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C. elegans Cell Survival Gene *ced-9* Encodes a Functional Homolog of the Mammalian Proto-Oncogene *bcl-2*

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Summary

The activity of the C. elegans gene *ced-9* is required to protect cells that normally survive from undergoing programmed cell death. Here we describe the cloning and molecular characterization of this gene. *ced-9* is an element of a polycistronic locus that also contains the gene *cyt-1*, which encodes a protein similar to cytochrome b_{560} of complex II of the mitochondrial respiratory chain. *ced-9* encodes a 280 amino acid protein showing sequence and structural similarities to the mammalian proto-oncogene *bcl-2*. Overexpression of *bcl-2* can mimic the protective effect of *ced-9* on C. elegans cell death and can prevent the ectopic cell deaths that occur in *ced-9* loss-of-function mutants. These results suggest that *ced-9* and *bcl-2* are homologs and that the molecular mechanism of programmed cell death has been conserved from nematodes to mammals.

Introduction

Programmed cell death plays an important role in animal development and homeostasis and occurs in a wide variety of tissues in both vertebrates and invertebrates (Glücksmann, 1950; Cohen, 1991; Ellis et al., 1991; Raff, 1992). In many tissues, cell death and cell proliferation are precisely balanced to maintain the proper number and types of cells, and disruption of this balance can result in disease (reviewed by Williams, 1991).

In the nematode Caenorhabditis elegans, 131 of the 1090 somatic cells generated during hermaphrodite development undergo programmed cell death (Sulston and Horvitz, 1977; Sulston et al., 1983). Genetic studies have led to the identification of 14 genes that are involved at various steps of this process; these genes can be placed into a genetic pathway for programmed cell death in C. elegans (reviewed by Ellis et al., 1991; Driscoll, 1992). Three of these genes are involved in the regulation and execution of all 131 deaths. The activities of two of these three genes, *ced-3* and *ced-4* (called *ced* for cell death abnormal), are required for cells to die (Ellis and Horvitz, 1986). In *ced-3* or *ced-4* mutants, essentially all cells that usually die instead survive, differentiate, and (in at least some cases) properly function (Ellis and Horvitz, 1986; Avery and Horvitz, 1987; White et al., 1991). Genetic mosaic analyses suggest that these two genes must be expressed by the cells scheduled to undergo programmed death for these cells to die (Yuan and Horvitz, 1990). The *ced-4* gene encodes a protein with no significant sequence similarity to any other protein in the data bases (Yuan and Horvitz, 1992). The *ced-3* gene encodes a homolog of the mammalian interleukin-1β-converting enzyme (Yuan et al., 1993), which suggests that the CED-3 protein acts as a protease to cause programmed cell death.

The third gene involved in the control of all programmed cell deaths, *ced-9*, negatively regulates the pathway for programmed cell death: a *ced-9* gain-of-function mutation prevents the deaths of cells that normally die, while mutations that inactivate *ced-9* cause cells that normally live to undergo programmed cell death (Hengartner et al., 1992). Thus, the function of *ced-9* is to prevent cells that normally survive from undergoing programmed cell death. The absence of *ced-9* function results in maternal-effect lethality, indicating that *ced-9* function is essential for C. elegans development.

The proto-oncogene *bcl-2* appears to function in mammals as *ced-9* functions in nematodes. *bcl-2* was discovered and molecularly cloned based on its involvement in a t(14;18) translocation that is observed in the majority of follicular lymphomas diagnosed in the United States (Fukuhara et al., 1979; Yunis et al., 1982). This translocation fuses the *bcl-2* locus to the immunoglobulin heavy chain gene, resulting in the overexpression of normal Bcl-2 protein in B cells (Tsujimoto et al., 1984; Bakhshi et al., 1985; Cleary et al., 1986; Tsujimoto and Croce, 1986; Seto et al., 1988). Overexpression of *bcl-2* prevents or delays significantly the programmed cell death (apoptosis) of a large variety of cells under various conditions that usually lead to cell death. For example, *bcl-2* protects interleukin-dependent lymphoid cell lines from apoptosis induced by interleukin withdrawal (Vaux et al., 1988; Nuñez et al., 1990) and thymocytes from apoptosis induced by glucocorticoids or by γ-irradiation (Sentman et al., 1991; Strasser et al., 1991). *bcl-2* also can protect neurons from apoptosis induced by trophic factor withdrawal (García et al., 1992; Allsopp et al., 1993; Batistatou et al., 1993) and can prevent apoptosis induced by c-*myc* in Rat-1 cells (Fanidi et al., 1992) and CHO cells (Bissonette et al., 1992). In tissues characterized by cell turnover, *bcl-2* is often expressed in progenitor or long-lived cells (Hockenbery et al., 1991). Moreover, signals that rescue lymph node germinal center cells from susceptibility to apoptosis induce *bcl-2* expression (Liu et al., 1991). Based on these observations, it has been suggested that *bcl-2* expression protects cells that should survive from apoptosis (Hockenbery et al., 1991).

Recently, a number of genes with some sequence similarity to *bcl-2* have been reported (Boise et al., 1993; Kozopas et al., 1993; Lin et al., 1993; Oltvai et al., 1993; reviewed by Williams and Smith, 1993). Two of these genes, *bax* and *bcl-x*, have effects on the regulation of apoptosis. The relatively low sequence similarities (30%–50% identity between various members) among these *bcl-2* homologs suggest that this gene family might be of ancient origin and not restricted to vertebrates.

Here we report the molecular characterization of the C.

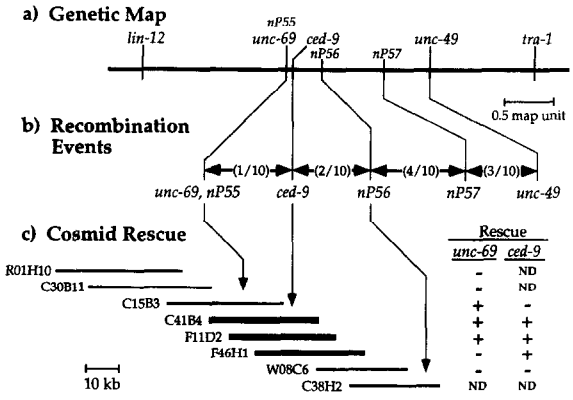


Figure 1. Genetic, Physical, and Functional Maps of the *ced-9* Region

(a) Genetic map. Relevant genes as well as the approximate positions of the N2/RC301 RFLPs *nP55*, *nP56*, and *nP57* used to map *ced-9* are shown.

(b) Number of recombination events observed between various markers in the *unc-69–unc-49* interval. Ten Unc-49 non-Unc-69 recombinants from *unc-69 ced-9 unc-49/+++* parents were analyzed for their genotypes for the genetic markers in this region; the intervals in which the ten recombination events occurred are indicated (see Experimental Procedures). We did not separate the *nP55* RFLP from *unc-69* in these experiments, suggesting that *unc-69* is to the right or close to the left of *nP55*.

(c) Cosmid rescue of the *unc-69* and *ced-9* mutant phenotypes. Cosmids situated between the *nP55* and *nP56* RFLPs (recognized by cosmids C15B3 and C38H2, respectively) were injected into *unc-69* or *unc-69 ced-9/++* animals. Established transgenic lines were scored for rescue of the *unc-69* (uncoordinated, coiled) and *ced-9* (sterile, maternal-effect lethal) phenotypes. Plus inducates rescue, minus, no rescue. ND, not determined.

