The TRA-1A Sex Determination Protein of C. elegans Regulates Sexually Dimorphic Cell Deaths by Repressing the egl-1 Cell Death Activator Gene

Barbara Conradt* and H. Robert Horvitz†
*Howard Hughes Medical Institute
™Department of Biology
Massachusetts Institute of Technology
Cambridge, Massachusetts 02319

Summary

The hermaphrodite-specific neurons (HSNs) of the nematode Caenorhabditis elegans are generated embryonically in both hermaphrodites and males but undergo programmed cell death in males. The gene egl-1 encodes a BH3-containing cell death activator that is required for programmed cell death in C. elegans. Gain-of-function (gf) mutations in egl-1 cause the inappropriate programmed cell death of the HSNs in hermaphrodites. These mutations lie 5.6 kb downstream of the egl-1 transcription unit and disrupt the binding of the TRA-1A zinc finger protein, the terminal global regulator of somatic sexual fate. This disruption results in the activation of the egl-1 gene in the HSNs not only in males but also in hermaphrodites. Our findings suggest that in hermaphrodites TRA-1A represses egl-1 transcription in the HSNs to prevent these neurons from undergoing programmed cell death.

Introduction

Programmed cell death is a fundamental feature of animal development, and the mechanisms of programmed cell death have been conserved through evolution (reviewed by Ellis et al., 1991; Jacobson et al., 1997). Programmed cell death is necessary for normal neurogenesis in both vertebrates and invertebrates (reviewed by Oppenheimer, 1991; Pettmann and Henderson, 1998) and can result in sexual dimorphism within the nervous system (Truman, 1984; Oppenheimer, 1991). For example, a greater extent of programmed cell death in the development of the female nervous system contributes to the formation of sexually dimorphic structures in the song control region of the zebrafish brain (Konishi and Akutagawa, 1985; Kim and DeVoogd, 1989) and in the preoptic area and the bulbocavernosus in mammals (Nordeen et al., 1985; Breedlove, 1986; Davis et al., 1996). In the moth Manduca sexta programmed cell death is required for the formation of the sexually dimorphic pattern of imaginal midline neurons (Thorn and Truman, 1994).

Sexual dimorphism also exists in the nervous system of the nematode Caenorhabditis elegans. The nervous system of the C. elegans hermaphrodite consists of 302 neurons, eight of which are found only in hermaphrodites. C. elegans males, on the other hand, have a total of 381 neurons, 87 of which are found only in males (reviewed by Hodgkin, 1988). Most of the sexually dimorphic neurons are generated by differential numbers of cell divisions or by differential cell fates. However, a few sex-specific neurons, such as the hermaphrodite-specific neurons (HSNs), are a consequence of sexually dimorphic programmed cell death. The HSNs, a pair of serotonergic motor neurons required for egg laying by hermaphrodites, are generated embryonically in both hermaphrodites and males but undergo programmed cell death in males shortly after they are formed (Sulston and Horvitz, 1977; Sulston et al., 1983).

Genetic analyses in C. elegans have lead to the identification of four genes, ced-3, ced-4, ced-9, and egl-1 (ced, cell-death defective; egl, egg-laying defective), that can mutate to block programmed cell death and thus appear to be components of a central programmed cell death pathway (reviewed by Metzstein et al., 1998). The gene ced-3 encodes a member of the caspase family of cysteine proteases (reviewed by Nicholson and Thornberry, 1997), ced-4 encodes a protein with similarities to the mammalian cell death activator Apaf1 (reviewed by Vaux, 1997), ced-9 encodes a Bcl2-like cell death inhibitor (reviewed by Adams and Cory, 1998), and egl-1 encodes a BH3 domain-containing protein similar in structure to the mammalian "BH3 domain-only" cell death activators, which form a subgroup within the family of Bcl2-like molecules (reviewed by Kelekar and Thompson, 1997; Adams and Cory, 1998). Genetically ced-3, ced-4, ced-9, and egl-1 appear to act in a simple pathway in which egl-1 negatively regulates ced-9, ced-9 negatively regulates ced-4, and ced-4 positively regulates ced-3 (reviewed by Metzstein et al., 1998). It has been proposed that the EGL-1 protein activates programmed cell death by binding to and thereby negatively regulating the cell death inhibitor protein CED-9, releasing CED-4 from CED-9 and resulting in the CED-4-dependent activation of the CED-3 caspase (Conradt and Horvitz, 1998; del Peso et al., 1998).

