Horvitz 1996b) and *ced-9* (Hengartner and Horvitz 1994b) appear to have both killing and protective functions. On the other hand, the proteins encoded by strong *ced-3* alleles, such as *ced-3*(*n2433*), may not only be defective in CED-3 activity but may also interfere with a CED-3-independent activity required for programmed cell death. Consistent with this hypothesis is our observation that the *ced-3*(*n2433*) allele can prevent cell death in a weakly semidominant fashion, suggesting that the *ced-3*(*n2433*) product can actively interfere with the cell-death process. Perhaps CED-3(*n2433*) can inhibit a second caspase capable of inducing programmed cell death. The mutant CED-3 protein might titrate an activator of or serve as a substrate for such a second caspase. In the latter case, the mutant CED-3 protein would act as a competitive inhibitor, similar to the proposed action of the baculovirus p35 protein in preventing programmed cell death in *C. elegans* (Xue and Horvitz 1995). Alternatively, heterodimer formation between the CED-3 mutant protein and a second caspase might prevent protease activity of the second caspase. Three caspase-related genes have been identified in *C. elegans* (Shaham 1998). If the product of any of these genes participates in programmed cell death, it might be inhibited by mutant CED-3 products as described above.

The null phenotype of *ced-3*: The issues discussed above highlight the importance of unambiguously establishing the phenotype caused by a complete absence of *ced-3* function. It seems likely that a mutant completely lacking *ced-3* function is viable and deficient in programmed cell death, as are the large number of *ced-3* mutants we have characterized. In support of this hypothesis, four *ced-3* alleles (*n1163*, *n1164*, *n1165*, and *n1286*) were isolated in noncomplementation screens—which could have identified lethal *ced-3* alleles—and all four when homozygous result in animals that are viable and deficient in programmed cell deaths. Furthermore, when *ced-3* function is inhibited using the method of RNA-mediated interference (Fire *et al.* 1998), the resulting animals are also deficient in programmed cell deaths (P. W. Reddien and H. R. Horvitz, unpublished observations).

However, 50 of the 52 *ced-3* alleles we studied contain mutations that could retain some *ced-3* function: missense mutation, nonsense mutations that could potentially allow synthesis of a fragment of the CED-3 protein, or splicing mutations. Furthermore, we examined *ced-3* mRNA size and level in 11 of these 50 *ced-3* mutants (*n717*, *n718*, *n1040*, *n1129*, *n1163*, *n1165*, *n1286*, *n1949*, *n2426*, *n2430*, and *n2433*) and found that all 11 produced *ced-3* mRNA of roughly the same size and abundance as in the wild type (data not shown). By contrast, nonsense, missense, and splicing mutants defective in other *C. elegans* genes often contain little or no RNA (Pulak and Anderson 1993). Thus, it is conceivable that most of the existing *ced-3* mutants generate CED-3 protein products that have either reduced or abnormal *ced-3* activity.

Nonetheless, two *ced-3* alleles (*n2452* and *n2888*) do seem like good candidates for being null alleles, based upon their molecular lesions. As discussed above, *ced-3*(*n2452*) animals are deleted for the entire C-terminal region of the 503-amino-acid CED-3 protein beyond amino acid 180. *ced-3*(*n2888*) animals have an arginine-to-opal nonsense mutation at codon 154, presumably resulting in a truncated CED-3 protein lacking the regions necessary for protease activity. Thus, assuming that CED-3 protease activity is essential for all *ced-3* function, *ced-3*(*n2452*) and *ced-3*(*n2888*) are both most likely null alleles. Isolation of complete deletions of the *ced-3* locus should help resolve this issue.

Whatever the *ced-3* null phenotype, it is clear from our studies that semidominant mutations in *ced-3* can prevent programmed cell death. It is therefore possible that similar mutations in human caspases also result in cell survival. Such cell survival, as in the case of *bcl-2*, could promote tumor formation. Thus, not only recessive mutations, but also dominant mutations in human caspase genes might predispose carriers to the development of cancer.