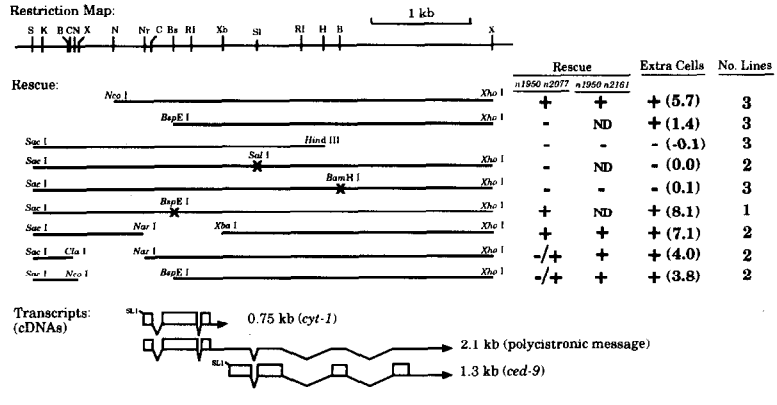
elegans *ced-9* gene. Analysis of *ced-9* genomic structure and transcripts suggests that *ced-9* might be an element of a complex genetic locus, sharing a promoter with an upstream gene encoding a mitochondrial cytochrome, so that at least some *ced-9* transcripts arise through the processing of a polycistronic message. We show that the CED-9 protein shows significant sequence and structural similarity to mammalian Bcl-2. We demonstrate that overexpression of either *ced-9* or human *bcl-2* prevents the normal cell deaths that occur during C. elegans development. We also show that *bcl-2* can rescue the *ced-9(lf)* (loss-of-function) mutant phenotype and can substitute for *ced-9* in preventing the deaths of cells that should normally survive. These results suggest that *bcl-2* is a functional homolog of *ced-9* and that the molecular mechanism responsible for programmed cell death has been conserved among species as diverse as nematodes and mammals.

Results

Cloning of *ced-9*

We previously reported that *ced-9* maps to the right arm of chromosome III, approximately 0.05 map units right of *unc-69* (Hengartner et al., 1992). This position places *ced-9* between the two cloned genes *lin-12* and *tra-1* (Figure 1a). The interval between these two genes, corresponding to approximately 2 Mb, was cloned by the C. elegans physical mapping project (Coulson et al., 1986, 1988, 1991). To define better the region containing *ced-9*, we mapped *ced-9* with respect to a series of restriction fragment length polymorphisms (RFLPs) that we identified between RC301, a strain isolated from the wild near Freiburg in the Federal Republic of Germany (R. Cassada, personal communication), and the common wild-type laboratory strain N2 (data not shown).

Mapping *ced-9* with respect to these RFLPs localized the gene to a roughly 60 kb interval between the RFLPs *nP55* and *nP56* (Figure 1b). We tested cosmids from this region for their abilities to rescue *ced-9(lf)* mutants: individual cosmids were used to generate transgenic lines through germline transformation (Mello et al., 1991), and transgenic *ced-9(lf)* animals were then tested for fertility and viability. Three overlapping cosmids were found to be able to rescue the *ced-9* mutant phenotype (Figure 1c).



Rescue: n1950 n2077	Rescue: n1950 n2161	Extra Cells	No. Lines
+	+	+ (5.7)	3
-	ND	+ (1.4)	3
-	-	- (-0.1)	3
-	ND	- (0.0)	2
-	-	- (0.1)	3
+	ND	+ (8.1)	1
+	+	+ (7.1)	2
-/+	+	+ (4.0)	2
-/+	+	+ (3.8)	2

Figure 2. The *ced-9* Locus

(Top) Restriction map of the *ced-9* region. Restriction sites shown: B, BamHI; Bs, BspMII; C, ClaI; H, HindIII; K, KpnI; N, NcoI; Nr, NarI; RI, EcoRI; S, SacI; SI, SalI; X, XhoI; Xb, XbaI. (Middle) Rescue of *ced-9* mutant phenotypes. Transgenic lines of various subclones from the *ced-9* region were tested for their abilities to rescue the maternal-effect lethality caused by the *ced-9(lf)* mutations *n1950 n2077* and *n1950 n2161*. An X indicates a frameshift mutation (see Experimental Procedures for details). Rescue results: plus, transgenic *ced-9(lf)* animals segregated viable and fertile progeny; minus, transgenic *ced-9(lf)* animals segregated no viable progeny; plus/minus, weak rescue with transgenic *ced-9(lf)* animals segregating a few viable, but mostly sterile, progeny; ND, not determined. Extra cells: animals of genotype *qC1/unc-69(e587) ced-9(n1950 n2077);nEx* from transgenic lines were scored for the presence of extra surviving cells in the anterior pharynx. The average scores for all lines analyzed are shown in parentheses. For comparison, wild-type animals show on average 0.05 extra cells and *ced-3* mutants show 14.2 extra cells in this region (see Figure 8). No. lines: number of lines analyzed for each construct.

(Bottom) Intron/exon structure of the transcripts from the *ced-9* region. Symbols: ORFs, boxes; untranslated regions, horizontal lines; direction of transcription, arrows. Both the 0.75 and 1.3 kb cDNAs sequenced showed an SL1 splice leader at their 5′ ends (SL1). The structure shown was deduced from the alignment of genomic and cDNA sequences.

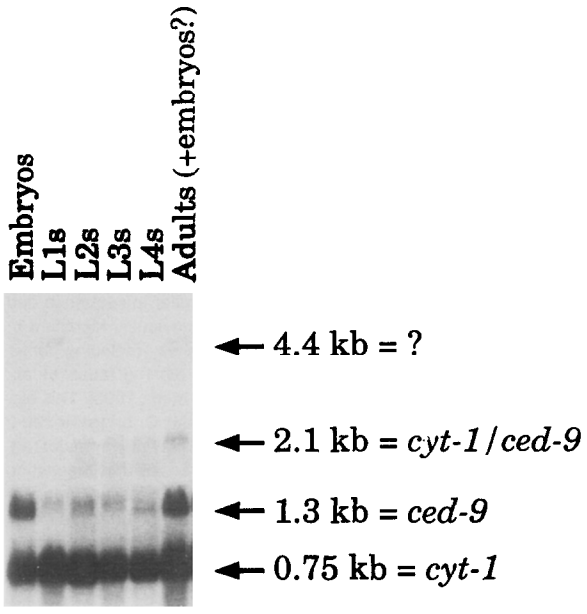


Figure 3. Transcripts from the *ced-9* Region

Phosphorimage of a poly(A)$^+$ developmental Northern blot probed with the hybrid 2.1 kb cDNA. Note that the adults used also contained embryos in utero. L1s to L4s, first through fourth larval stages, respectively.

By testing various subclones from the region common to these three cosmids, we identified a 3.7 kb NcoI–XhoI fragment (Figure 2) that efficiently rescued both the sterility and the maternal-effect lethality of *ced-9(n1950 n2077)* mutants. Further deletions into this fragment from either the right or the left abolished or greatly diminished the rescuing activity of the fragment (Figure 2).

The *ced-9* Region Encodes Four Transcripts

To identify potential *ced-9* transcripts, we used various DNA fragments from the rescuing region to probe Northern blots. Hybridization of a developmental Northern blot to a 4.2 kb ClaI–XhoI rescuing fragment revealed four distinct transcripts (Figure 3). The most abundant transcript, about 0.75 kb in size, was expressed at a constant level throughout development (based upon levels of expression of the actin gene *act-1* [Files et al., 1983], which was used as a control; data not shown). A transcript of about 1.3 kb was highly enriched in embryos. This transcript is also more abundant in adults, although we do not know whether adults indeed have higher levels of the 1.3 kb transcript or whether the observed enrichment is a result of contamination of the adult population by embryos in utero. Finally, we detected two relatively rare transcripts of 2.1 kb and 4.4 kb, the expression of which varied little throughout development.