Sexual fate in C. elegans is determined by the ratio of the number of X chromosomes to the number of autosomes (X:A ratio) (reviewed by Meyer, 1997). This primary signal is transmitted through a cascade of interacting genes and determines the level of activity of the terminal, global control gene of somatic sex determination, tra-1 (tra, transformer) (reviewed by Hodgkin, 1988; Meyer, 1997). tra-1 activity is regulated posttranslationally (de Bono et al., 1995), and tra-1 acts cell autonomously to promote female development of the soma (Hodgkin, 1987; Hunter and Wood, 1990). The presence of two X chromosomes (XX) results in high levels of tra-1 activity and, as a consequence, in the formation of an animal with a female soma, specifically, a hermaphrodite. C. elegans hermaphrodites are basically female animals that produce sperm during the L4 stage.) The presence of only one X chromosome (XO) results in low levels of tra-1 activity and, therefore, in the development of an animal with a male soma, that is, a male (Hodgkin, 1987).
The tra-1 gene encodes a DNA-binding protein, TRA-1A, with five zinc fingers most similar to the zinc fingers of the gene products of the segment polarity gene cubitus interruptus (cii) and the pair-rule gene odd-paired of Drosophila and the vertebrate Gli genes, which together form a subfamily within the family of Kruppel-like zinc finger DNA-binding proteins and transcription factors (Zarkower and Hodgkin, 1992; Benedyk et al., 1994). TRA-1A protein binds DNA in vitro in a sequence-specific manner, and it has been proposed that TRA-1A controls sexual fate by transcriptionally activating female-specific and/or by transcriptionally repressing male-specific genes required for sexual differentiation (Zarkower and Hodgkin, 1993). A number of genes have been identified that when mutated result in sex-specific defects (e.g., the mab genes [mab, male abnormal]; Hodgin, 1983), and that therefore might be targets of TRA-1A. However, none of these genes has been shown to be a direct target of TRA-1A.

In this paper, we present data indicating that in the sexually dimorphic HSNs, the cell death activator gene egl-1 is under the direct control of the C. elegans sex determination pathway. egl-1(gf) mutations cause the inappropriate deaths of the HSNs in hermaphrodites (Trent et al., 1983; Ellis and Horvitz, 1986). These egl-1(gf) mutations disrupt a TRA-1A-binding site 5.6 kb downstream of the egl-1 transcription unit and result in the inappropriate activation of the egl-1 gene in the HSNs in hermaphrodites.

**Results**

Dominant gain-of-function (gf) mutations in the egl-1 gene cause the HSNs to inappropriately undergo programmed cell death in hermaphrodites (Trent et al., 1983; Ellis and Horvitz, 1986). Seven such egl-1 mutations have been identified in screens for egg laying-defective mutants (Trent et al., 1983; Desai and Horvitz, 1989; J. Yuan et al., unpublished observations). In a screen for suppressors of the egg laying defect caused by the egl-1(gf) mutation n1084, we identified the mutation n3082 (Conradt and Horvitz, 1998). This mutation suppresses the egl-1(n1084)-induced programmed cell deaths of the HSNs, because it results in the loss of function of a gene encoding a BH3-containing cell death activator required for programmed cell death (Conradt and Horvitz, 1998). The n3082 mutation is linked to the egl-1(n1084gf) mutation, which raised the possibility that the n3082 and n1084 mutations affect the same gene. However, we did not find any mutations in the coding region or in the 5' and 3' untranslated regions of the BH3 gene in animals carrying any of the seven egl-1(gf) mutations. We show below that the egl-1(gf) mutations reside in regulatory regions of the BH3-containing cell death activator gene defined by n3082.

