Molecular Cell, Vol. 4, 309–319, September, 1999, Copyright ©1999 by Cell Press

The *C. elegans* Cell Death Specification Gene *ces-1* Encodes a Snail Family Zinc Finger Protein

Mark M. Metzstein and H. Robert Horvitz*
Howard Hughes Medical Institute
Department of Biology
Massachusetts Institute of Technology
Cambridge, Massachusetts 02139

Summary

The *ces-1* and *ces-2* genes of *C. elegans* control the programmed deaths of specific neurons. Genetic evidence suggests that *ces-2* functions to kill these neurons by negatively regulating the protective activity of *ces-1*. *ces-2* encodes a protein closely related to the vertebrate PAR family of bZIP transcription factors, and a *ces-2*/*ces-1*-like pathway may play a role in regulating programmed cell death in mammalian lymphocytes. Here we show that *ces-1* encodes a Snail family zinc finger protein, most similar in sequence to the *Drosophila* neuronal differentiation protein Scratch. We define an element important for *ces-1* regulation and provide evidence that CES-2 can bind to a site within this element and thus may directly repress *ces-1* transcription. Our results suggest that a transcriptional cascade controls the deaths of specific cells in *C. elegans*.

Introduction

Programmed cell death (apoptosis) is an important cellular process in all animals and is used during development to sculpt tissues and organs, in the nervous system to refine contacts between neurons and targets, in the immune system to select for functional lymphocytes and to select against self-reactive cells, and in tissue homeostasis to balance cell proliferation in maintaining constant cell numbers (reviewed by Lockshin et al., 1998). Failure to activate programmed cell death can result in cellular overproliferation and oncogenesis, while ectopic activation may be the underlying cause of degenerative diseases (reviewed by Rudin and Thompson, 1997). Recently much has been learned about the molecular machinery used by cells to kill themselves (reviewed by Pettmann and Henderson, 1998).

Genetic analysis of the nematode *Caenorhabditis elegans* has helped identify and analyze genes involved in programmed cell death (reviewed by Metzstein et al., 1998). Single-gene mutations in *C. elegans* that lead to general defects in the process of programmed cell death have been identified, and the cloning of the genes defined by these mutations has revealed the molecular nature of key cell death regulators. Many of the molecules important for programmed cell death have been conserved throughout evolution.

*To whom correspondence should be addressed (e-mail: horvitz@mit.edu).

How are the activities of proteins involved in the general process of programmed cell death regulated in specific cell types during development? This question has been addressed using systems in which clearly defined developmental cues lead to the deaths of specific cells. For example, programmed cell death is induced in many larval tissues during insect metamorphosis in response to the hormone ecdysone (Schwartz, 1992; Robinow et al., 1993; Jiang et al., 1997), and the changes in expression of some components of the general cell death machinery have been examined in cells that die in response to ecdysone (Robinow et al., 1997). However, the genes that regulate such changes have yet to be identified.

The *ces* (cell death specification) genes are candidate developmental regulators of programmed cell death in *C. elegans* (Ellis and Horvitz, 1991). Mutations in the known *ces* genes prevent the deaths of only a few of the cells that undergo programmed cell death in wild-type animals. By contrast, mutations in general components of the cell death process, that is, *egl-1*, *ced-9*, *ced-4*, and *ced-3*, can affect the death process in all cells that normally die. The gene *ces-2* is required for the deaths of the sister cells of the two NSM neurons (Ellis and Horvitz, 1991). In wild-type animals, the two NSM sisters undergo programmed cell death during embryogenesis, while in animals with reduced *ces-2* activity the NSM sisters frequently survive. *ces-2* encodes a basic leucine zipper (bZIP) transcription factor similar in sequence and in binding specificity to the vertebrate PAR (proline and acid–rich) subfamily of bZIP proteins (Metzstein et al., 1996). The function of *ces-2* may be evolutionarily conserved, since an oncogenic fusion protein consisting of the activation domain of the transcription factor E2A and the bZIP domain of the PAR family member HLF (hepatic leukemia factor) has been implicated in the regulation of programmed cell death in human pro-B cells (Inaba et al., 1996).

Genetic evidence suggests that *ces-2* does not directly regulate components of the general cell death machinery. Rather, *ces-2* is thought to function by negatively regulating a cell-specific survival activity encoded by the gene *ces-1* (Ellis and Horvitz, 1991). Like *ces-2* loss-of-function (lf) mutations, dominant, gain-of-function (gf) mutations of *ces-1* block the deaths of the NSM sisters. *ces-1*(gf) mutations also block the deaths of another neural cell type, the I2 sisters, which seem to be unaffected by *ces-2* mutations. In *ces-1*(lf) mutants, the NSM sisters and I2 sisters die, just as they do in wild-type animals. However, in such mutants the NSM sister deaths are independent of *ces-2* activity: NSM sisters die in *ces-1*(lf) *ces-2*(lf) double-mutant animals. These deaths still require the activity of general cell death components (Ellis and Horvitz, 1991; Conradt and Horvitz, 1998). These data suggest that the function of *ces-2* is to negatively regulate the activity of *ces-1* and that *ces-1* activity in turn can block the programmed cell death of the NSM sisters.

To elucidate further how programmed cell death is controlled in the NSM sister cells as well as how the

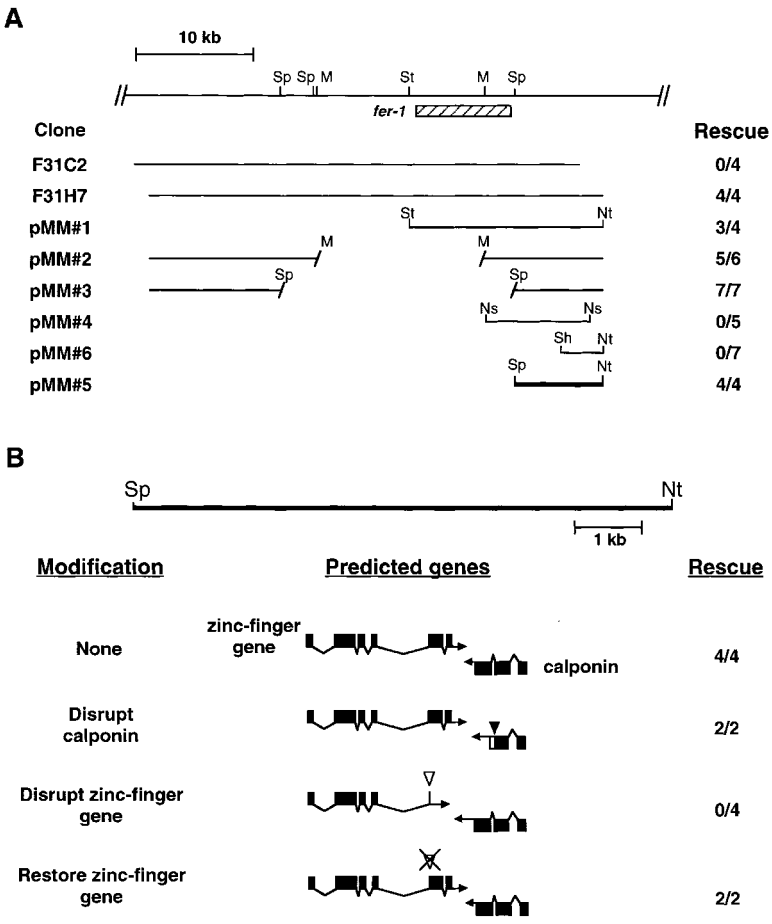

Figure 1. Molecular Cloning of *ces-1*

(A) Physical map around *ces-1* indicating cosmids and clones assayed for *ces-1* rescue. The extent of the *fer-1*-coding sequences is shown as a hatched box (Achanzar and Ward, 1997). The minimal rescuing fragment (pMM#5) is shown in bold. Restriction enzyme cleavage sites are indicated: M, MluI; Ns, NsiI; Nt, NotI; Sh, SphI; Sp, SpeI; St, StuI. The NotI sites on subclones derive from the cosmid vector. pMM#2 and pMM#3 were derived from the cosmid F31H7 by deleting internal fragments.