We also used the rescuing 4.2 kb ClaI–XhoI fragment to probe a C. elegans cDNA library. Three distinct classes of cDNAs were isolated, corresponding in size to the 0.75 kb, 1.3 kb, and 2.1 kb transcripts detected on Northern blots, which we will hereafter also refer to as the upstream, downstream, and polycistronic transcripts, respectively (see below). We determined the sequence of one cDNA of each class as well as of the entire rescuing 4.2 kb genomic fragment. Both the 0.75 kb and 1.3 kb cDNAs had an SL1 trans-spliced leader (Krause and Hirsh, 1987) at their 5′ ends, suggesting that we had isolated full-length cDNAs. The deduced intron/exon structures of the three classes of cDNAs are shown in Figures 2 and 4. The three cDNA classes are related to each other in an unusual way. The 0.75 kb upstream and 1.3 kb downstream transcripts do not share any common sequence and are predicted to encode two completely distinct proteins. However, the genomic regions encoding these transcripts are in very close physical proximity: the polyadenylation site of the 0.75 kb upstream transcript overlaps with the SL1 splice acceptor site (5′ end) of the downstream 1.3 kb transcript (Figure 4). Therefore, only 1 or 2 nt (Figure 4) separate the genomic regions that encode these two transcripts. The longer 2.1 kb transcript contains both the upstream and downstream transcripts: the 2.1 kb transcript has the same 5′ end as the 0.75 kb transcript and uses the same polyadenylation site as the 1.3 kb transcript. Thus, the 2.1 kb transcript contains the open reading frames (ORFs) of both the 0.75 and 1.3 kb transcripts but is expected to direct expression of only the first (upstream) protein, since the two ORFs are separated by several stop codons and are in different reading frames.

We confirmed the existence of the polycistronic upstream–downstream transcript by performing reverse transcriptase–polymerase chain reaction (RT–PCR) experiments using total C. elegans RNA and primers flanking the upstream/downstream junction. As expected, we could amplify a fragment of DNA corresponding in size to the expected fusion product (data not shown). Substitution of the upstream primer with either SL1 or SL2 splice leader primers confirmed that most of the downstream message was trans-spliced to SL1. Quantification of the intensity of the bands obtained with the SL1 and SL2 primers suggested that the SL1-spliced form of the downstream transcript is at least 20 times more abundant than the SL2-spliced form (data not shown).

We confirmed that the 0.75 kb, 1.3 kb, and 2.1 kb transcripts previously identified on Northern blots correspond to the three classes of cDNAs isolated by probing separate Northern blots using either the 0.75 kb or the 1.3 kb cDNA as a probe. As expected, the 0.75 kb probe detected the 0.75 kb and 2.1 kb transcripts but not the 1.3 kb transcript, while the 1.3 kb probe detected the 1.3 kb and 2.1 kb transcripts but not the 0.75 kb transcript (data not shown). We have not yet determined the nature of the 4.4 kb transcript.

Identification of the *ced-9* Transcript

To determine which of the two identified ORFs corresponds to the *ced-9* gene, we introduced frameshift mutations into each ORF and tested the resulting constructs for their abilities to rescue the sterility and lethality of *ced-9(lf)* mutants. Both mutations introduced into the downstream ORF abolished the rescuing activity of the construct; by contrast, a mutation in the upstream ORF had no effect (see Figure 2). Moreover, deletion of the entire upstream

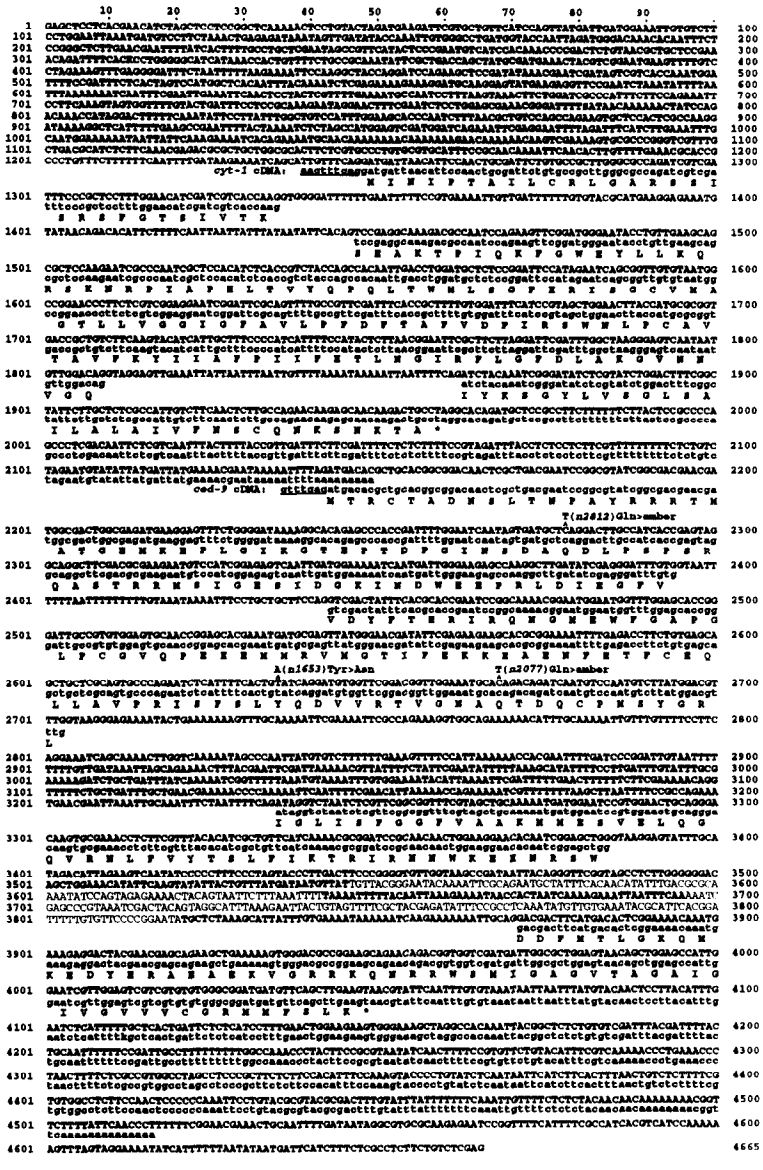


Figure 4. Sequence of the *ced-9* Locus

Sequence of the rescuing SacI–XhoI 4.65 kb fragment. The sequences of the cDNAs of the upstream *cyt-1* and downstream *ced-9* transcripts are aligned with the genomic sequence and shown in lowercase letters. Both sequenced cDNAs have SL1 spliced leader sequences at their 5′ end (underlined). The nucleotide changes observed in three *ced-9* mutants are indicated. The second intron of *ced-9* contains an imperfect inverted repeat (shown in lighter type; residues 3546–3641 and 3696–3820). This repeat is similar in sequence and structure to elements previously identified in various C. elegans genes, including *fem-1* (Spence et al., 1990), *hlh-1* (Krause et al., 1990), and *ced-3* (Yuan et al., 1993). This element is not present in the C. briggsae *ced-9* gene, arguing against its playing any major regulatory role in *ced-9* expression and suggesting that its appearance in the C. elegans gene (or its disappearance from the C. briggsae gene) is recent, possibly as a result of transposition.

ORF except for the first 13 residues (the deletion extended from the NarI site to the XbaI site) did not prevent rescue of *ced-9* mutants.

To confirm these observations, we also determined the sequences of several *ced-9* mutants. We found changes in the downstream ORF in three distinct *ced-9(lf)* mutants (Figure 4). For example, the strong *ced-9* mutation *n2077*, which we previously identified as a potential null allele (Hengartner et al., 1992), was associated with a C to T transition at position 2668, resulting in a premature amber stop codon at position 160 of the downstream ORF. Consistent with this observation, we have found that the amber suppressor mutation *sup-7(st5)X* weakly suppressed the sterility (but not the lethality) caused by *ced-9(n1950 n2077)* mutations (M. O. H. and H. R. H., unpublished data). These results strongly suggest that *ced-9* corresponds to the downstream ORF, which can encode a protein of 280 amino acids and about 32 kd.