**n1084 Maps about 0.04 Map Units Left of n3082**

The egl-1(gf) mutation n1084 and the n3082 mutation both map to linkage group V between the genetic markers rol-4 (rol, roller) and unc-76 (unc, uncoordinated), which are 2.7 map units apart (Conradt and Horvitz, 1998). To determine how tightly the two mutations are linked, we attempted to separate them using the egl-1(n1084gf) n3082 chromosome, which we identified in our egl-1(n1084gf) suppressor screen, in a four factor-mapping experiment. Starting with a strain of genotype rol-4 unc-76/egl-1(n1084gf) n3082, we screened for Rol non-Unc and Unc non-Rol progeny. Among 70 identified Unc non-Rol progeny, one was of the phenotype Egl Unc non-Rol. The genotype of the recombinant chromosome of this animal was egl-1(n1084gf) unc-76, indicating that the recombination event had occurred between egl-1(n1084gf) and n3082. Among 137 identified Rol non-Unc progeny, none had an Egl phenotype. These findings indicate that the egl-1(n1084gf) mutation maps to the left of n3082 on the genomic map or downstream of n3082 on the physical map (Figure 1). The frequency of recombination between egl-1(n1084gf) and n3082 detected among the Unc non-Rol progeny (1/70) suggests that egl-1(n1084gf) and n3082 are approximately 0.04 map units apart and therefore tightly linked.

**Identification of egl-1(gf) Mutations**

These map data suggest that the egl-1(gf) mutations might affect downstream regulatory regions of the BH3 gene defined by n3082. The egl-1(gf) mutations cause a dominant phenotype. We therefore reasoned that DNA fragments that span the transcription unit of the BH3 gene and in addition carry an egl-1(gf) mutation downstream of this transcription unit might be capable of phenocopying the egl-1(gf) phenotype when introduced into hermaphrodites. To test this hypothesis, we used...
the polymerase chain reaction (PCR) to amplify DNA fragments from animals carrying egl-1(gf) mutations and analyzed these fragments for their abilities to induce the killing of the HSNs in hermaphrodites. We chose to amplify a 7.8 kb fragment that includes the transcription unit of the BH3 gene and 5.8 kb of its downstream region (Figure 2A). A wild-type genomic fragment of this length almost completely rescued the cell death defect caused by the n3082 mutation and hence appears to include most regulatory regions of the BH3 gene (Conradt and Horvitz, 1998). We amplified the 7.3 kb fragment from the wild-type (N2) C. elegans, from egl-1(n1084gf)n3082 animals, and from two strains carrying different egl-1(gf) mutations, n1084 or n986. Using germline transformation, we introduced these fragments into egl-1(n1084gf)n3082 animals, which are cell death defective (Ced) and have HSNs. Thus, we could score the fragments not only for their abilities to induce HSN killing but also for their abilities to rescue the Ced phenotype of egl-1(n1084gf)n3082 animals as a control. The fragment amplified from wild-type animals rescued the Ced phenotype of the egl-1(n1084gf)n3082 animals but did not induce the HSNs to undergo programmed cell death in hermaphrodites (Figure 2B). The fragment derived from egl-1(n1084gf)n3082 animals, as expected, failed to rescue the Ced phenotype and did not induce HSN killing. By contrast, the fragments amplified from egl-1(n1084gf) or egl-1(n986gf) animals rescued the Ced phenotype and induced HSN killing in hermaphrodites (Figure 2B). The observed HSN killing was suppressed by a ced-9(gf) mutation, which blocks programmed cell death, confirming that the HSNs died by programmed cell death (data not shown). These results demonstrate that the 7.8 kb fragments amplified from egl-1(gf) mutants were able to phenocopy the egl-1(gf) dominant phenotype and therefore included the dominant egl-1(gf) mutations.

We then tested a 5.0 kb fragment that spans only the proximal 3.0 kb of the region downstream of the transcription unit of the BH3 gene (Figure 2A). Amplified from egl-1(gf) animals, these fragments still rescued the Ced phenotype of egl-1(n1084gf)n3082 animals but were unable to induce HSN killing in hermaphrodites (Figure 2C). Furthermore, a 7.8 kb fragment composed of the 3‘ 3.6 kb amplified from egl-1(n986gf) animals and the 5‘ 4.2 kb from the wild-type strain (Figure 2A) was capable of inducing HSN killing (Figure 2D), while a fragment containing the wild-type 3‘ 3.6 kb and the egl-1(n986gf) 5‘ 4.2 kb (Figure 2A) failed to do so (Figure 2D). These results suggest that the egl-1(gf) mutations are likely to be located in the 3‘ region of the 7.8 kb fragment.