(B) pMM#5, showing the intron/exon structure of *ces-1* and the calponin homolog (*cpn-1*) and modified clones assayed for rescuing activity. Exons are shown as closed boxes, and the direction of transcription is indicated by arrows. Closed arrowhead, calponin frameshift mutation. Open arrowhead, *ces-1* oligonucleotide inserted. Open arrowhead with X, *ces-1* oligonucleotide removed. Open box, coding sequence frameshifted from original sequence.

general components of programmed cell death are regulated, we have molecularly characterized the *ces-1* gene.

Results

ces-1 Mapping and Rescue

ces-1 had been mapped genetically on linkage group I between the genes *fer-1* and *sup-17* (Ellis and Horvitz, 1991), both of which had been placed on the *C. elegans* physical map (Waterston et al., 1997). Three-factor mapping placed *ces-1* very close to *fer-1* (Ellis and Horvitz, 1991; our unpublished results). We used the suppression by *ces-1*(lf) of the *ces-2*(lf) phenotype of NSM sister survival as an assay of *ces-1* activity in transformation rescue experiments: we predicted that extra copies of the *ces-1* gene would cause the NSM sisters to survive in a *ces-1*(lf) *ces-2*(lf) background. We transformed *ces-1*(lf) *ces-2*(lf) animals with a cosmid (F31H7) that contained *fer-1* and found that it was able to rescue *ces-1* (Figure 1A). We then generated a series of subclones of this rescuing cosmid, assayed these subclones for *ces-1*-rescuing activity, and narrowed the rescuing activity to a 7.7 kb subclone, pMM#5 (Figure 1A).

We noted that *ces-1*(lf) *ces-2*(lf) animals with a *ces-1* transgene showed NSM sister survival, which is characteristic of both *ces-1*(gf) and *ces-2*(lf) animals, and also showed I2 sister cell survival, characteristic of *ces-1(*gf) but not *ces-2*(lf) animals (Ellis and Horvitz, 1991). NSM sister and I2 sister survival can also be observed in *ces-1*(+) *ces-2*(+) animals carrying a *ces-1* transgene (Table 1). We interpret the survival the NSM sisters and I2 sisters to be a result of *ces-1* overexpression (from the

Table 1. *ces-1* Gain-of-Function Phenotype Is Induced by *ces-1*-Containing Arrays

Genotype	Line Number	Percent Survival	
		NSM Sisters	I2 Sisters
Wild type[a]		0%	9%
Transgene(s)			
ces-1 + *unc-76*	1	33%	24%
	2	21%	22%
	3	41%	40%
unc-76	1	1%	4%
	2	0%	4%
	3	0%	1%

Numbers represent the percentage of NSM sister or I2 sister cells observed in each of the transgenic lines. At least 100 sides were scored for each line. Transgenes consist of: *ces-1* + *unc-76*, pMM#5 + pU76-16B; *unc-76*, pU76-16B. The transgenes were injected into and maintained in *unc-76(e911)* animals. The survival of the NSM sisters and I2 sisters in such transgenic animals requires the CES-1 protein, as survival is not observed in animals carrying arrays with a disruption of the *ces-1*-coding frame or arrays containing only *ces-1* regulatory regions (our unpublished results).

[a]Data from Ellis and Horvitz (1991).

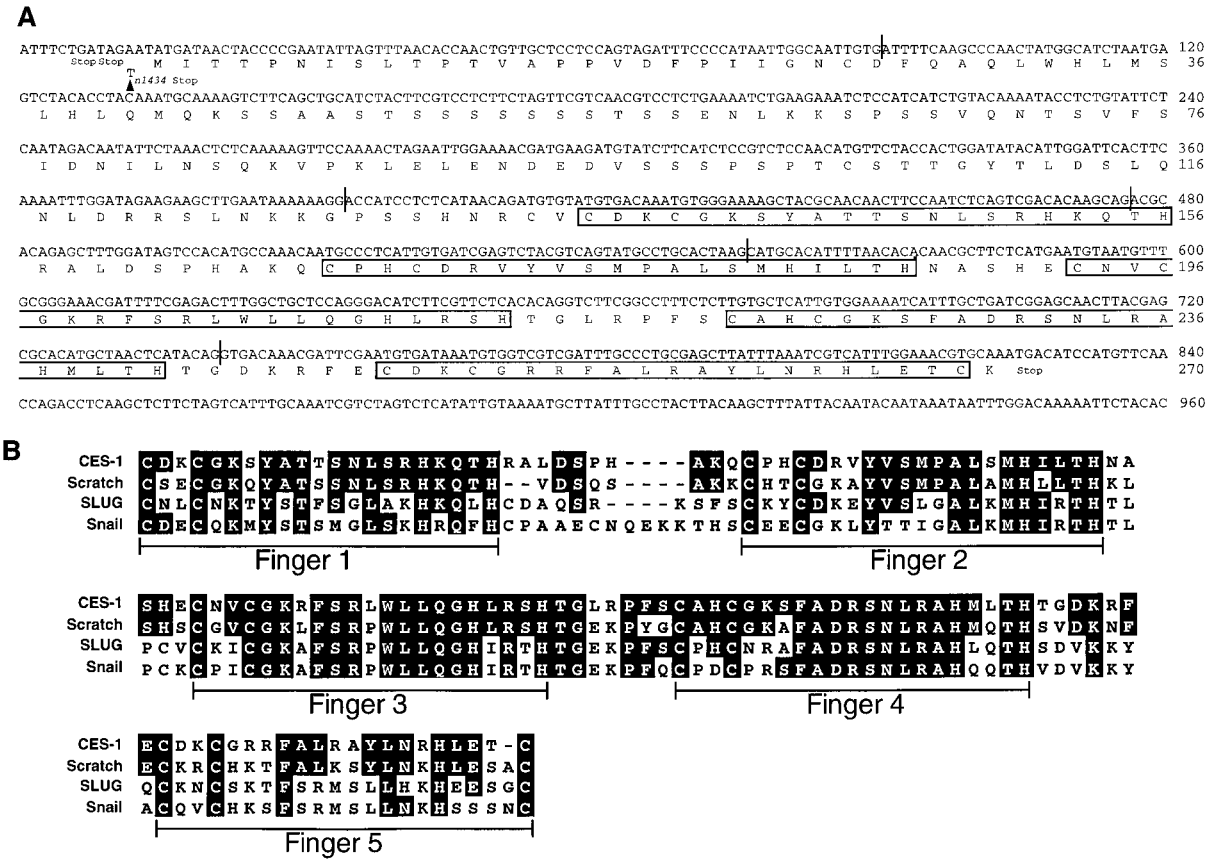

Figure 2. *ces-1* Sequence Analysis

(A) *ces-1* composite transcript sequence, derived from analysis of genomic DNA, cDNAs, and RACE products. Nucleotides (above) and predicted protein sequence (below) are numbered on the right. Two in-frame stop codons before the first initiation codon are marked. Intron positions are marked with vertical lines. Zinc finger motifs are boxed. The *ces-1* loss-of-function allele *n1434* is marked with an arrowhead. *ces-1* cDNAs have a poly(A) tail immediately following this sequence (not shown).

(B) Alignment of the zinc finger domains of CES-1 (amino acids 167-270), Scratch (amino acids 469-601; Roark et al., 1995), Snail (amino acids 247-385; Boulay et al., 1987), both from *Drosophila*, and human SLUG (amino acids 130–264; Cohen et al., 1998; A. T. Look, personal communication), the closest known mammalian homolog of CES-1. Residues identical between CES-1 and any of the three other proteins are highlighted in black, and the individual zinc fingers are indicated by brackets.

multiple copies on the extrachromosomal array), suggesting that the survival phenotype seen in *ces-1*(gf) mutants could be a consequence of overexpression or ectopic expression of a wild-type gene product.

ces-1 Encodes a Snail Family Zinc Finger Protein

We determined the sequence of the 7.7 kb minimal rescuing fragment and analyzed the sequence with the programs BLAST (Altschul et al., 1990) and GENEFINDER (Favello et al., 1995). These analyses suggested that the fragment contained two complete transcription units. One of the transcription units encodes a protein with similarity to C_2H_2 zinc finger proteins (Klug and Schwabe, 1995), while the other encodes a member of the calponin family of smooth muscle proteins (el-Mezgueldi, 1996).