The Upstream ORF Encodes a Mitochondrial Protein

Comparison of the sequence of the protein predicted to be encoded by the upstream ORF with the sequences in available protein data bases revealed the upstream protein to be similar to a number of previously identified proteins (Figure 5), including bovine cytochrome b_{560} (Yu et al., 1992) (30% identity over the entire length of the protein), the product of the Saccharomyces cerevisiae *YKL4* gene (Abraham et al., 1992) (29% identity), the hypothetical product of *ORF137* in the mitochondrial genome of the liverwort Merchantia polymorpha (Oda et al., 1992) (29%), and the *sdhC* gene of Escherichia coli (Wood et al., 1984) (19%). Cytochrome b_{560} is an inner mitochondrial membrane protein involved in system II (succinate dehydrogenase) of the mitochondrial electron transport chain, which is responsible for transferring electrons from succinate to ubiquinone (coenzyme Q) (Lehninger, 1982; Stryer, 1988).

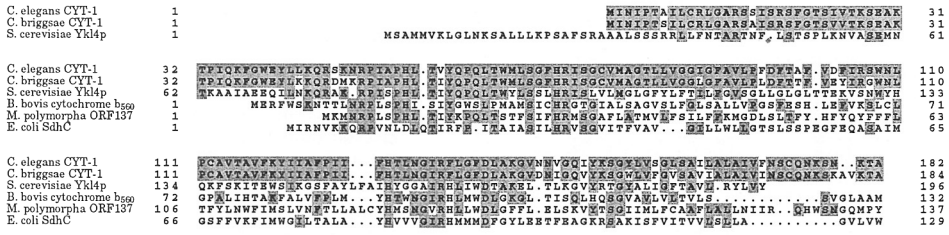


Figure 5. The Upstream ORF Encodes a Presumptive C. elegans Cytochrome b_{560}

Alignment of the C. elegans and C. briggsae CYT-1 sequences with those of bovine cytochrome b_{560} (Yu et al., 1992) and the products of the S. cerevisiae *YKL4* (Abraham et al., 1992), liverwort mitochondrial *ORF137* (Oda et al., 1992), and E. coli *sdhC* (Wood et al., 1984) genes. Residues identical to those of the C. elegans protein are stippled. Gaps made in individual protein sequences to optimize the alignments are indicated by elipses.

The E. coli *sdhC* gene also encodes a subunit of succinate dehydrogenase. The S. cerevisiae gene *YKL4* probably encodes the yeast cytochrome b_{560}, since *YKL4* is required for normal mitochondrial respiratory function (Abraham et al., 1992). Because these results suggest that the upstream ORF encodes a C. elegans mitochondrial cytochrome b_{560} homolog, we named this gene *cyt-1* (for cytochrome).

Regulation of *ced-9* Expression

While the *cyt-1* ORF is not required for rescue of *ced-9 (n1950 n2077)* mutants, deletion of a segment further upstream substantially diminished rescuing activity (see Figure 2, middle) suggesting that this region contains an element important for *ced-9* expression. One possibility is that this region contains an enhancer involved in *ced-9* expression. An alternative is that *ced-9* and *cyt-1* share a common promoter. This latter model, which would also explain our detection of bicistronic transcripts containing both ORFs, predicts that at least some *ced-9* transcripts arise from a 2.1 kb bicistronic message that is trans-spliced at the *ced-9* 5′ end to the SL1 splice leader to generate the mature *cyt-1* and *ced-9* transcripts (Figure 6).

CED-9 Is Homologous to Bcl-2

A search of protein data bases with the predicted CED-9 protein sequence revealed that the known protein most similar to CED-9 is the product of the mammalian proto-oncogene *bcl-2*: the CED-9 and human Bcl-2 proteins show 23% identity over their entire sequences (Figure 7a). Interestingly, the proposed function of the *bcl-2* oncogene is equivalent to the function we proposed for *ced-9* (Hengartner et al., 1992): *bcl-2* is thought to be required to prevent cells from undergoing programmed cell death (reviewed by Korsmeyer, 1992). These similarities in sequence and biological function suggest that *bcl-2* could be a vertebrate homolog of *ced-9*.

The low overall degree of similarity between *ced-9* and *bcl-2* prompted us to seek further evidence that these two proteins are homologous. We found that *ced-9* and *bcl-2* share a number of additional features. First, the C-terminal halves of the two proteins show similar Kyte–Doolittle hydrophobicity plots (data not shown), including the hydrophobic tail (Figure 7a), thought to be important for *bcl-2* localization, function, or both (Alnemri et al., 1992; Tanaka et al., 1992; Hockenbery et al., 1993). Second, the last *ced-9* intron is in the same position of the ORF as are the last introns of the human, mouse, and chicken *bcl-2* genes (Negrini et al., 1987; Seto et al., 1988; Eguchi et al., 1992), as well as the human *bcl-x* (Boise et al., 1993) and *bax* (Oltvai et al., 1993) genes (Figure 7a).

Caenorhabditis briggsae *ced-9*

If *ced-9* and *bcl-2* are homologs, then the residues that are conserved between these two genes are likely also to be conserved between C. elegans *ced-9* and the *ced-9* gene of a species more closely related than vertebrates. Furthermore, we would expect the regions found to be more conserved among various mammalian *bcl-2* genes also to be more conserved between *ced-9* and its closer homologs. To test these predictions, we cloned and determined the sequence of the *ced-9* gene from the nematode C. briggsae. The genomic structure of the C. briggsae

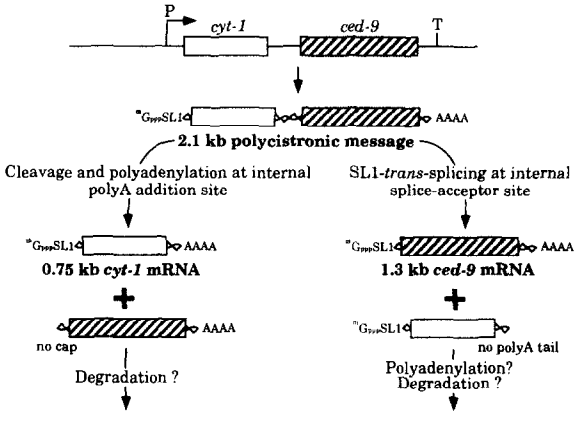


Figure 6. Model for the Generation of Mature *ced-9* and *cyt-1* mRNAs

In this model, *ced-9* and *cyt-1* are part of a complex locus and share a promoter. The mature *ced-9* transcript is obtained by SL1 trans-splicing from a bicistronic message. Alternatively, cleavage at the *cyt-1* polyadenylation site destroys the *ced-9* splice acceptor site and leaves the *ced-9* message uncapped and susceptible to degradation. The *cyt-1* transcript might be obtained either from the bicistronic message (as shown) or directly through early termination of transcription. P, promoter; T, transcription terminator; mGppp, methyl-guanosine cap; AAAA, poly(A) tail.