We determined the nucleotide sequence of the 7.8 kb fragment for each of the seven egl-1(gf) mutants and the wild-type transcription unit of the BH3 gene. Data shown are averages ± standard deviations of results obtained from 12 independent transgenic lines. The 7.8 kb chimeric fragment N2-n3082 was injected into animals of the genotype egl-1(n1084) unc-76(e911). In this case n3082 is in trans to n1084.) Data represent averages ± standard deviations of results obtained from 11 independent transgenic lines.

Figure 2. DNA Fragments Carrying egl-1(gf) Mutations Can Induce HSN Killing

(A) DNA fragments that were amplified by PCR are shown aligned with the physical map of the egl-1 region. (B) Fragments (7.8 kb) amplified from egl-1(gf) animals induced HSN killing. The fragments indicated were amplified by PCR and introduced by germline transformation into hermaphrodites of genotype ced-1(e1735); egl-1(n1084 n3082) unc-76(e911), as described in Experimental Procedures. Rescue of the Ced phenotype was analyzed by counting the number of cell corpses in the heads of transgenic larvae at the first larval stage using Nomarski optics, as described (Conradt and Horvitz, 1998). HSN survival was scored in transgenic animals at the first larval stage using Nomarski optics, as described (Conradt and Horvitz, 1998). Percentages reflect the abilities of various 7.8 kb fragments to rescue the Ced phenotype of the injected animals (black columns) and to induce HSN killing (hatched columns). The data are averages ± standard deviations of results obtained from six (N2), eight (n1084 n3082), four (n1084), and 19 (n986) independent transgenic lines. (C) Fragments (5.0 kb) amplified from egl-1(gf) animals failed to induce HSN killing. The fragments indicated were generated by PCR, introduced into ced-1(e1735); egl-1(n1084 n3082) unc-76(e911) animals, and analyzed for their abilities to cause killing as described in (B). The data are averages ± standard deviations of results obtained from six (N2), four (n1084 n3082), one (n1084), and four (n986) independent transgenic lines. (D) The 3‘ 3.6 kb of fragments amplified from egl-1(gf) animals was sufficient to induce HSN killing. The 7.8 kb chimeric fragments indicated were made as described in Experimental Procedures. Transgenic lines were generated using ced-1(e1735); egl-1(n1084 n3082) unc-76(e911) animals and analyzed as described in (B). The data are averages ± standard deviations of results obtained from four (n986-N2) and seven (N2-n986) independent transgenic lines. (E) The egl-1(gf) mutations were required in cis to a functional transcription unit of the BH3 gene to induce HSN killing. The fragments indicated were generated as described in Experimental Procedures, and data were collected as described in (B). The 7.8 kb n1084 n3082 fragment was injected into animals of the genotype unc-76(e911) to generate transgenic lines. (In this case n1084 was in trans to the wild-type transcription unit of the BH3 gene.) Data shown are averages ± standard deviations of results obtained from 12 independent transgenic lines. The 7.8 kb chimeric fragment N2-n3082 was injected into animals of the genotype egl-1(n1084) unc-76(e911). (In this case n3082 is in trans to n1084.) Data represent averages ± standard deviations of results obtained from 11 independent transgenic lines.
found DNA lesions in all seven mutants. As expected, these lesions are at the 3' end of the 7.8 kb fragment, clustered within five bp of each other 5631 to 5635 bp downstream of the stop codon of the BH3 gene. We found a G-to-A transition at bp +5631 in animals carrying the egl-1(gf) mutations n487, n1084, or n1796, a G-to-A transition at bp +5634 in animals carrying the egl-1(gf) mutations n986, n987, or n2164, and a G-to-A transition at bp +5635 in animals carrying the egl-1(gf) mutation n2248 (Table 1).