To determine which, if either, of these two candidates was responsible for *ces-1*-rescuing activity, we specifically disrupted each. We found that a construct with a disruption of the calponin family gene was capable of rescuing the *ces-1* mutant phenotype (Figure 1B), suggesting that the calponin gene does not correspond to *ces-1*. We named the calponin-encoding gene *cpn-1* (calponin). By contrast, a construct with a disruption of the zinc finger gene was unable to rescue the *ces-1* mutant phenotype (Figure 1B). Furthermore, restoration of the zinc finger gene restored *ces-1*-rescuing activity (Figure 1B). These data demonstrate that *ces-1* encodes a zinc finger containing protein. Although the subclone pMM#4 and the cosmid F31C2 both contain the entire *ces-1*-coding region (Figure 1A) and at least as much sequence 5′ to *ces-1* as the rescuing subclone pMM#5, both failed to rescue. We suspect that there is an enhancer element 3′ to *ces-1*, beyond the calponin transcription unit, required for proper *ces-1* expression.

We isolated cDNAs corresponding to the *ces-1* gene, determined their sequences, and used RACE (rapid amplification of cDNA ends) to identify the 5′ end of the transcript (Figure 2A). These analyses revealed the presence of a single long open reading frame predicted to encode a 270–amino acid 30 kDa protein with four C_2H_2 and one C_2HC zinc finger at the C terminus (Figure 2A). Both in overall structure and in sequence similarity within the zinc fingers, CES-1 is a member of the Snail

family of C_2H_2 transcription factors, defined originally by the *Drosophila* developmental gene *snail* (Boulay et al., 1987) (Figure 2B). The predicted CES-1 protein is 46% identical to Snail within the zinc finger regions, with the third and fourth fingers each being 71% identical (Figure 2B). Within its zinc finger regions, CES-1 is particularly similar to the *Drosophila* Snail family member Scratch (Figure 2B) (Roark et al., 1995): CES-1 and Scratch are more similar to each other than either is to any other protein. Outside the zinc finger domain, CES-1 is not similar in primary sequence to Scratch or to any other protein.

Analysis of *ces-1* Alleles

There exist three independently isolated *ces-1* gain-of-function alleles (*n703*, *n1895*, and *n1896*) and two *ces-1* loss-of-function alleles (*n703 n1406* and *n703 n1434*); the loss-of-function alleles were isolated by the reversion of the gain-of-function allele *n703* (Ellis and Horvitz, 1991). All five alleles were generated with the mutagen ethyl methanesulphonate (EMS). The gain-of-function alleles behave similarly to each other. The two loss-of-function alleles also behave similarly to each other. We have characterized the molecular lesions in all five alleles.

First, we analyzed Southern blots of genomic DNA from each of the alleles, using the minimal 7.7 kb rescuing fragment as a probe (data not shown). The allele *n703 n1406* showed a polymorphic pattern for all restriction enzymes tested. Using a series of probes from the *ces-1* region, we further characterized *n703 n1406* and found that this allele consists of a complex rearrangement involving a deletion of about 1 kb of DNA in the 3′ region of the *ces-1* gene and an associated insertion of more than 10 kb of DNA next to the deletion. We have not identified the source of the inserted material. The deletion disrupts the second and eliminates the third, fourth, and fifth zinc fingers. This rearrangement probably also disrupts *cpn-1*. We have not observed any phenotype associated with a loss of function of *cpn-1*.

To identify the molecular lesions in the remaining four alleles, we determined the sequences of *ces-1* exons, the majority of introns (all but the large fourth intron), 5′ and 3′ UTRs, and about 50 base pairs (bp) 5′ of the transcription start of *ces-1* in *ces-1* mutant animals. The loss-of-function allele *n703 n1434* contains a single base change, a C-to-T transition converting an asparagine CAA codon at position 40 to a TAA stop codon (Figure 2A). This alteration is predicted to result in a truncated protein of only 39 amino acids lacking all five of the zinc fingers. Based upon these molecular studies, both *n703 n1406* and *n703 n1434* are likely to eliminate *ces-1* function.

None of the gain-of-function alleles had any mutations within the *ces-1*-coding regions, 5′ or 3′ UTRs, or those introns examined. To identify the sites of these mutations, we used the polymerase chain reaction (PCR) to amplify DNA fragments from wild-type and *n703* genomic DNA and then used chemical cleavage of mismatched DNA (CCM) (Smooker and Cotton, 1993; Aroian et al., 1994) to detect base differences between these PCR products. We performed this analysis on DNA fragments of 0.9 kb located 5′ of the transcription start site, of 1.2 kb spanning the large fourth intron, and of 1.2 kb located 3′ of the 3′ end of the *ces-1* transcript (which includes *cpn-1*). We identified a mismatch using a probe generated from DNA located 5′ of the transcription start site (data not shown). We determined the sequence of this region in DNA from the wild type and from the three gain-of-function mutant *n703*, *n1895*, and *n1896* animals. We found that all of the gain-of-function alleles contain an identical G-to-A transition mutation located 601 bp before the transcription start site as compared to the wild type. Since the *ces-1*(gf) mutations are not within the *ces-1* transcript and are a considerable distance from the putative transcription start site, it is likely that these mutations are in a *cis*-regulatory sequence.

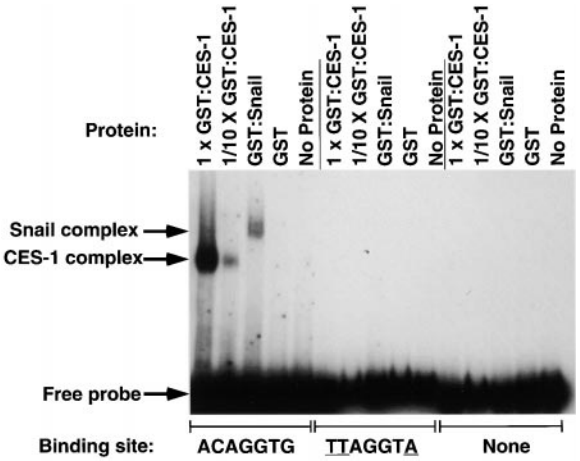

Figure 3. CES-1 Binds to Snail Family Consensus Sites

An electrophoretic mobility shift assay performed using bacterially produced CES-1 and Snail proteins. 1 × GST:CES-1, GST:Snail, and GST, about 80 ng of protein; 1/10 × GST:CES-1, about 8 ng of protein. The probes were fragments from a plasmid polylinker into which oligonucleotides containing test binding sites were cloned. In the first set of five reactions, the probe contained an optimal Snail-binding site; in the second set, a site altered at three positions (underlined); and in the final set, the equivalent fragment of the polylinker without any cloned oligonucleotides.

CES-1 Binds to Consensus Snail-Binding Sites

To determine whether CES-1 is similar to Snail family proteins in biochemical function as well as in sequence, we examined the DNA-binding properties of recombinant CES-1 protein. We produced in *E. coli* a protein (GST:CES-1) consisting of the C-terminal half of CES-1 (which includes all five zinc fingers) fused at its N terminus to glutathione S-transferase (GST). We used this protein in electrophoretic mobility shift experiments to assay binding of CES-1 to a double-stranded oligonucleotide containing the sequence ACAGGTG, known to be bound by other Snail family proteins (Ip et al., 1992; Hayashi et al., 1993; Mauhin et al., 1993) (Figure 3). We found that GST:CES-1 and GST:Snail, a protein consisting of the C-terminal of Snail fused to GST (Ip et al., 1992), can bind to this oligonucleotide (Figure 3). This binding is specific: when the oligonucleotide was mutated such that three consensus bases were altered to nonconsensus bases or when the site was not present