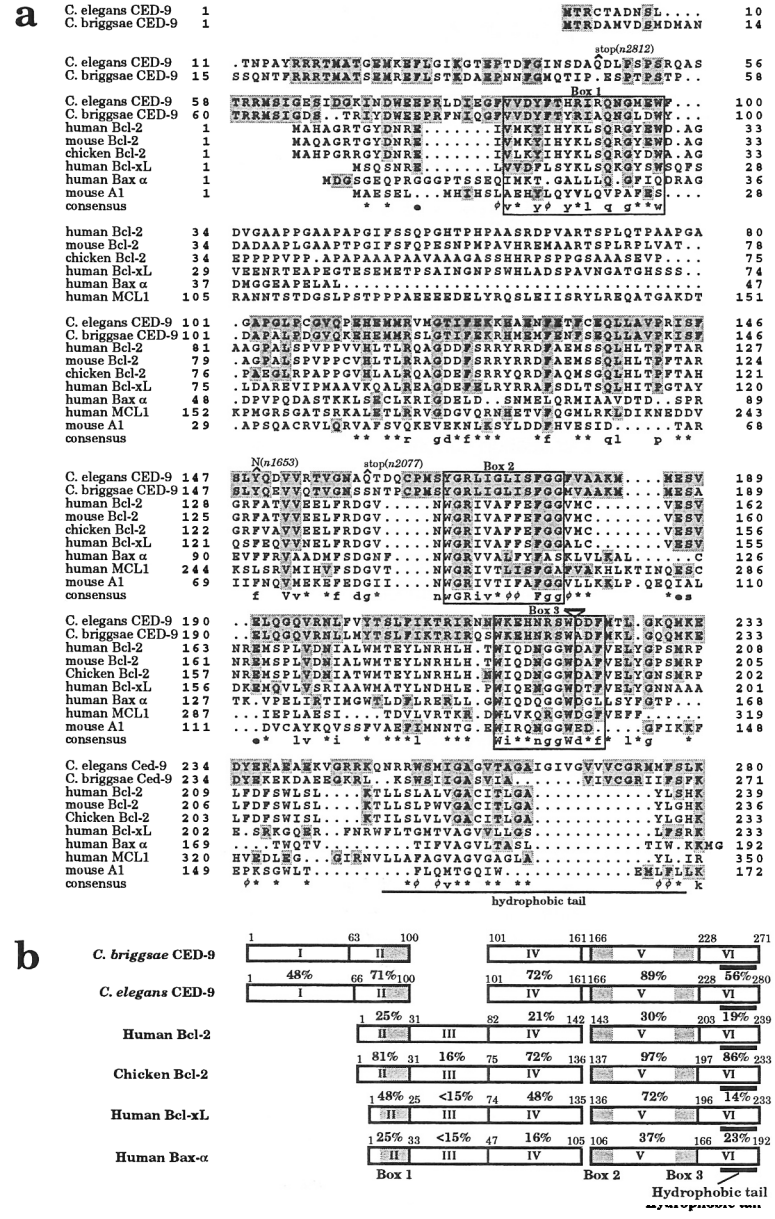


Figure 7. CED-9 and Bcl-2 Proteins Are Homologous

(a) Alignment of the C. elegans and C. briggsae CED-9 proteins with human (Cleary et al., 1986), murine (Negrini et al., 1987), and chicken (Eguchi et al., 1992) Bcl-2; human Bcl-xL (Boise et al., 1993), Bax-α (Oltvai et al., 1993), and MCL1 (Kozopas et al., 1993); and mouse A1 (Lin et al., 1993). Residues identical to those of the C. elegans protein are stippled. Gaps made in individual protein sequences to optimize the alignment are indicated by elipses. Consensus line: capital letter, 9 of 9 residues at this position are identical; lowercase letter, at least 6 of 9 identities; asterisk, at least 6 of 9 similar (for this purpose, the following sets of amino acids are considered similar: G, A, C, S, T; E, D, Q, N; R, K, H; V, M, L, I; F, Y, W); Φ, at least 6 of 9 hydrophobic (last two groups in the previous list). The position of the conserved last intron is indicated by a closed bar and a triangle above the line. (The positions of introns in MCL1 and A1 have not been reported.) Deduced amino acid changes caused by the *ced-9* mutations *n1653*, *n2077*, and *n2812* are indicated above the line. Boxes identify three conserved stretches. The conserved C-terminal hydrophobic tail is indicated by a line. Only the second half of the MCL1 protein sequence is shown, as no significant similarity could be detected in the first half.

(b) Extent of identities among CED-9, Bcl-2, Bcl-xL, and Bax. The proteins, aligned as in (a), have been divided somewhat arbitrarily into regions, and the percent identities in each region between adjacent proteins are indicated. Small numbers above the bars refer to amino acid residue numbers. Positions of the three conserved boxes (stippling) and the hydrophobic tail (underlined) are indicated.

ced-9 gene is similar to that of C. elegans *ced-9*: the two genes have identical intron–exon patterns, and the C. briggsae *cyt-1* gene (see Figure 5 for the protein sequence) is located just upstream of the C. briggsae *ced-9* gene in an arrangement very similar to that in C. elegans (data not shown).

Conserved Regions between CED-9 and Bcl-2

The predicted C. briggsae CED-9 protein is 66% identical to C. elegans CED-9 and is as similar to the mammalian Bcl-2 proteins as is C. elegans CED-9 (Figure 7a). The C. elegans and C. briggsae proteins are most conserved in the region most similar between CED-9 and Bcl-2 (region V in Figure 7b). The similarity (allowing for conservative substitutions) is particularly striking in two stretches of residues in this region (Figure 7, boxes 2 and 3). These regions are also those most conserved among the vertebrate members of the *bcl-2* family (Cleary et al., 1986; Negrini et al., 1987; Eguchi et al., 1992; Boise et al., 1993; Kozopas et al., 1993; Lin et al., 1993; Oltvai et al., 1993; reviewed by Williams and Smith, 1993). The level of conservation in the first half of the protein appears more limited. A short conserved stretch of residues (Figure 7a, box 1) can be found in most, but not all, members of the family. The particularly high degree of conservation in these "boxes" suggests that these regions play crucial roles in the structure or function (or both) of these proteins.

Overexpression of Wild-Type *ced-9* Prevents Cell Death

We noted during our germline transformation experiments that the genomic fragments that could rescue *ced-9(lf)* mutants not only prevented the ectopic programmed cell deaths normally observed in these mutants (Hengartner

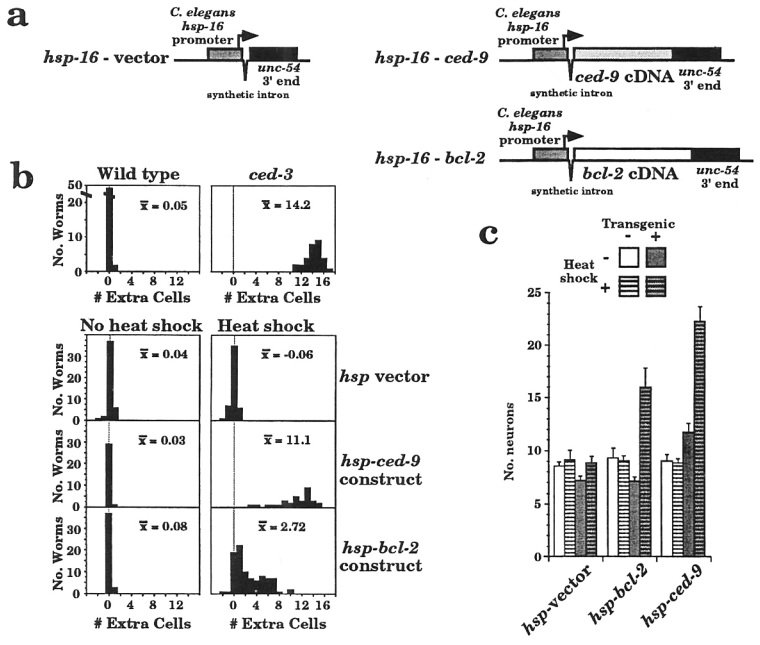


Figure 8. Overexpression of *ced-9* or *bcl-2* Prevents Cell Death in C. elegans

(a) Structures of the heat shock constructs used. Human *bcl-2* or C. elegans *ced-9* cDNAs were placed under the control of a C. elegans *hsp16* promoter (Perry et al., 1993).