The egl-1(gf) Mutations and n3082 Are Allelic

The 7.8 kb fragment amplified from egl-1(n1084gf) animals but not from egl-1(n1084gf) n3082 animals induced the HSNs to undergo programmed cell death (Figure 2B). This result indicates that an intact coding region of the BH3 gene is required for the ability of the egl-1(gf) mutations to induce HSN killing. Furthermore, the fact that the 7.8 kb fragment amplified from wild-type animals did not cause HSN deaths when injected into egl-1(n1084gf) n3082 animals (Figure 2B), or likewise, the fact that a 7.8 kb fragment amplified from egl-1(n1084gf) n3082 animals failed to cause HSN killing in wild-type (+/+) animals (Figure 2E) demonstrates that the egl-1(gf) mutations and an intact coding region of the BH3 gene must be in cis to cause HSN killing. Furthermore, the n3082 mutation failed to suppress the egl-1(gf) mutation n1084 when trans: a chimeric 7.8 kb fragment carrying only the n3082 mutation (Figure 2A) was unable to suppress HSN killing when introduced into egl-1(n1084gf) animals (Figure 2E). These observations indicate that the n3082 mutation and the egl-1(gf) mutation n1084 affect the same transcription unit, the transcription unit encoding the BH3 containing cell death activator, and therefore are allelic. The egl-1 gene and the BH3 gene, hence, are the same and hereafter will be referred to as egl-1. n3082 consequently represents a loss-of-function (lf) mutation in the egl-1 gene, egl-1(n1084 n3082lf).

The egl-1(gf) Mutations Disrupt a Conserved Putative TRA-1A-Binding Site

The egl-1(gf) mutations are located within a stretch of nucleotides the sequence of which is highly similar to the core sequence of a DNA-binding site for the terminal, global regulator of somatic sex in C. elegans, TRA-1A (Zarkower and Hodgkin, 1993) (Table 1). This putative TRA-1A-binding site is located 5629 to 5637 bp downstream of the egl-1 stop codon. The three different G-to-A transitions found in the seven egl-1(gf) mutants are within this core sequence and are predicted to severely affect the ability of TRA-1A to bind (Zarkower and Hodgkin, 1993). To determine whether this putative TRA-1A-binding site has been conserved through evolution, we cloned the egl-1 locus from C. briggsae, a related Caenorhabditis species (our unpublished observations). We found an identical nine bp core sequence (and additional conserved areas) 3794 to 3802 bp downstream of the predicted stop codon of the C. briggsae egl-1 gene (Table 1). This result suggests that this putative TRA-1A-binding site is important for the transcriptional regulation of a target gene. Our finding that this site is required in cis for the proper regulation of egl-1 in the HSNs suggests that the egl-1 gene represents a target of this conserved putative TRA-1A-binding site.

Table 1. The egl-1(gf) Mutations Are Single-Base Changes in a Putative Conserved TRA-1-Binding Site

<table>
<thead>
<tr>
<th>Core</th>
<th>TRA-1-binding site</th>
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<tbody>
<tr>
<td>C. briggsae egl-1 locus</td>
<td>TTTGNNNNTGGGTGGTC</td>
</tr>
<tr>
<td>n986, n987, n2164</td>
<td>CTCCTAACCGGATGGTC</td>
</tr>
<tr>
<td>n1084, n1796</td>
<td>CTCCTAACCGGATGGTC</td>
</tr>
<tr>
<td>n487</td>
<td>CTCCTAACCGGATGGTC</td>
</tr>
<tr>
<td>C. elegans egl-1 locus</td>
<td>TTTGNNNNTGGGTGGTC</td>
</tr>
<tr>
<td>+3801 bp</td>
<td>+3793 bp</td>
</tr>
<tr>
<td>+5645 bp</td>
<td>+5629 bp</td>
</tr>
</tbody>
</table>

The consensus sequence of the TRA-1-binding site was determined by in vitro selection by Zarkower and Hodgkin (1993). The sequences of the putative TRA-1-binding site in the egl-1 locus of C. elegans and C. briggsae were determined by the C. elegans Sequencing Consortium. The sequences of the TRA-1-binding sites of the egl-1 locus of egl-1(gf) mutants were determined as described in Experimental Procedures.