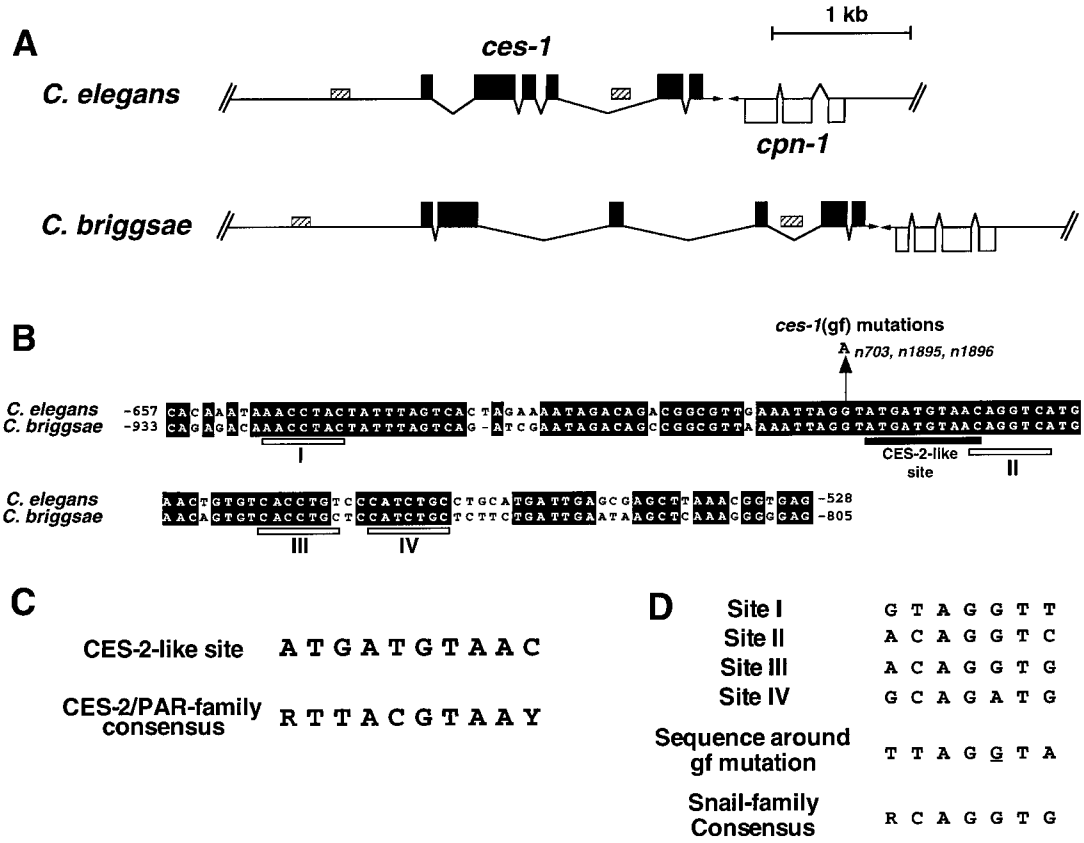

Figure 4. Analysis of the *ces-1* Regulatory Region Conserved in *C. briggsae*

(A) Comparison of the *ces-1* locus in *C. elegans* and *C. briggsae*. Closed boxes, *ces-1* exons; open boxes, *cpn-1* exons; hatched boxes, conserved upstream and fourth intron elements; arrows, direction of transcription. The identity within the upstream element between the two nematode species is 82% (106/130 bp), and within the fourth intron element the identity is 70% (109/156 bp), while elsewhere outside coding regions the identity in any 100 bp region is less than 30% (and not greater than 50% measured in any 40 bp region).

(B) Alignment of the conserved upstream region. Numbering is with respect to the putative *ces-1* initiation codon in the two species. Identities are highlighted in black. Open bars, sites similar to Snail family consensus-binding sites; closed bar, site similar to CES-2/PAR family consensus-binding site. Also indicated is the base change (G-to-A) found in the *ces-1* gain-of-function alleles *n703*, *n1895*, and *n1896*.

(C) Alignment between the CES-2-like binding site in the *ces-1* upstream element and the CES-2/PAR family consensus. Y = T or C; R = A or G.

(D) Alignment among Snail family-like binding sites in the *ces-1* upstream element and the Snail family consensus sequence (the complements of sites I, III, and IV are shown for alignment). All of these sites are conserved between *C. elegans* and *C. briggsae*, except for the first base in site III (A in *C. elegans*, G in *C. briggsae*). Also shown is the sequence around the *ces-1*(gf) mutations. The underlined base is the one mutated to an A by the gf mutations.

in the oligonucleotide, neither GST:CES-1 nor GST:Snail bound the mutant site (Figure 3).

Analysis of *C. briggsae ces-1*

Genetic evidence suggests that *ces-1* acts downstream of and is negatively regulated by the gene *ces-2* (Ellis and Horvitz, 1991). Since *ces-2* encodes a transcription factor, we wanted to determine whether this negative regulation might be mediated by direct transcriptional repression. To identify regions of the *ces-1* locus important in gene regulation, we used phylogenetic comparison of genomic sequence. By low-stringency hybridization to a *C. elegans ces-1* cDNA probe, we isolated clones from a λ genomic library made from the closely related nematode *Caenorhabditis briggsae*. We determined about 6 kb of sequence of a subclone derived from one of the λ clones and compared this sequence to our *C. elegans* sequence (Figure 4A). We found that this *C. briggsae* clone included a homolog of *cpn-1*, providing evidence that the hybridizing clone contained the *C. briggsae* equivalent of *ces-1* rather than some other closely related *snail* family gene. The *C. briggsae* CES-1 protein shows a pattern of conservation typical of Snail family members: the N terminus is not particularly highly conserved (56% identity compared to *C. elegans* CES-1), while the C-terminal zinc fingers are highly conserved (97% identity) (alignments not shown).

When we compared the sequence of noncoding regions around the *C. elegans* and *C. briggsae ces-1* genes, we found that there is almost no conservation between the two species, consistent with what has been observed for other genomic regions (Fitch and Thomas, 1997). Two regions are exceptions.

First, there is a stretch of moderate conservation within the *ces-1* fourth intron (Figure 4A) (70% identity over a 156 bp region, alignment not shown). This region

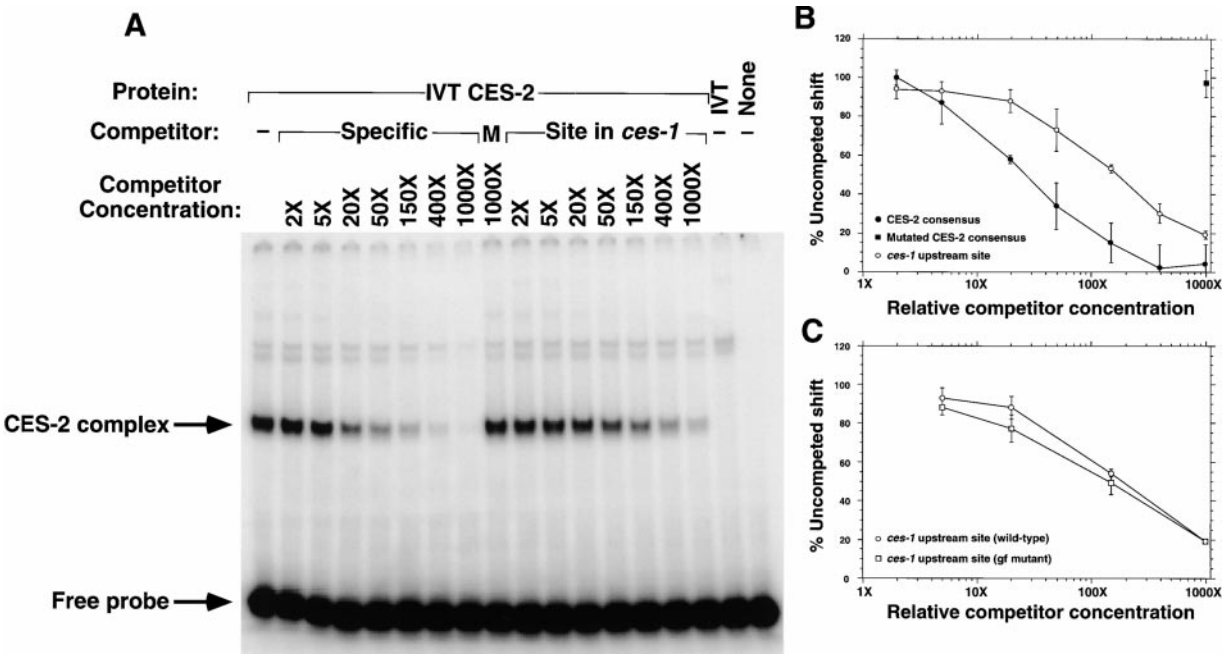

Figure 5. Binding of CES-2 to a Site in the Conserved *ces-1* Upstream Element

(A) An electrophoretic mobility shift assay performed using the bZIP domain of CES-2 made by in vitro transcription/translation (IVT CES-2). The first lane shows the shift of labeled probe containing an optimal CES-2-binding site. (Specific) Lanes contain increasing amounts of unlabeled competitor of the same sequence as the labeled probe. The fold excess of unlabeled probe is shown above the gel. (M) Lane shows competition with 1000-fold excess of a mutated competitor that has four bases different from the optimal binding site. (Site in *ces-1*) The competitor used contained the site found in the conserved element upstream of *ces-1*. The doublet above the CES-2 complex indicates a shift caused by an unknown component in the in vitro transcription/translation reaction, as it was seen without adding the *ces-2* cDNA to the reaction (IVT).