(b) Number of extra cells observed in the anterior pharynx of animals transgenic for various *hsp* constructs: no heat shock, control animals raised at 20°C; heat shock, animals subjected to a 45 min heat shock during embryogenesis (see Experimental Procedures). For reference, the number of extra cells observed in nontransgenic, nonheat-shocked wild type (no extra cells; x = 0.05) and *ced-3(n2433)* (no cell death; x = 14.2) are shown.

(c) Number of neuronal cells in the posterior–ventral nerve cord (between the vulva and the preanal ganglion) in *ced-9(n1950 n2077)* L3 and L4 larvae transgenic (stippled bars) for the three *hsp* constructs under control conditions (nonheat shock, open bars) or following a 45 min heat shock during the L1 stage (lined bars). The results obtained for nontransgenic siblings of the transgenic animals are indicated by open bars (the extrachromosomal arrays used are sometimes lost during meiosis, resulting in the generation of both transgenic and nontransgenic progeny from a transgenic mother). Values shown are averages ± SEM for at least ten animals. Similar results were obtained in the anterior–ventral cord (data not shown).

et al., 1992) but also prevented many of the cell deaths that occur during normal development. To quantitate this effect, we determined the number of surviving cells in the anterior pharynx. In wild-type animals there are 49 cell nuclei in the anterior pharynx, whereas in *ced-3* and *ced-4* animals there are 12–14 additional nuclei (Hengartner et al., 1992). These extra nuclei correspond exactly in position as well as in number to the cells that fail to die in *ced-3* and *ced-4* mutants. Transgenic animals carrying *ced-9* extrachromosomal arrays showed a smaller but significant number (one to eight) of additional cells in this region (see Figure 2, middle). Different transgenic lines showed this effect to different degrees (data not shown), which might be a consequence of different levels of overexpression of the CED-9 protein.

To confirm that these extra cells resulted from overexpression of the CED-9 protein, we put a full-length *ced-9* cDNA under the control of a C. elegans heat shock promoter (Figure 8a). Under normal conditions, animals transgenic for this construct and nontransgenic control animals showed no extra cell survival. Following a heat shock pulse, however, we observed an average of 11.1 ± 0.5 extra cells in transgenic but not in control animals (Figure 8b). These results indicate that overexpression of wild-type CED-9 protein is sufficient to prevent the programmed cell deaths that occur during normal development.

bcl-2 Prevents Normal Programmed Cell Deaths in C. elegans

Overexpression of *bcl-2* prevents the programmed cell deaths of a variety of mammalian cells (Korsmeyer, 1992). We reasoned that if *bcl-2* is the mammalian homolog of *ced-9*, this mammalian gene might substitute for *ced-9* and therefore also prevent programmed cell deaths in C. elegans. To test this hypothesis, we constructed an *hsp–bcl-2* construct analogous to the *hsp–ced-9* construct mentioned above. Animals transgenic for this *hsp–bcl-2* construct showed a significant number (2.7 ± 0.3) of additional cells following heat shock (Figure 8b). Therefore, like *ced-9*, *bcl-2* can prevent, albeit less efficiently, the cell deaths that occur during normal C. elegans development. These results confirm and extend similar previous observations of Vaux et al. (1992), as we have directly observed extra surviving cells rather than inferring their presence from the absence of cell corpses.

bcl-2 Prevents the Ectopic Cell Deaths in *ced-9(lf)* Mutants

The observation that *bcl-2* can prevent the normal programmed cell deaths that occur during C. elegans development indicates that *bcl-2* can interact with the C. elegans cell death pathway, but gives no information as to where in the pathway this interaction occurs. The sequence similarity between *ced-9* and *bcl-2* suggests that *bcl-2* acts at the same step as *ced-9* normally acts, i.e., that *bcl-2* mimics the effect of *ced-9* on C. elegans programmed cell death. If so, then *bcl-2* might be able to substitute for *ced-9*. To test this hypothesis, we determined whether the *bcl-2* transgene could prevent the ectopic programmed cell deaths observed in mutants deficient in *ced-9* activity. We previously observed widespread cell death of the postembryonically derived neurons of the ventral nerve cord in *ced-9(lf)* mutants (Hengartner et al., 1992). We therefore asked whether *ced-9(lf)* animals transgenic for the *hsp–bcl-2* construct would show increased cell survival in the

ventral nerve cord following heat shock. We found that heat-shocked *hsp–bcl-2* transgenic animals had almost twice as many neurons in the ventral cord as heat-shocked animals transgenic for a *hsp*–vector construct or as non-heat-shocked animals (Figure 8c). These results indicate that *bcl-2* can prevent the ectopic cell deaths that occur in *ced-9(lf)* mutants and therefore that *bcl-2* interacts with the cell death pathway either at the same step or downstream of *ced-9*, supporting the hypothesis that *bcl-2* can substitute for *ced-9*. However, we cannot formally exclude the alternative possibility that *bcl-2* acted in this experiment by potentiating residual *ced-9* activity contributed maternally or zygotically.

Discussion

ced-9 Is an Element of a Polycistronic Locus

ced-9 appears to be an element of a complex locus that includes the gene *cyt-1*, which encodes a C. elegans cytochrome b_{560} homolog. The existence of a *cyt-1–ced-9* bicistronic message and the requirement for sequences upstream of *cyt-1* for *ced-9* expression suggest that at least some *ced-9* mRNA arises from bicistronic message that is processed by trans-splicing (Figure 6). An alternative possibility is that the region 5′ of *cyt-1* contains an enhancer required for *ced-9* expression and that the bicistronic message we detected (by Northern, RT–PCR, and cDNA analyses) is an aberrant end product rather than an intermediate.

Polycistronic loci in C. elegans have previously been reported by Blumenthal and colleagues (Spieth et al., 1993). For all six polycistronic loci described by these authors, the downstream gene was trans-spliced mainly to an SL2 splice leader, rather than to the common SL1 splice leader found in the vast majority of cases in which trans-splicing has been reported. While we could detect rare SL2–*ced-9* transcripts by RT–PCR, a major proportion of the *ced-9* message appeared to be SL1 spliced. We suggest that this difference might be a consequence of the short distance between the upstream *cyt-1* and downstream *ced-9* genes. In all the cases reported by Spieth et al. (1993), the upstream and downstream genes are separated by at least 100 bp, whereas the *cyt-1* and *ced-9* mature transcripts are separated by only a few base pairs. Spieth et al. (1993) suggested that cleavage at the polyadenylation site might act (directly or indirectly) to signal the SL2 leader to splice to the downstream gene; for example, the SL2 spliceosome might recognize and trans-splice to an uncapped message. However, in the case of *cyt-1–ced-9*, cleavage at the *cyt-1* (upstream) polyadenylation site would destroy the *ced-9* (downstream) SL splice acceptor site. Thus, trans-splicing to *ced-9* could occur only on an intact polycistronic message, thereby causing an SL1 leader to be used. If so, *cyt-1–ced-9* might be the exception that confirms the rule proposed by Spieth et al. (1993).

Our proposed model would allow for an added level of regulation. Since the *cyt-1* polyadenylation site overlaps the *ced-9* SL1 trans-splice acceptor site (Figure 6), cleavage at the polyadenylation site in the bicistronic message would destroy the *ced-9* splice acceptor site, producing an RNA lacking a methyl-guanosine cap. Such an RNA would be translated poorly and rapidly degraded, as the cap structure is essential for efficient translation and mRNA stability (reviewed by Kozak, 1991). Competition between polyadenylation and trans-splicing might therefore determine the fraction of the bicistronic message that produces functional *ced-9* message and hence the amount of CED-9 protein in the cell.

Is *cyt-1* Involved in Programmed Cell Death in C. elegans?