The egl-1(gf) Mutations Cause the Inappropriate Activation of egl-1 in the HSNs in Hermaphrodites

To determine the expression of the egl-1 gene in the HSNs and the effect that the egl-1(gf) mutations in the conserved putative TRA-1A-binding site have on this expression, we made a green fluorescent protein (GFP) reporter construct in which the expression of the gfp gene is under the control of the 5' (1.0 kb upstream of the 5' UTR of egl-1) and 3' (5.6 kb downstream of the 3' UTR of egl-1) regulatory regions of the egl-1 gene (Pegl-1:gfp) (Figure 1). This reporter construct was introduced into animals carrying the ced-3(gf) mutation n717. ced-3 acts downstream of egl-1 genetically, and ced-3(gf) mutations block most if not all programmed cell deaths that occur during development, including the deaths of the HSNs in males. This experimental design allowed us to analyze the expression of the reporter construct in the HSNs in both sexes. While GFP was present in a number of cells, we failed to detect the protein in the HSNs in hermaphrodites carrying extrachromosomal arrays of the reporter construct. None of the HSNs (n = 60) in transgenic hermaphrodites were positive for GFP. By contrast, we detected GFP in 87% of the HSNs (n = 60) in transgenic males (Figure 3). The egl-1 gene therefore appears to be expressed in the HSNs in males, in which the HSNs normally undergo programmed cell death, but not in hermaphrodites, in which the HSNs normally survive.

To determine the effect of the egl-1(gf) mutations on the expression of egl-1 in the HSNs, we introduced the G-to-A transition found in animals carrying the egl-1(gf)
DNA fragments as a probe, and unlabeled wild-type or mutant DNA fragments as competitors. A 10-fold excess of the wild-type competitor was sufficient to reduce TRA-1A binding to the wild-type probe by 50% (Figures 4B and 4C). TRA-1A binding to the wild-type probe was almost completely abolished in the presence of a 100-fold excess of wild-type competitor (Figures 4B and 4C). These observations are in agreement with data obtained by others using a truncated TRA-1A protein (Zarkower and Hodgkin, 1993). Competitor carrying the egl-1(gf) mutation n1084 reduced TRA-1A binding to the wild-type probe by 50% when present at a 500-fold excess (Figures 4B and 4C). Fragments carrying the mutations n2164 or n2248 were less effective competitors than the n1084-containing fragment. At a 500-fold excess, the n2164- and n2248-containing fragments reduced TRA-1A binding to the wild-type probe only by about 25% (Figures 4B and 4C). This result shows that TRA-1A binds to the TRA-1A-binding sites carrying the three different G-to-A transitions, although with reduced affinities, and that TRA-1A has a higher affinity for the n1084-containing binding site than for the n2164- or n2248-containing binding sites.

The Different egl-1(gf) Mutations Have Different Effects on HSN Survival In Vivo
To determine whether the three different G-to-A transitions also behave differently in vivo, we determined their effects on HSN survival in hermaphrodites. Compared to 100% HSN survival in wild-type hermaphrodites, 0% of the HSNs survived in hermaphrodites homozygous for any of the three egl-1(gf) mutations egl-1(n1084gf), egl-1(n2164gf), and egl-1(n2248gf) (Table 2). However, 34% of the HSNs survived in hermaphrodites heterozygous for the mutation n1084 (egl-1(n1084gf)), and 11% and 9% of the HSNs survived in hermaphrodites heterozygous for the mutations n2164 and n2248, respectively (egl-1(n2164gf)+/−, egl-1(n2248gf)+/−). The survival rates of the HSNs in animals heterozygous for n1084, n2164, and n2248 therefore correlate with the affinities with which TRA-1A binds to the mutant TRA-1A-binding sites in vitro. These results are consistent with the hypothesis that TRA-1A acts as a transcriptional repressor of the egl-1 gene in the HSNs.
	ra-1 Specifies the Cell Death Fate of the HSNs and Acts as a Negative Regulator of egl-1
To determine whether the tra-1 gene plays a role in specifying the cell death fate of the HSNs, we analyzed the survival of the HSNs in animals carrying either ff or gf mutations in the tra-1 gene. tra-1(ff) mutations lead to the masculinization of the soma of hermaphrodites (XX) (Hodgkin, 1987). Compared to 100% survival in wild-type XX animals, only 11% of the HSNs survived in XX animals homozygous for the strong tra-1(ff) mutation e1099 (Table 3). Maternal effects have not been reported for the tra-1 gene. However, that 11% of the HSNs survived in essentially male tra-1(e1099ff) XX animals might be a consequence of the presence of maternally derived active TRA-1A protein during embryogenesis, when the HSNs normally die.