(B) Quantitation of the CES-2 electrophoretic mobility shift assays. The graph shows the amount of shift complex as the percentage of the shift seen with no added competitor. The percentage given is the average of two or three experiments, and the error bars represent ±1 SD from the mean.

(C) Comparison of competition of an optimal CES-2-binding site with the site found upstream of *ces-1* in a wild-type context or gain-of-function mutant context. No difference in binding between these two sites was apparent.

seems to be noncoding, as there are no significant open reading frames or suitable splice donor or acceptor sites.

Second, upstream of the *ces-1* transcription start site, there is a region with striking conservation between *C. elegans* and *C. briggsae* (Figure 4A). In a region of 130 bp (−657 to −528 bp with respect to the *C. elegans* transcription start site), there are 106 bases that are identical between the two species (82% identity, Figures 4A and 4B). This region spans the site of the *ces-1*(gf) mutations. Included in this region is a site with high similarity to the CES-2-binding site consensus sequence (Metzstein et al., 1996) (Figure 4B). Half of this site (GTAAC) is a perfect match to the CES-2-binding consensus (GTAAY), while the other half matches in three of five positions (ATGAT versus RTTAC) (Figure 4C). This putative CES-2-binding site is completely conserved in the *C. briggsae* sequence.

Also within the conserved region are four sites with high similarity to CES-1/Snail family consensus-binding sites (containing at least five identities within the seven-base Snail-binding consensus; Hayashi et al., 1993; Mauhin et al., 1993) (Figures 4B and 4D), suggesting the possibility that *ces-1* may autoregulate. With the exception of a single, conservative substitution within site III, these sites are also conserved in the *C. briggsae* sequence. The sequence flanking the site of the *ces-1*(gf) mutations also has some similarity to the Snail consensus-binding sequence (four identities within the seven-base sequence) (Figure 4D), and the gain-of-function mutations reduce this similarity. However, we could not demonstrate binding by CES-1 or Snail protein to a probe containing the wild-type version of this site in vitro (Figure 3), and it is not clear whether this particular site can be bound by either CES-1 or other *C. elegans* Snail family members in vivo.

CES-2 Can Bind to a Site in the *ces-1* Upstream Element

To determine whether CES-2 protein can bind the candidate CES-2-binding site upstream of *ces-1*, we used electrophoretic mobility shift assays. We produced a protein consisting of the C-terminal half of CES-2, which includes the entire CES-2 bZIP domain, using in vitro transcription/translation (IVT CES-2). We used IVT CES-2 to shift an oligonucleotide probe containing an optimal CES-2 consensus-binding site (Metzstein et al., 1996) (Figure 5A). This shift required CES-2 protein, as it was not observed in a control experiment using the in vitro transcription/translation mix alone (Figure 5A). We then used various unlabeled double-stranded oligonucleotides as competitors. We found that the optimal binding

Table 2. *ces-1*(gf) Requires *ced-9* to Block Programmed Cell Death in the NSM Sister Cells

Genotype	NSM Sister Survival (%)
ces-1(+); ced-9(+); ced-3(n2427)	10 ± 5
ces-1(n703); ced-9(+); ced-3(n2427)	88 ± 6
ces-1(+); ced-9(n2812); ced-3(n2427)	59 ± 8
ces-1(n703); ced-9(n2812); ced-3(n2427)	71 ± 8

Each number represents the percentage of NSM sister cells observed in each of the strains. The errors in the data are 95% confidence limits calculated using the binomial distribution (Zar, 1974). At least 100 sides were scored for each genotype. All the strains also contained *dpy-17(e164)* as a *cis*-linked marker for the *ced-9* mutation.

site competed the labeled probe for binding (Figures 5A and 5B). This competition was specific: an oligonucleotide competitor that contained a mutated binding site with four base changes (M in Figure 5A) did not compete for binding, even at a 1000-fold higher molar ratio than the labeled probe (Figures 5A and 5B). The putative CES-2-binding site upstream of *ces-1*, which differs from a CES-2 optimal site at two bases, competed for binding with the labeled probe (Figures 5A and 5B), although at a somewhat lower efficiency than did the optimal site. Hence, we conclude that CES-2 can bind, at least in vitro, the site upstream of *ces-1*.

Next we tested whether the *ces-1*(gf) mutation can affect the binding of IVT CES-2 to the binding site in the *ces-1* upstream element. We found no significant difference in binding between the wild-type and mutated version of the site (Figure 5C), suggesting that the *ces-1*(gf) mutations do not act by disrupting CES-2 binding.

ces-1 Requires Functional *ced-9* to Block the Deaths of the NSM Sisters

Previous observations established that a gain-of-function mutation in the cell death–protecting gene *ced-9* allowed the NSM sisters to survive even in *ces-1*(lf) animals (M. Hengartner and H. R. H., unpublished results). This finding indicated that *ces-1* acts upstream or parallel to *ced-9*. To help distinguish between these alternatives, we examined whether *ced-9* activity is required for *ces-1*(gf) activity to block programmed cell deaths. Specifically, we tested whether *ces-1*(gf) could still block the deaths of the NSM sisters in a *ced-9*(null) background. Because *ced-9* loss-of-function mutations are lethal (Hengartner et al., 1992), we used a weak *ced-3*(lf) mutation, *n2427*, to suppress the lethality of the *ced-9*(null) mutation. First, we tested whether the effect of *ced-3(n2427)* on NSM sister survival can be distinguished from that of *ces-1*(gf) (Table 2). In a *ces-1(+); ced-3(n2427)* mutant, we found that approximately 10% of NSM sister cells survive. By contrast, we found that the *ces-1(n703); ced-3(n2427)* strain has approximately 88% NSM sister survival. Hence, as in a *ced-3*(+) background, in a *ced-3(n2427)* background *ces-1(n703)* can block death of the NSM sisters.

Is this activity of *ces-1* dependent on the activity of *ced-9*? In a *ces-1(+); ced-9(n2812); ced-3(n2427)* strain, we found that 59% of NSM sisters survive. This increase over the *ced-3(n2427)* strain has been interpreted as a cell-killing activity of *ced-9* (Hengartner and Horvitz, 1994). We found that a *ces-1(n703); ced-9(n2812); ced-3(n2427)* strain had only a slightly greater survival of NSM sisters (71%) than the *ces-1(+); ced-9(n2812); ced-3(n2427)* strain. Hence, we conclude that in the absence of *ced-9* activity, *ces-1(n703)* can no longer efficiently block programmed cell death of the NSM sister cells.

Discussion

A Zinc Finger Protein Controls the Deaths of Certain Neurons in *C. elegans*

The cell death specification gene *ces-1* encodes a Snail family zinc finger transcription factor that is particularly closely related in sequence to the *Drosophila* Snail family protein Scratch. Scratch is expressed in all developing neurons during *Drosophila* embryogenesis and is thought to promote neural differentiation by blocking nonneuronal cell fates, since loss-of-function mutations in *scratch* lead to a slight neuronal hypoplasia (Roark et al., 1995). Like *scratch*, the *ces-1* gene can be considered to act to promote neuronal fates, since *ces-1*(gf) mutations (and presumably overexpression of wild-type *ces-1*) causes the NSM sisters and I2 sisters to become neurons (Ellis and Horvitz, 1991). By analogy to *ces-1*, which acts to prevent the NSM sisters from undergoing programmed cell death, *scratch* might also act to prevent neuronal cells from undergoing programmed cell death. In other words, the nonneuronal fate blocked by *scratch* could be programmed cell death, and the neurons missing in *scratch* animals could be missing because they underwent programmed cell death.