Why might *cyt-1* and *ced-9* share a promoter? The conservation of the presumptive *cyt-1–ced-9* operon in C. briggsae is consistent with the hypothesis that there has been evolutionary pressure to maintain the two genes in this arrangement. One possibility is that a constitutive *cyt-1* promoter allows for a basal level of *ced-9* to be constantly expressed. Since protection from programmed cell death in C. elegans appears to be an active process, all surviving cells might need a basal level of CED-9 protein to protect them from activating the cell death program. Additional levels of regulation would presumably then lead to the observed variations in the relative abundances of *cyt-1* and *ced-9* transcripts during development.

Alternatively, polycistronic messages might allow the concerted regulation of genes involved in a common biological function, as is commonly observed in prokaryotes (Gottesman and Neidhardt, 1983). Indeed, as Spieth et al. (1993) noted, several of the polycistronic gene pairs they described seem to be related in this way. For example, the genes *lin-15A* and *lin-15B* are both involved in the regulation of *C. elegans* vulval development (Ferguson and Horvitz, 1989). One speculative possibility would thus be that *cyt-1* plays a role in programmed cell death in C. elegans. In this context, it is interesting to note that the *bcl-2* oncogene has been reported to be localized to mitochondria (Hockenbery et al., 1990; see also Chen-Levy et al., 1989; Alnemri et al., 1992), which is also where cytochrome b_{560} is localized (Yu et al., 1992). Furthermore, recent observations suggest that Bcl-2 might function in an antioxidant pathway to prevent apoptosis at sites of free radical generation, such as mitochondria (Hockenbery et al., 1993; Veis et al., 1993; Kane et al., 1993).

One argument against the involvement of cytochrome b_{560} in programmed cell death stems from recent results of Raff and colleagues (Jacobson et al., 1993), who showed that Bcl-2 can block apoptosis in cells that lack mitochondrial DNA. While these observations indicate that neither programmed cell death nor the protective effect of Bcl-2 requires a complete and functional electron transport chain, this study does not address the issue of whether cytochrome b_{560} itself or a more extensive part of the electron transport chain could be involved in cell death. For example, system II of the electron transport chain, of which cytochrome b_{560} is a component, does not contain any mitochondrially encoded subunits (Wolstenholme, 1992) and thus might be totally functional in a cell lacking mitochondrial DNA.

ced-9 Is Homologous to *bcl-2*

The gene *ced-9* is required to protect cells that should survive from programmed cell death. We have shown here that *ced-9* is similar to mammalian *bcl-2* at the levels of sequence, structure, and function. While the overall degree of identity between the two proteins is only 23%, a number of observations suggest that *ced-9* is indeed an invertebrate member of the *bcl-2* gene family. First, CED-9 is more closely related to Bcl-2 than to any other known protein. Second, the CED-9 and Bcl-2 proteins show similar hydropathy plots for their C-terminal halves, with CED-9 possessing the C-terminal hydrophobic tail important for Bcl-2 function. Third, the last intron in both genes is at the identical position in their respective ORFs; this intron is also found in *bcl-x* and *bax*. This observation suggests that this intron was present in the gene before vertebrates and nematodes diverged. Fourth, the regions in which *ced-9* and *bcl-2* are most similar to each other (boxes 1, 2, and 3 in Figure 7) are the regions most conserved among *bcl-2* and its vertebrate relatives and between C. elegans *ced-9* and its C. briggsae homolog. These regions probably identify domains that are important for *ced-9/bcl-2* function. Fifth, *ced-9* and *bcl-2* have similar biological effects: overexpression of each gene prevents the deaths of cells that would normally die. Sixth, *bcl-2* can act like *ced-9* in C. elegans: overexpression of *bcl-2* prevents the deaths of cells that should normally die, and *bcl-2* can substitute for *ced-9* in preventing the deaths of cells that should normally live (in mutants that lack endogenous *ced-9* activity). Taken together, these observations strongly suggest that *ced-9* and *bcl-2* are members of the same gene family. A more difficult question is whether *ced-9* and *bcl-2* are direct homologs. While CED-9 is slightly more similar to Bcl-2 than to the other known members of the family, we cannot exclude the possibility that there is an as yet to be discovered vertebrate *bcl-2* homolog to which *ced-9* will show greater similarity. Alternatively, C. elegans might not have as many *bcl-2* family members as vertebrates, so *ced-9* could be the only *ced-9/bcl-2* family member in C. elegans.

All Metazoans Might Share a *ced-9/bcl-2* Pathway of Programmed Cell Death

The involvement of *ced-9/bcl-2* family members in the control of programmed cell death in both C. elegans and mammals has important implications concerning the nature of programmed cell death. Our observations suggest that the biological phenomenon of programmed cell death predates the separation of nematodes and vertebrates and thus is of very ancient origin. It therefore seems reasonable to propose that not only *ced-9* but also the entire pathway in which *ced-9* acts has been conserved through evolution. If so, there may well be a molecular mechanism for programmed cell death common to all metazoans.

Experimental Procedures

Mutations and Strains

All mutations were generated in a Bristol N2 background, which was used as the standard wild-type strain, except where noted. The following mutations were used: LGIII: *unc-69(e587)*, *ced-9(n1950)*, *ced-9 (n1950 n2077)*, *ced-9(n1950 n2161)*, *ced-9(n1653ts)*, *ced-9(n2812)*, *unc-49(e382)*; LGIV: *ced-3(n2433)*; LGX: *sup-7(st5)*; and wild polymorphic strains: AB2, RC301, TR679, EM1002, N62, and RW7000.

All *ced-9* mutations except *n2812* were described by Desai et al. (1988) and Hengartner et al. (1992). *unc-69(e587)* and *unc-49(e382)* were described by Brenner (1974) and *sup-7(st5)* by Waterston (1981). The mutations *ced-9(n2812)*, isolated by S. Shaham (personal communication), and *ced-3(n2433)*, isolated by us (unpublished data), will be described elsewhere. The *ced-9(n1950 n2077)*, *ced-9(n1950 n2161)*, and *ced-9(n2812)* mutations were maintained in balanced strains in trans to LGIII balancer *qC1*, which carries the mutations *dpy-19(e1259ts,mat) glp-1(q339)* (J. Austin and J. Kimble, personal communication). Strains were maintained as described previously (Brenner, 1974). All strains were grown at 20°C. Some strains were obtained from the Caenorhabditis Genetics Center.

Cell Counts

Animals were anesthetized with 30 mM NaN_3 (Avery and Horvitz, 1987) and observed using Nomarski optics microscopy (Sulston and Horvitz, 1977). Average numbers are shown with (if appropriate) the SEM, as determined by the StatView II program (Abacus Concepts, Incorporated, Berkeley, California).

RFLP Mapping

Various cosmids from the *lin-12–tra-1* interval were tested for their abilities to detect RFLPs on genomic Southern blots of the common laboratory strain N2 and of various strains isolated from the wild (AB2, RC301, TR679, BO, and RW7000). We determined the position of *ced-9* with respect to these RFLPs as described by Ruvkun et al. (1989). In brief, we obtained N2–RC301 recombinants in the *ced-9* region by mating RC301 males with *unc-69(e587) ced-9(n1950) unc-49(e382)* hermaphrodites to generate *unc-69 ced-9 unc-49* (N2)/+++ (RC301) heterozygotes. From these animals, Unc-49 non-Ced-9 non-Unc-69 and Unc-49 Ced-9 non-Unc-69 recombinants were picked. Progeny homozygous for each recombinant chromosome were maintained and their genotypes at the various RFLP loci were determined by genomic Southern blots.