Dominant gf mutations in tra-1 cause the synthesis of a TRA-1A protein that is active in both hermaphrodites
and males, resulting in the feminization of the soma of males (XO) (Hodgkin, 1987). While 0% of the HSNs survived in wild-type X0 animals, 82% of the HSNs survived in X0 animals heterozygous for the tra-1(gf) mutation e1575 (tra-1(e1575gf)/+ (Table 3), (That 18% of the HSNs in tra-1(e1575gf) animals died presumably reflects the incomplete feminization of such animals (Hodgkin, 1987), which might have been amplified by the fact that these animals were also heterozygous for a tra-2(lf) mutation.) Thus, both if and gf alleles of tra-1 affected the cell death fate of the HSNs.

To test whether the ability of tra-1 to specify the cell death fate of the HSNs is dependent on egl-1, we analyzed animals carrying mutations in both genes. Ninety-six percent of the HSNs survived in XX animals that carry the egl-1(lf) mutation n3082, but only 11% of the HSNs survived in tra-1(e1099lf) XX animals. In XX animals carrying both mutations, tra-1(e1099lf); egl-1(n1084 n3082lf), 88% of the HSNs were present, suggesting that the egl-1(lf) mutation can suppress the lf mutation in tra-1 (Table 3). Furthermore, only 1% of the HSNs

Table 2. The Three egl-1(gf) Mutations Have Different Effects on HSN Survival in Hermaphrodites

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Percent of HSNs Present (n)</th>
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<tbody>
<tr>
<td>+/+</td>
<td>100 (50)</td>
</tr>
<tr>
<td>egl-1(ln1084gf) +</td>
<td>0 (50)</td>
</tr>
<tr>
<td>egl-1(ln1084gf)/+</td>
<td>34 (82)</td>
</tr>
<tr>
<td>egl-1(n2164gf) +</td>
<td>0 (40)</td>
</tr>
<tr>
<td>egl-1(n2164gf)/+</td>
<td>11 (80)</td>
</tr>
<tr>
<td>egl-1(n2248gf) +</td>
<td>0 (42)</td>
</tr>
<tr>
<td>egl-1(n2248gf)/+</td>
<td>9 (88)</td>
</tr>
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</table>

The presence of HSNs was scored by Nomarski optics (Sulston and Horvitz, 1977). The complete genotypes of the animals scored were as follows: +/unc-76(e111), egl-1(ln1084) +/egl-1(ln1084) unc-76(e111), egl-1(ln1084)/+/unc-76(e111), egl-1(n2164)+/egl-1(n2164) unc-76(e111), egl-1(n2164)/+/unc-76(e111), egl-1(n2248)+/egl-1(n2248) unc-76(e111), egl-1(n2248)/+/unc-76(e111), n, number of HSNs.

Table 3. egl-1 Mutations Suppress the Effects of tra-1 Mutations on the Fate of the HSNs

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Percent of HSNs Present (n)</th>
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<tr>
<td></td>
<td>XX</td>
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<tr>
<td>+/+</td>
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<tr>
<td>egl-1(ln1084 n3082lf)</td>
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</tr>
<tr>
<td>egl-1(n2164gf)/+</td>
<td>11 (80)</td>
</tr>
<tr>
<td>tra-1(e1099lf)</td>
<td>11 (46)</td>
</tr>
<tr>
<td>tra-1(e1099lf);</td>
<td>88 (46)</td>
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<tr>
<td>egl-1(n1084 n3082lf)</td>
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<td>tra-1(e1575gf)/+</td>
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<tr>
<td>egl-1(n2164gf)/+</td>
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</tbody>
</table>