The *scratch* gene is thought to function redundantly with transcription factors that directly promote neuronal fates, such as the bHLH (basic helix-loop-helix)-encoding gene *deadpan* (Bier et al., 1992; Roark et al., 1995). Like *scratch*, *deadpan* is expressed in all developing neurons, and loss-of-function of *deadpan* leads to a slight loss of neuronal function (Bier et al., 1992). Unlike single mutants for either gene, animals mutant for both *scratch* and *deadpan* have profound neuronal loss (Roark et al., 1995). These data have led to a model in which either promotion of a neuronal fate, by *deadpan*, or inhibition of nonneuronal fates, by *scratch*, is sufficient for neuronal differentiation (Roark et al., 1995). A similar model might explain why *ces-1* loss-of-function mutants appear to be wild type in phenotype: *ces-1* might function to block programmed cell death in some or all developing *C. elegans* neurons; however, promotion of neuronal fates is sufficient to overcome the tendency of neurons to undergo programmed cell death in *ces-1*(lf) mutants. bHLH proteins expressed during *C. elegans* neurogenesis have been identified (Krause et al., 1997), but their loss-of-function phenotypes have not yet been described.

The Nature of *ces-1* Gain-of-Function Mutations

Our molecular analysis suggests that the *ces-1* gain-of-function phenotype may be caused by overexpression of an otherwise wild-type gene product, since extrachromosomal arrays carrying the wild-type *ces-1* locus show a *ces-1* gain-of-function phenotype and the gain-of-function mutations do not alter the *ces-1* transcript.

However, earlier gene dosage studies had indicated that wild-type *ces-1* antagonizes the gain-of-function alleles (Ellis and Horvitz, 1991). Furthermore, our molecular analysis suggests that the two *ces-1* loss-of-function alleles completely eliminate function; both are expected to eliminate zinc fingers, which should be necessary for CES-1 function. Again, this result is inconsistent with gene dosage studies, which suggested that the *ces-1* loss-of-function alleles have residual activity (Ellis and Horvitz, 1991). These inconsistencies between the molecular and genetic data could be explained by the presence of interacting loci in the deficiencies and duplications used for genetic dosage studies. Such loci could have had a modulatory effect on *ces-1* activity and hence confounded the genetic analysis.

Surprisingly, all three *ces-1* gain-of-function mutations are identical, suggesting either that only this base can mutate to generate viable animals with a Ces-1 phenotype or that this particular base is very sensitive to EMS mutagenesis. We favor the latter alternative. This mutation has been isolated at a frequency of about 1/10,000 genomes screened (Ellis and Horvitz, 1991). While slightly lower than the loss-of-function frequency for a typical gene (Brenner, 1974; Meneely and Herman, 1979; Greenwald and Horvitz, 1980), this frequency is much higher than the mutagenesis rate for an average base pair by EMS, which is estimated to be between 1/150,000 and 1/500,000 genomes (Anderson, 1995; de Bono et al., 1995). If other base changes could cause a *ces-1* gain-of-function phenotype, these changes most likely would be too rare to have been detected.

Very few gain-of-function mutations caused by single-base changes in regulatory DNA are known in animals. The only previous example of such a mutation described in *C. elegans* is in the *her-1* gene. In this case, a single-base change leads to the upregulation of the *her-1* transcript and a gain-of-function phenotype (Trent et al., 1988; Perry et al., 1994). The site affected is predicted to be a binding site for the SDC-1 zinc finger protein, which genetically acts upstream of and as a negative regulator of *her-1* (Villeneuve and Meyer, 1987; Nonet and Meyer, 1991). In *Drosophila*, there is also one clear example: two alleles of the Antennapedia complex, *Hab-1* and *Hab-2*, appear to result from the same single-base change, which disrupts a binding site for the transcriptional repressor Krüppel and as a consequence leads to the misexpression of the *abd-A* gene (Shimell et al., 1994).

How does the *ces-1* gain-of-function mutation cause the NSM sisters and I2 sisters to survive? By analogy with the mutations described above, the *ces-1* mutation seems most likely to lead to the loss of binding of a transcriptional repressor, allowing either overexpression of *ces-1* in cells in which *ces-1* normally functions or misexpression in cells in which *ces-1* is not normally expressed. The CES-2 protein is a candidate for being such a repressor. CES-2 might function specifically in the NSM sister cells, since *ces-2* loss-of-function mutations lead to NSM sister cell survival. However, our results indicate that the *ces-1* gain-of-function mutation does not significantly reduce the ability of CES-2 protein to bind in vitro. It is possible that in a cellular context this mutation does affect CES-2 binding: chromatin structure or other proteins might modulate CES-2 binding specificity (Suckow and Hollenberg, 1998). For instance, since many bZIP proteins function as heterodimers in vivo, the binding affinity of CES-2 might be modulated by a bZIP partner (Kerppola and Curran, 1993). Finally, *ces-1* gain-of-function mutations lead to survival of both NSM sisters and I2 sisters, whereas *ces-2* loss-of-function mutations lead only to NSM sister survival. These observations suggest that the *ces-1* gain-of-function mutations disrupt the binding of another, possibly I2 sister–specific, regulatory factor.

Another candidate for being a *ces-1*-negative regulatory factor is the CES-1 protein itself, since the *ces-1* upstream element contains a number of sites with high similarity to Snail family consensus-binding sites and can bind CES-1 protein (our unpublished observations). Although the sequence around the site of the gain-of-function mutations has some similarity to a Snail family consensus-binding site, CES-1 does not seem to bind to this site in vitro. Again, one possibility is that such binding can be detected only in a cellular context. Autoregulation is not unusual for eukaryotic transcription factors (Bateman, 1998), and, for transcriptional repressors, may be involved in modulating the temporal expression of the gene.

Targets of CES-1

What genes are likely to be regulated by CES-1 to control programmed cell death in the NSM sisters and I2 sisters? Our data suggest that *ces-1* requires the activity of the *ced-9* gene to block programmed cell death in the NSM sisters. These results suggest that *ces-1* functions to block programmed cell death in the NSM sisters by potentiating the activity of *ced-9*. Since both *ces-1* and *ced-9* function to block programmed cell death, CES-1 is predicted either to transcriptionally upregulate *ced-9* or a gene (or genes) that potentiates the activity of *ced-9* or to transcriptionally downregulate a gene (or genes) that antagonizes the activity of *ced-9*. A candidate for the latter gene class is *egl-1*. Genetically *egl-1* acts downstream of *ces-1* and upstream of *ced-9* to antagonize *ced-9* activity (Conradt and Horvitz, 1998). Numerous Snail family–like binding sites are present in genomic regions around *egl-1*. However, since recognition sites for Snail family proteins are only seven base pairs in length, it is not possible to predict whether any of these sites are actually bound by CES-1 protein in vivo. It will be important in future experiments to test whether *egl-1* is indeed a target of CES-1 transcriptional regulation and thus a link between cell death specification and cell death execution in *C. elegans*.

Evolutionary Conservation of *ces-1*

In mammalian cells, the E2A:HLF oncogene, a transcriptional activator with the same target specificity as the CES-2/PAR family (Hunger et al., 1994; Inaba et al., 1994; Metzstein et al., 1996), has been proposed to upregulate a CES-1-like activity and thus block programmed cell death in pro-B cells (Inaba et al., 1996; Metzstein et al., 1996). According to this model, the *ces-1* gene could be a direct transcriptional target of the CES-2 protein. In this paper, we provide support for this model, since we

have identified in *ces-1* regulatory DNA an evolutionarily conserved site that can be bound by the CES-2 protein.

Further evidence has emerged that the *ces-2/ces-1*-like pathway may be evolutionarily conserved. Inukai et al. (1999) (this issue of *Molecular Cell*) have identified a zinc finger protein similar in sequence to CES-1. This protein, called SLUG, shows properties expected of a human CES-1 homolog: the transcription of SLUG is regulated by the E2A:HLF CES-2-like oncogene, and enforced expression of SLUG blocks programmed cell death in human pro-B cells. These data suggest that a transcriptionally regulated *ces-1*-like activity may be at least in part responsible for blocking programmed cell death in leukemic pro-B cells.

Experimental Procedures

General Methods

C. elegans was raised using standard methods (Brenner, 1974). We followed standard methods for DNA manipulation (Ausubel et al., 1993).

ces-1 Rescue and Gene Disruptions

To assay *ces-1* rescue, we used standard procedures (Mello and Fire, 1995) to inject test DNA into animals of the genotype *unc-55(e402) ces-1(n703 n1406) ces-2(n732*ts*); unc-76(e911)* using the *unc-76* rescuing plasmid pU76-16B (Bloom and Horvitz, 1997) as a transformation marker. We established transgenic lines (non-Unc-76) at 20° and grew the lines at 25° for at least one generation to score the temperature-sensitive Ces-2 phenotype of NSM sister survival. The appearance of NSM sisters was scored by direct observation using Nomarski differential interference contrast microscopy, as previously described (Ellis and Horvitz, 1991). Lines containing pU76-16B alone had 2.7% NSM sister survival (n = 330 possible NSM sisters, in three lines). We considered lines with greater than 30% of the NSM sisters surviving to be rescued (*ces-2(n732*ts*)* animals had 37% NSM sister survival at 25°). Cosmids were injected at 20 ng/μl each. Plasmids and pU76-16B were injected at 50 ng/μl each. For characterization of the *ces-1*(gf) phenotype induced by a *ces-1* transgene, we injected animals of the genotype *unc-76(e911)* with pU76-16B with and without the *ces-1* minimal rescuing fragment pMM#5. We established and scored these transgenic lines at 20°.