Germline Transformation and *ced-9* Rescue

DNAs were microinjected into the mitotic germline of hermaphrodites, according to the method developed by Mello et al. (1991). For rescue experiments, the relevant DNA was injected at concentrations of 5–25 μg/ml. pRF4, a plasmid containing the *rol-6(su1006)* allele, was coinjected as a dominant marker to identify transgenic animals. Since *ced-9(lf)* mutants are almost sterile and produce only inviable progeny (Hengartner et al., 1992), we injected heterozygotes of genotype *qC1/unc-69(e587) ced-9(n1950 n2077)*, in which *unc-69* was used as a linked marker to identify the *ced-9* chromosome. Non-Unc non-Dpy Rol F1s were picked to establish stably transmitting roller lines. From these animals, Rol Unc-69 individuals were picked and tested for rescue of the *ced-9(lf)*-associated sterility and maternal-effect lethality. A line was considered to be rescued if the transgenic homozygous *ced-9(lf)* animals could generate enough viable progeny to create a self-perpetuating strain.

Molecular Biology

Standard molecular biology protocols (see Sambrook et al., 1989) were followed except where noted. All plasmid subcloning was done into pBluescript (Stratagene, La Jolla, California). Nested deletions of the 4.7 kb rescuing genomic SacI–XhoI fragment were prepared using the ExoIII–S1 method and the DNA sequences of both strands determined by T7 polymerase (Sequenase, U. S. Biochemicals, Cleveland, Ohio) following the protocol suggested by the manufacturer. A 4.2 kb ClaI–XhoI rescuing genomic fragment (residues 477–4665 in Figure 4) was used to probe a λZAP cDNA library provided by R. Barstead and R. Waterston. From approximately 300,000 plaques, we isolated 11 cDNAs. The sequences present at the ends of the inserts were determined for all 11 clones. Eight cDNAs come from the 0.75 kb transcript, two (one incomplete) from the 1.3 kb *ced-9* transcript and one from the 2.1 kb polycistronic transcript. The DNA sequence of one cDNA from each class was then determined (one strand only). Frameshift mutations in the rescuing fragments were introduced into the ORFs by cutting at unique restriction sites (BstEII, SalI, and

BamHI). The 4 bp 5′ overhangs created were filled using Klenow polymerase and the blunt ends were self-ligated, creating a 4 bp insertion at the restriction site. The DNA sequence of the region overlapping the filled restriction site was determined to confirm that the desired frameshift mutation was obtained.

ced-9 Expression

The *cyt-1–ced-9* fusion cDNA was used to probe a poly(A)$^+$ developmental Northern blot (provided by A. Rougvie). The blot was visualized and band intensities quantified using a Fuji Film phosphorimager. Additional probings with the separate *cyt-1* and *ced-9* cDNAs were done using mixed stage poly(A)$^+$ RNA.

RT-PCR

The following oligonucleotides were used for RT–PCR: SL1 primer, GTTTAATTACCCAAGTTTGAG; SL2 primer, GGTTTTAACCCAGTTACTCAAG; upstream (*cyt-1*) primer (to detect the polycistronic transcript), CTGGACTTTCGGCTATTC; downstream primer, TCCCATAACTCGCATCAT. Thirty cycles of amplification reactions using AmpliTaq (Perkin-Elmer Cetus, Norwalk, Connecticut) were performed as suggested by the manufacturer. After one round of amplification, only the SL1 and *cyt-1* upstream primers gave bands of the correct sizes (379 and 617 bp, respectively) on ethidium bromide–stained gels. We can exclude the possibility that these products arose from genomic DNA because such products would include the small first *ced-9* intron and be about 50 bp larger. The separated PCR products were transferred to a nylon membrane (Nitran, Schleicher & Schuell, Keene, New Hampshire) and hybridized to a *ced-9* cDNA probe. Under these conditions, a small amount of SL2 product could also be detected. Quantification of the strength of the SL1 and SL2 bands using a Molecular Dynamics phosphorimager indicated that the SL1 band was at least 20 times stronger than the SL2 band.

Identification of *ced-9* Mutations

Exons from *ced-9* mutants were amplified by asymmetric PCR (McCabe, 1990) using primers that flanked the ORFs. The primers used were as follows: exons I and II, TTTACTTTTACCGTTGAT and TTTGCTGATTTCCTGAAG; exon III, TTTGTGGAAAATACATTA and TTGTGAAATAGCATTCTG; exon IV, CTAAAGCATTATTTGTGA and ATCTGATGGTGAAACAGC. In addition to the PCR primers, the following primers were used for sequencing: exons I and II, TCAGGACTTGCCATCACC, ACGAAATGATGCGAGTTA, TTTTCTGCCACCTTTCTG, TCCCATAACTCGCATCAT, and TCGGTGCGTGAAATAGTC; exon III, TTCCGCCAGAAATGAAC and TTATCGGCTTACCAACAC; exon IV, GTGTGTGGGCGGATGATG, ACTTTTCCGTGTTCTGTA, CGTGATGGCGAAAATG, CACGAAAAGAGACAGTTA, and TCCCACTTCTTCCAGTTC.

Cloning C. briggsae *ced-9*

Degenerate oligonucleotides from the region conserved between *ced-9* and *bcl-2* were used to amplify a *ced-9* fragment from C. briggsae genomic DNA. TNGGNYTNATHTCNTTYGGNGG and TTYTCNYTNTAYCARGAYGTNGT were used as upstream primers, and ACCCARCTNCKRTTRTGYTCNWTC was used as a downstream primer (N: A, C, G, or T; Y: C or T; R: A or G; K: G or T; W: A or T; H: A, C, or T). PCR products were cloned into pCR II (Invitrogen, San Diego, California), and the sequence of the insert was determined. Fragments showing sequence similarity to *ced-9* were used to probe a C. briggsae genomic library (gift of D. Baillie) in λCharon4. Positive phage were purified, and EcoRI insert fragments that hybridized under low stringency to C. elegans *ced-9* or *cyt-1* probes were subcloned into pBluescript and their DNA sequence determined using an Applied Biosystems 373A sequencer according to the protocols of the manufacturer.

ced-9 and *bcl-2* Transgenic Nematodes

To generate the *hsp–ced-9* construct, a full-length 1.1 kb *ced-9* cDNA in pBluescript I SK(−) was digested with SacI and KpnI and cloned into the heat shock vectors pPD49.78 and pPD49.83 (Perry et al., 1993; referred to throughout the text as *hsp*–vector) previously digested with SacI and KpnI. For *hsp–bcl-2*, a 1.9 kb cDNA clone in pBluescript SK(−) (Seto et al., 1988) was digested with XbaI and KpnI and cloned into pPD49.78 and pPD49.83 previously digested with NheI and KpnI. The pPD49.78 and pPD49.83 vectors contain the promoter of two distinct but related 16 kd C. elegans heat shock proteins that show different and (to a large extent) complementary patterns of expression (Stringham et al., 1992). For this reason, to maximize the number of cells that expressed the transgenes, we always injected both constructs (at 100 ng/μl each). pRF4 (at 50 ng/μl) was coinjected as a dominant marker to identify transgenic animals. All subsequent experiments were performed using transgenic lines carrying integrated arrays or extrachromosomal arrays showing a high frequency of transmission.

Heat Shock Experiments

To study the embryonic deaths in the anterior pharynx, we transferred gravid hermaphrodites of the appropriate genotype to fresh petri plates and allowed these animals to lay eggs for 1 hr. The plates were then transferred to 33°C for 45 min, after which the plates were returned to 20°C. After a 75 min recovery period, the adult animals were removed from the plates, and the eggs remaining on the plates were kept for analysis. Cells in the anterior pharynx were scored at the L3 and L4 larval stages. To study the postembryonic deaths in the ventral nerve cord, L1 larvae were transferred to 33°C for 45 min, after which the plates were returned to 20°C. Neurons present in the ventral nerve cord were scored at the late L3 larval stage, approximately 15–24 hr after the Pn.a descendants should have died, using Nomarski optics.

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GenBank Accession Numbers

The accession numbers for the sequences reported in this paper are L26545 (the *ced-9* locus) and L26546 (the C. briggsae genomic sequence).