We thank Linda Huang and members of the Horvitz laboratory for helpful comments about the manuscript and Gillian Stanfield and Michael Hengartner for isolating some of the *ced-3* alleles we studied. S.S. was supported by a William Keck Foundation fellowship and a fellowship from the Glaxo Research Institute. B.D. was supported by the MIT Undergraduate Research Opportunity Program (UROP) and by the Howard Hughes Medical Institute. P.W.R. was supported by a National Science Foundation fellowship and a National Institutes of Health training grant. H.R.H. is an Investigator of the Howard Hughes Medical Institute.

LITERATURE CITED

Alnemri, E. S., D. J. Livingston, D. W. Nicholson, G. Salvesen, N. A. Thornberry *et al.*, 1996 Human ICE/CED-3 protease nomenclature. Cell **87:** 171.

Anderson, P., 1995 Mutagenesis. Methods Cell Biol. **48:** 31–58.

Boldin, M. P., T. M. Goncharov, Y. V. Goltsev and D. Wallach, 1996 Involvement of MACH, a novel MORT1/FADD-interacting protease, in Fas/APO-1- and TNF receptor-induced cell death. Cell **85:** 803–815.

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

Cerretti, D. P., C. J. Kozlosky, B. Mosley, N. Nelson, K. V. Ness *et al.*, 1992 Molecular cloning of the interleukin-1β converting enzyme. Science **256:** 97–100.

Chalfie, M., Y. Tu, G. Euskirchen, W. W. Ward and D. C. Prasher,

1994 Green fluorescent protein as a marker for gene expression. Science **263:** 802–805.

Chinnaiyan, A. M., D. Chaudhary, K. O'Rourke, E. V. Koonin and V. M. Dixit, 1997 Role of CED-4 in the activation of CED-3. Nature **388:** 728–729.

Clark, D. V., T. M. Rogalski, L. M. Donati and D. L. Baillie, 1988 The *unc-22*(IV) region of *Caenorhabditis elegans*: genetic analysis of lethal mutations. Genetics **119:** 345–353.

Coulondre, C., and J. H. Miller, 1977 Genetic studies of the *lac* repressor. Mutagenic specificity in the *lacI* gene of *E. coli*. J. Mol. Biol. **117:** 577–606.

Ellis, H. M., and H. R. Horvitz, 1986 Genetic control of programmed cell death in the nematode *C. elegans*. Cell **44:** 817–829.

Ellis, R. E., D. Jacobson and H. R. Horvitz, 1991 Genes required for the engulfment of cell corpses during programmed cell death in *Caenorhabditis elegans*. Genetics **129:** 79–94.

Enari, M., R. V. Talanian, W. W. Wong and S. Nagata, 1996 Sequential activation of ICE-like and CPP32-like proteases during Fas-mediated apoptosis. Nature **380:** 723–726.

Faucheu, C., A. Diu, A. W. Chan, A. M. Blanchet, C. Miossec *et al.*, 1995 A novel human protease similar to the interleukin-1 beta converting enzyme induces apoptosis in transfected cells. EMBO J. **14:** 1914–1922.

Fernandes-Alnemri, T., G. Litwack and E. S. Alnemri, 1994 CPP32, a novel human apoptotic protein with homology to *Caenorhabditis elegans* cell death protein Ced-3 and mammalian interleukin-1 beta-converting enzyme. J. Biol. Chem. **269:** 30761–30764.

Fernandes-Alnemri, T., G. Litwack and E. S. Alnemri, 1995a *Mch2*, a new member of the apoptotic *Ced-3*/*Ice* cysteine protease gene family. Cancer Res. **55:** 2737–2742.

Fernandes-Alnemri, T., A. Takahashi, R. Armstrong, J. Krebs, L. Fritz *et al.*, 1995b Mch3, a novel human apoptotic cysteine protease highly related to CPP32. Cancer Res. **55:** 6045–6052.

Fernandes-Alnemri, T., R. C. Armstrong, J. Krebs, S. M. Srinivasula, L. Wang *et al.*, 1996 *In vitro* activation of CPP32 and Mch3 by Mch4, a novel human apoptotic cysteine protease containing two FADD-like domains. Proc. Natl. Acad. Sci. USA **93:** 7464–7469.