To disrupt the calponin family member, we digested the 7.7 kb minimal rescuing construct at a unique NcoI site located in the second exon of the calponin, filled in the overhang with Klenow fragment polymerase, and religated the ends. This procedure introduced a 4 bp insertion into the calponin gene, creating a frameshift mutation about halfway through the coding sequence. The altered gene is predicted to encode the first 82 amino acids of the original 192–amino acid protein followed by 19 amino acids of unrelated sequence and then a stop codon. The C-terminal region, which should be missing in the modified product, includes highly conserved and functionally important domains of the calponin family (reviewed by el-Mezgueldi, 1996). To disrupt the zinc finger gene, we inserted an oligonucleotide linker containing an in-frame stop codon at an SphI site within the second (of five) predicted fingers of the putative zinc finger gene. We restored the zinc finger gene by digestion with SphI, removing the oligonucleotide, and religated the construct.

ces-1 Sequence and cDNAs

We determined the sequence of the 7.7 kb *ces-1* minimal rescuing fragment by a shotgun procedure using an ABI 373A sequencer. The sequence was identical to that obtained by the *C. elegans* genome sequencing project (bases 26315–34044 of cosmid F43G9; Waterston et al., 1997). We isolated cDNAs encoded in the rescuing fragment from a mixed stage λZAP library and an embryonic λgt11 library (Barstead and Waterston, 1989; Okkema and Fire, 1994). For RACE, we used the 5′ RACE system (GIBCO-BRL Life Technologies).

ces-1 Allele Analysis

To identify base changes, we used PCR to amplify fragments using genomic DNA isolated from wild-type and mutant animals as templates. The fragments were purified using agarose gels and then used directly in sequencing reactions.

To scan for mutations not in *ces-1*-coding sequences, we applied a CCM procedure (Smooker and Cotton, 1993) using hydroxylamine to modify mismatched cytosine residues.

Protein Production

We made a GST:CES-1 zinc finger fusion protein (henceforth called GST:CES-1) construct by cloning a 463 bp ApoI/EcoRV fragment from a *ces-1* cDNA (encoding amino acids 117–270 of CES-1) into plasmid pGEX-4T-3 (Pharmacia) digested with EcoRI/SmaI. We produced and purified GST:CES-1 protein and GST:Snail protein from *E. coli*, following the protocol used for GST:Snail, essentially as described by Ip et al. (1992).

We constructed a CES-2 bZIP in vitro transcription/translation construct by first cloning a 313 bp NlaIV/EcoRV fragment from a full-length *ces-2* cDNA (encoding amino acids 109–210 of CES-2) (Metzstein et al., 1996) into plasmid pAS1 (Bai and Elledge, 1997) digested with SmaI. We then cloned a 330 bp NdeI/SalI fragment from this construct into plasmid pCITE4a (Novagen) digested with NdeI/SalI. We produced protein in vitro using the TnT coupled reticulocyte system (Promega) and T7 RNA polymerase, according to the manufacturer's protocols.

DNA Binding Assays

Assays were performed in a total volume of 20 μl containing 20,000–30,000 CPM probe, 1× buffer, and various amounts of protein. For GST:CES-1, the 1× buffer comprised: 25 mM HEPES (pH 7.5), 100 mM KCl, 10 μg/ml poly dI·dC, 10% glycerol, 3 mM $MgCl_2$, 20 μg/ml BSA, 1 mM EDTA, 0.05% Nonidet NP-40, and 1 mM DTT. For IVT CES-2, the 1× buffer comprised: 10 mM HEPES (pH 7.5), 133 mM KCl, 150 μg/ml poly dI·dC, 10% glycerol, 500 μg/ml BSA, 0.1 mM EDTA, and 0.25 mM DTT. Reactions were incubated at either 20° or 30° for 30 min and then separated using 6% polyacrylamide gels made up in 1× TGE + 2.5% glycerol (1× TGE = 25 mM Tris, 190 mM glycine, 1 mM EDTA) and using 1× TGE as the running buffer. Gels were run at room temperature at 35 mA, dried, and exposed and quantitated on a phosphorimager detection system. The sequences of the probes used for testing CES-2 binding were: optimal, ATTACGTAAT; mutant, AACACGTGTT; site in *ces-1*, 5′ RTATGATGTAAC (where R is G for the wild-type site, and R is A for the *ces-1* gain-of-function site).

Acknowledgments

We thank Gillian Stanfield, Rajesh Ranganathan, and Peter Reddien for comments concerning the manuscript; Bill Achanzar and Sam Ward for providing the map location of *fer-1* prior to publication; Giovanni Lesa and Paul Sternberg for their CCM protocol; Terry Snutch and David Baillie for their *C. briggsae* genomic library; Susan Gray and Michael Levine for the GST:Snail clone; Tania Baker for use of her phosphorimaging system; Bart Jones and Tom Look for HLF bandshift protocols; Laird Bloom for pU76-16B; Bob Barstead and Peter Okkema for cDNA libraries; and Alan Coulson for cosmids. We are particularly grateful to Beth James for DNA sequence determinations, Joel Pomerantz for bandshift advice, and Tom Look for communicating results prior to publication. This work was supported by United States Public Health Service Research grant GM24663 to H. R. H. M. M. M. was supported by a Howard Hughes Medical Institute predoctoral fellowship. H. R. H. is an Investigator of the Howard Hughes Medical Institute.

Received April 13, 1999; revised June 17, 1999.

References

Achanzar, W.E., and Ward, S. (1997). A nematode gene required for sperm vesicle fusion. J. Cell Sci. *110*, 1073–1081.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. (1990). Basic local alignment search tool. J. Mol. Biol. *215*, 403–410.

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

Aroian, R.V., Lesa, G.M., and Sternberg, P.W. (1994). Mutations in the *Caenorhabditis elegans let-23* EGFR-like gene define elements important for cell-type specificity and function. EMBO J. *13*, 360–366.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. (1993). Current Protocols in Molecular Biology (New York: Greene and Wiley-Interscience).

Bai, C., and Elledge, S.J. (1997). Gene identification using the yeast two-hybrid system. Methods Enzymol. *283*, 141–156.

Barstead, R.J., and Waterston, R.H. (1989). The basal component of the nematode dense-body is vinculin. J. Biol. Chem. *264*, 10177–10185.

Bateman, E. (1998). Autoregulation of eukaryotic transcription factors. Prog. Nucleic Acid Res. Mol. Biol. *60*, 133–168.

Bier, E., Vaessin, H., Younger-Shepherd, S., Jan, L.Y., and Jan, Y.N. (1992). *deadpan*, an essential pan-neural gene in *Drosophila*, encodes a helix-loop-helix protein similar to the *hairy* gene product. Genes Dev. *6*, 2137–2151.

Bloom, L., and Horvitz, H.R. (1997). The *Caenorhabditis elegans* gene *unc-76* and its human homologs define a new gene family involved in axonal outgrowth and fasciculation. Proc. Natl. Acad. Sci. USA *94*, 3414–3419.

Boulay, J.L., Dennefeld, C., and Alberga, A. (1987). The *Drosophila* developmental gene *snail* encodes a protein with nucleic acid binding fingers. Nature *330*, 395–398.

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

Cohen, M.E., Yin, M., Paznekas, W.A., Schertzer, M., Wood, S., and Jabs, E.W. (1998). Human SLUG gene organization, expression, and chromosome map location on 8q. Genomics *51*, 468–471.