Fire, A., 1986 Integrative transformation of *Caenorhabditis elegans*. EMBO J. **5:** 2673–2680.

Fire, A., S. W. Harrison and D. Dixon, 1990 A modular set of *lacZ* fusion vectors for studying gene expression in *Caenorhabditis elegans*. Gene **93:** 189–198.

Fire, A., S. Xu, M. K. Montgomery, S. A. Kostas, S. E. Driver *et al.*, 1998 Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature **391:** 806–811.

Fraser, A. G., and G. I. Evan, 1997 Identification of a *Drosophila melanogaster* ICE/CED-3-related protease, drICE. EMBO J. **16:** 2805–2813.

Gagliardini, V., P. Fernandez, R. K. K. Lee, H. C. A. Drexler, R. J. Rotello *et al.*, 1994 Prevention of vertebrate neuronal death by the *crmA* gene. Science **263:** 826–828.

Hakem, R., A. Hakem, G. S. Duncan, J. T. Henderson, M. Woo *et al.*, 1998 Differential requirement for caspase 9 in apoptotic pathways in vivo. Cell **94:** 339–352.

Hay, B. A., T. Wolff and G. M. Rubin, 1994 Expression of baculovirus p35 prevents cell death in *Drosophila*. Development **120:** 2121–2129.

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

Hengartner, M. O., and H. R. Horvitz, 1994a C. elegans cell survival gene *ced-9* encodes a functional homolog of the mammalian proto-oncogene *bcl-2*. Cell **76:** 665–676.

Hengartner, M. O., and H. R. Horvitz, 1994b Activation of *C. elegans* cell death protein CED-9 by an amino-acid substitution in a domain conserved in Bcl-2. Nature **369:** 318–320.

Hengartner, M. O., R. E. Ellis and H. R. Horvitz, 1992 *Caenorhabditis elegans* gene *ced-9* protects cells from programmed cell death. Nature **356:** 494–499.

Heusel, J. W., R. L. Wesselschmidt, S. Shresta, J. H. Russell and T. J. Ley, 1994 Cytotoxic lymphocytes require granzyme B for the rapid induction of DNA fragmentation and apoptosis in allogeneic target cells. Cell **76:** 977–987.

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

Humke, E. W., J. Ni and V. M. Dixit, 1998 ERICE, a novel FLICE-activatable caspase. J. Biol. Chem. **273:** 15702–15707.

Inohara, N., T. Koseki, Y. Hu, S. Chen and G. Nunez, 1997 CLARP, a death effector domain-containing protein interacts with caspase-8 and regulates apoptosis. Proc. Natl. Acad. Sci. USA **94:** 10717–10722.

Kuida, K., J. A. Lippke, G. Ku, M. W. Harding, D. J. Livingston *et al.*, 1995 Altered cytokine export and apoptosis in mice deficient in interleukin-1 beta converting enzyme. Science **267:** 2000–2003.

Kuida, K., T. S. Zheng, S. Na, C. Kuan, D. Yang *et al.*, 1996 Decreased apoptosis in the brain and premature lethality in CPP32-deficient mice. Nature **384:** 368–372.

Kuida, K., T. F. Haydar, C.-Y. Kuan, Y. Gu, C. Taya *et al.*, 1998 Reduced apoptosis and cytochrome c-mediated caspase activation in mice lacking caspase 9. Cell **94:** 325–337.

Kumar, S., M. Kinoshita, M. Noda, N. G. Copeland and N. A. Jenkins, 1994 Induction of apoptosis by the mouse *Nedd-2* gene, which encodes a protein similar to the product of the *Caenorhabditis elegans* cell death gene *ced-3* and the mammalian IL-1β-converting enzyme. Genes Dev. **8:** 1613–1626.

Mello, C., and A. Fire, 1995 DNA transformation. Methods Cell Biol. **48:** 451–482.

Mello, C. C., J. M. Kramer, D. Stinchcomb and V. Ambros, 1991 Efficient gene transfer in *C. elegans*: extrachromosomal maintenance and integration of transforming sequences. EMBO J. **10:** 3959–3970.

Milligan, C. E., D. Prevette, H. Yaginuma, S. Homma, C. Cardwell *et al.*, 1995 Peptide inhibitors of the ICE protease family arrest programmed cell death of motoneurons in vivo and in vitro. Neuron **15:** 385–393.

Miura, M., H. Zhu, R. Rotello, E. A. Hartwieg and J. Yuan, 1993 Induction of apoptosis in fibroblasts by IL-1 beta-converting enzyme, a mammalian homolog of the C. elegans cell death gene *ced-3*. Cell **75:** 653–660.

Molineaux, S. M., F. J. Casano, A. M. Rolando, E. P. Peterson, G. Limjuco *et al.*, 1993 Interleukin 1 beta (IL-1 beta) processing in murine macrophages requires a structurally conserved homologue of human IL-1 beta converting enzyme. Proc. Natl. Acad. Sci. USA **90:** 1809–1813.

Munday, N. A., J. P. Vaillancourt, A. Ali, F. J. Casano, D. K. Miller *et al.*, 1995 Molecular cloning and pro-apoptotic activity of ICErelII and ICErelIII, members of the ICE/CED-3 family of cysteine proteases. J. Biol. Chem. **270:** 15870–15876.

Muzio, M., A. M. Chinnaiyan, F. C. Kischkel, K. O'Rourke, A. Shevchenko *et al.*, 1996 FLICE, a novel FADD homologous ICE/CED-3–like protease, is recruited to the CD95 (Fas/Apo-1) death-inducing signaling complex. Cell **85:** 817–827.

Nicholson, D. W., A. Ali, N. A. Thornberry, J. P. Vaillancourt, C. K. Ding *et al.*, 1995 Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. Nature **376:** 37–43.

Pulak, R., and P. Anderson, 1993 mRNA surveillance by the *Caenorhabditis elegans smg* genes. Genes Dev. **7:** 1885–1897.

Ray, C. A., R. A. Black, S. R. Kronheim, T. A. Greenstreet, P. R. Sleath *et al.*, 1992 Viral inhibition of inflammation: cowpox virus encodes an inhibitor of the interleukin-1β converting enzyme. Cell **69:** 597–604.

Rotonda, J., D. W. Nicholson, K. M. Fazil, M. Gallant, Y. Gareau *et al.*, 1996 The three-dimensional structure of apopain/CPP32, a key mediator of apoptosis. Nat. Struct. Biol. **3:** 619–625.

Sambrook, J., E. F. Fritsch and T. Maniatis, 1989 *Molecular Cloning: A Laboratory Manual*, Ed. 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Savage, C., M. Hamelin, J. G. Culloti, A. Coulson, D. G. Albertson *et al.*, 1989 *mec-7* is a beta tubulin gene required for the production of 15 protofilament microtubules in *Caenorhabditis elegans*. Genes Dev. **3:** 870–881.

Shaham, S., 1998 Identification of multiple *Caenorhabditis elegans* caspases and their potential roles in proteolytic cascades. J. Biol. Chem. **273:** 35109–35117.

Shaham, S., and H. R. Horvitz, 1996a Developing *Caenorhabditis elegans* neurons may contain both cell-death protective and killer activities. Genes Dev. **10:** 578–591.

Shaham, S., and H. R. Horvitz, 1996b An alternatively spliced C. elegans *ced-4* RNA encodes a novel cell-death inhibitor. Cell **86:** 201–208.

Shi, L., C. M. Kam, J. C. Powers, R. Aebersold and A. H. Greenberg, 1992a Purification of three cytotoxic lymphocyte granule serine proteases that induce apoptosis through distinct substrate and target cell interactions. J. Exp. Med. **176:** 1521–1529.

Shi, L., R. P. Kraut, R. Aebersold and A. H. Greenberg, 1992b A natural killer cell granule protein that induces DNA fragmentation and apoptosis. J. Exp. Med. **175:** 553–566.