Conradt, B., and Horvitz, H.R. (1998). The *C. elegans* protein EGL-1 is required for programmed cell death and interacts with the Bcl-2-like protein CED-9. Cell *93*, 519–529.

de Bono, M., Zarkower, D., and Hodgkin, J. (1995). Dominant feminizing mutations implicate protein-protein interactions as the main mode of regulation of the nematode sex-determining gene *tra-1*. Genes Dev. *9*, 155–167.

el-Mezgueldi, M. (1996). Calponin. Int. J. Biochem. Cell Biol. *28*, 1185–1189.

Ellis, R.E., and Horvitz, H.R. (1991). Two *C. elegans* genes control the programmed deaths of specific cells in the pharynx. Development *112*, 591–603.

Favello, A., Hillier, L., and Wilson, R.K. (1995). Genomic DNA sequencing methods. Methods Cell Biol *48*, 551–569.

Fitch, D.H.A., and Thomas, W.K. (1997). Evolution. In *C. elegans* II, R.L. Riddle, T. Blumenthal, B.J. Meyer, and J. R. Priess, eds. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press), pp. 815–850.

Greenwald, I.S., and Horvitz, H.R. (1980). *unc-93(e1500)*: a behavioral mutant of *Caenorhabditis elegans* that defines a gene with a wild-type null phenotype. Genetics *96*, 147–164.

Hayashi, S., Hirose, S., Metcalfe, T., and Shirras, A.D. (1993). Control of imaginal cell development by the *escargot* gene of *Drosophila*. Development *118*, 105–115.

Hengartner, M.O., and Horvitz, H.R. (1994). 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., Ellis, R.E., and Horvitz, H.R. (1992). *Caenorhabditis elegans* gene *ced-9* protects cells from programmed cell death. Nature *356*, 494–499.

Hunger, S.P., Brown, R., and Cleary, M.L. (1994). DNA-binding and transcriptional regulatory properties of hepatic leukemia factor (HLF) and the t(17;19) acute lymphoblastic leukemia chimera E2A-HLF. Mol. Cell. Biol. *14*, 5986–5996.

Inaba, T., Shapiro, L.H., Funabiki, T., Sinclair, A.E., Jones, B.G., Ashmun, R.A., and Look, A.T. (1994). DNA-binding specificity and trans-activating potential of the leukemia-associated E2A-hepatic leukemia factor fusion protein. Mol. Cell. Biol. *14*, 3403–3413.

Inaba, T., Inukai, T., Yoshihara, T., Seyschab, H., Ashmun, R.A., Canman, C.E., Laken, S.J., Kastan, M.B., and Look, A.T. (1996). Reversal of apoptosis by the leukaemia-associated E2A-HLF chimaeric transcription factor. Nature *382*, 541–544.

Inukai, T., Inoue, A., Kurosawa, H., Goi, K., Shinjyo, T., Ozawa, K., Mao, M., Inaba, T., and Look, A.T. (1999). *SLUG*, a *ces-1*-related zinc finger transcription factor gene with antiapoptotic activity, is a downstream target of the E2A-HLF oncoprotein. Mol. Cell *4*, this issue, 343–352.

Ip, Y.T., Park, R.E., Kosman, D., Bier, E., and Levine, M. (1992). The *dorsal* gradient morphogen regulates stripes of *rhomboid* expression in the presumptive neuroectoderm of the *Drosophila* embryo. Genes Dev. *6*, 1728–1739.

Jiang, C., Baehrecke, E.H., and Thummel, C.S. (1997). Steroid regulated programmed cell death during *Drosophila* metamorphosis. Development *124*, 4673–4683.

Kerppola, T.K., and Curran, T. (1993). Selective DNA bending by a variety of bZIP proteins. Mol. Cell. Biol. *13*, 5479–5489.

Klug, A., and Schwabe, J.W. (1995). Protein motifs 5. Zinc fingers. FASEB J. *9*, 597–604.

Krause, M., Park, M., Zhang, J.M., Yuan, J., Harfe, B., Xu, S.Q., Greenwald, I., Cole, M., Paterson, B., and Fire, A. (1997). A *C. elegans* E/Daughterless bHLH protein marks neuronal but not striated muscle development. Development *124*, 2179–2189.

Lockshin, R.A., Zakeri, Z., and Tilly, J. (1998). When Cells Die: a Comprehensive Evaluation of Apoptosis and Programmed Cell Death (New York: Wiley-Liss).

Mauhin, V., Lutz, Y., Dennefeld, C., and Alberga, A. (1993). Definition of the DNA-binding site repertoire for the *Drosophila* transcription factor SNAIL. Nucleic Acids Res. *21*, 3951–3957.

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

Meneely, P.M., and Herman, R.K. (1979). Lethals, steriles and deficiencies in a region of the X chromosome of *Caenorhabditis elegans*. Genetics *92*, 99–115.

Metzstein, M.M., Hengartner, M.O., Tsung, N., Ellis, R.E., and Horvitz, H.R. (1996). Transcriptional regulator of programmed cell death encoded by *Caenorhabditis elegans* gene *ces-2*. Nature *382*, 545–547.

Metzstein, M.M., Stanfield, G.M., and Horvitz, H.R. (1998). Genetics of programmed cell death in *C. elegans*: past, present and future. Trends Genet. *14*, 410–416.

Nonet, M.L., and Meyer, B.J. (1991). Early aspects of *Caenorhabditis elegans* sex determination and dosage compensation are regulated by a zinc-finger protein. Nature *351*, 65–68.

Okkema, P.G., and Fire, A. (1994). The *Caenorhabditis elegans* NK-2 class homeoprotein CEH-22 is involved in combinatorial activation of gene expression in pharyngeal muscle. Development *120*, 2175–2186.

Perry, M.D., Trent, C., Robertson, B., Chamblin, C., and Wood, W.B. (1994). Sequenced alleles of the *Caenorhabditis elegans* sex-determining gene *her-1* include a novel class of conditional promoter mutations. Genetics *138*, 317–327.

Pettmann, B., and Henderson, C.E. (1998). Neuronal cell death. Neuron *20*, 633–647.

Roark, M., Sturtevant, M.A., Emery, J., Vaessin, H., Grell, E., and Bier, E. (1995). *scratch*, a pan-neural gene encoding a zinc finger protein related to *snail*, promotes neuronal development. Genes Dev. *9*, 2384–2398.

Robinow, S., Talbot, W.S., Hogness, D.S., and Truman, J.W. (1993). Programmed cell death in the *Drosophila* CNS is ecdysone-regulated and coupled with a specific ecdysone receptor isoform. Development *119*, 1251–1259.

Robinow, S., Draizen, T.A., and Truman, J.W. (1997). Genes that induce apoptosis: transcriptional regulation in identified, doomed neurons of the *Drosophila* CNS. Dev. Biol. *190*, 206–213.

Rudin, C.M., and Thompson, C.B. (1997). Apoptosis and disease: regulation and clinical relevance of programmed cell death. Annu. Rev. Med. *48*, 267–281.

Schwartz, L.M. (1992). Insect muscle as a model for programmed cell death. J. Neurobiol. *23*, 1312–1326.

Shimell, M.J., Simon, J., Bender, W., and O'Connor, M.B. (1994). Enhancer point mutation results in a homeotic transformation in *Drosophila*. Science *264*, 968–971.

Smooker, P.M., and Cotton, R.G. (1993). The use of chemical reagents in the detection of DNA mutations. Mutat. Res. *288*, 65–77.

Suckow, M., and Hollenberg, C.P. (1998). The activation specificities of wild-type and mutant Gcn4p in vivo can be different from the DNA binding specificities of the corresponding bZip peptides in vitro. J. Mol. Biol. *276*, 887–902.

Trent, C., Wood, W.B., and Horvitz, H.R. (1988). A novel dominant transformer allele of the sex-determining gene *her-1* of *Caenorhabditis elegans*. Genetics *120*, 145–157.

Villeneuve, A.M., and Meyer, B.J. (1987). *sdc-1*: a link between sex determination and dosage compensation in C. elegans. Cell *48*, 25–37.

Waterston, R.H., Sulston, J.E., and Coulson, A.R. (1997). The Genome. In *C. elegans* II, R.L. Riddle, T. Blumenthal, B.J. Meyer, and J.R. Priess, eds. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press), pp. 23–46.

Zar, J.H. (1974). The binomial distribution. In Biostatistical Analysis (Englewood Cliffs, N.J.: Prentice-Hall), pp. 281–300.

GenBank Accession Number

The accession numbers for the sequences reported in this paper are AF169201 (*C. elegans ces-1*) and AF176377 (*C. briggsae ces-1*).