Song, Z., K. McCall and H. Steller, 1997 DCP-1, a Drosophila cell death protease essential for development. Science **275:** 536–540.

Srinivasula, S. M., T. Fernandes-Alnemri, J. Zangrilli, N. Robertson, R. C. Armstrong *et al.*, 1996 The Ced-3/interleukin 1beta converting enzyme-like homolog Mch6 and the lamin-cleaving enzyme Mch2alpha are substrates for the apoptotic mediator CPP32. J. Biol. Chem. **271:** 27099–27106.

Sulston, J., and J. Hodgkin, 1988 Methods, pp. 587–606 in *The Nematode Caenorhabditis elegans*, edited by W. B. Wood and the Community of C. elegans Researchers. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Tewari, M., L. T. Quan, K. O'Rourke, S. Desnoyers, Z. Zeng *et al.*, 1995 Yama/CPP32β, a mammalian homolog of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly (ADP-Ribose) polymerase. Cell **81:** 801–809.

Thornberry, N. A., H. G. Bull, J. R. Calaycay, K. T. Chapman, A. D. Howard *et al.*, 1992 A novel heterodimeric cysteine protease is required for interleukin-1β processing in monocytes. Nature **356:** 768–774.

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

Van de Craen, M., P. Vandenabeele, W. Declercq, I. Van den Brande, G. Van Loo *et al.*, 1997 Characterization of seven murine caspase family members. FEBS Lett. **403:** 61–69.

Walker, N. P., R. V. Talanian, K. D. Brady, L. C. Dang, N. J. Bump *et al.*, 1994 Crystal structure of the cysteine protease interleukin-1 beta-converting enzyme: a $(p20/p10)_2$ homodimer. Cell **78:** 343–352.

Wang, L., M. Miura, L. Bergeron, H. Zhu and J. Yuan, 1994 Ich-1, an *Ice/ced-3*-related gene, encodes both positive and negative regulators of programmed cell death. Cell **78:** 739–750.

Wang, S., M. Miura, Y. K. Jung, H. Zhu, V. Gagliardini *et al.*, 1996 Identification and characterization of Ich-3, a member of the interleukin-1beta converting enzyme (ICE)/Ced-3 family and an upstream regulator of ICE. J. Biol. Chem. **271:** 20580–20587.

Way, J. C., and M. Chalfie, 1988 *mec-3*, a homeobox-containing gene that specifies differentiation of the touch receptor neurons in *C. elegans*. Cell **54:** 5–16.

Wilson, K. P., J. A. Black, J. A. Thomson, E. E. Kim, J. P. Griffith *et al.*, 1994 Structure and mechanism of interleukin-1 beta converting enzyme. Nature **370:** 270–275.

Xue, D., and H. R. Horvitz, 1995 Inhibition of the *Caenorhabditis elegans* cell-death protease CED-3 by a CED-3 cleavage site in baculovirus p35 protein. Nature **377:** 248–251.

Xue, D., S. Shaham and H. R. Horvitz, 1996 The *Caenorhabditis elegans* cell-death protein CED-3 is a cysteine protease with substrate specificities similar to those of the human CPP32 protease. Genes Dev. **10:** 1073–1083.

Yuan, J., and H. R. Horvitz, 1990 Genetic mosaic analyses of *ced-3* and *ced-4*, two genes that control programmed cell death in the nematode *C. elegans*. Dev. Biol. **138:** 33–41.

Yuan, J., S. Shaham, S. Ledoux, H. M. Ellis and H. R. Horvitz, 1993 The C. elegans cell death gene *ced-3* encodes a protein similar to mammalian interleukin-1β-converting enzyme. Cell **75:** 641–652.

Zou, H., W. J. Henzel, X. Liu, A. Lutschg and X. Wang, 1997 Apaf-1, a human protein homologous to C. elegans CED-4, participates in cytochrome c-dependent activation of caspase-3. Cell **90:** 405–413.

Communicating editor: R. K. Herman