The presence of HSNs was scored as described by Sulston and Horvitz (1977). The complete genotypes of the animals scored were as follows: +/unc-76(e911), egl-1(ln1084 n3082)/+egl-1(ln1084 n3082) unc-76(e911), egl-1(ln2164)+/+ unc-76(e911), tra-1(e1099), tra-1(e1099); egl-1(ln1084 n3082), tra-2(e1095)/--; tra-1(e1735)/--; him-5(e1490)/--; axIs36, tra-2(e1095)/--; tra-1(e1735)/--; him-5(e1490)/egl-1(n2164); axIs36. ND, not determined.
A

\[ \text{tra-1} \rightarrow \text{egl-1} \rightarrow \text{ced-9} \rightarrow \text{ced-4} \rightarrow \text{ced-3} \rightarrow \text{Cell death} \]

1. served TRA-1A-binding site near the egl-1 locus and Horvitz, 1986. (2) These gf mutations affect a conserved pathway of programmed cell death (Trent et al., 1983; Ellis et al., 1983; Horvitz, 1986). (3) The egl-1(gf) mutation is able to suppress transcription in these neurons (Figure 5A). The inability of TRA-1A to repress egl-1, as occurs in wild-type animals, may be a consequence of a disrupted site downstream of the egl-1 transcription unit negatively regulates an HSN-specific activator of egl-1 transcription (see below), resulting in the repression of the locus and HSN survival. In wild-type males (X0), the level of TRA-1A activity is too low to negatively regulate the HSN-specific activator, which allows the activation of the egl-1 gene and the deaths of the HSN. In egl-1(gf) hermaphrodites, TRA-1A activity is high but TRA-1A is unable to negatively regulate the HSN-specific activator because its binding site is disrupted, resulting in inappropriate activation of egl-1 in the HSNs in hermaphrodites and their deaths in the sex in which they normally survive. In egl-1(n1084 n3082) hermaphrodites (egl-1(1lf)), the egl-1 gene is also appropriately activated, but because the egl-1 coding region carries a mutation, the HSNs in hermaphrodites do not survive. In wild-type males (X0), the level of TRA-1A activity is too low to negatively regulate the HSN-specific activator, which allows the activation of the egl-1 gene and the deaths of the HSN. In egl-1(gf) hermaphrodites, TRA-1A activity is high but TRA-1A is unable to negatively regulate the HSN-specific activator because its binding site is disrupted, resulting in inappropriate activation of egl-1 in the HSNs in hermaphrodites and their deaths in the sex in which they normally survive. In egl-1(n1084 n3082) hermaphrodites (egl-1(1lf)), the egl-1 gene is also appropriately activated, but because the egl-1 coding region carries a mutation, the HSNs in hermaphrodites do not survive. (4) The tra-1 gene acts genetically as a negative regulator of egl-1 in the HSNs.

B

<table>
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<th>Genotype</th>
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<td>HIGH</td>
<td>ON</td>
<td>+</td>
</tr>
</tbody>
</table>

Figure 5. tra-1 is a Negative Regulator of egl-1 in the HSNs. The transcriptional regulation of F23B12.1 in the HSNs involves another factor, such as the neural-specific activator of egl-1 expression, and that TRA-1 specifically blocks this activator.

Discussion

TRA-1A Represses egl-1 Transcription in the HSNs

Based on the following observations we propose that the egl-1 gene is regulated at the transcriptional level in the sexually dimorphic HSNs and that TRA-1A acts as a repressor of egl-1 transcription in these neurons. (1) egl-1(gf) mutations cause the deaths of the HSNs in hermaphrodites by inappropriately activating the pathway of programmed cell death (Trent et al., 1983; Ellis and Horvitz, 1986). (2) These gf mutations affect a conserved TRA-1A-binding site near the egl-1 transcription unit, and this site is required in cis to the egl-1 transcription unit for the proper regulation of the egl-1 gene in the HSNs. (3) The egl-1(gf) mutations strongly reduce the ability of TRA-1A to bind to this site in vitro and cause the inappropriate transcriptional activation of the egl-1 gene in vivo. (4) The tra-1 gene acts genetically as a negative regulator of egl-1 in the HSNs.
DNA-binding sites that act at a distance have also been characterized for the Drosophila Ci protein and the vertebrate Gli protein, two other members of the TRA-1A/Ci/Gli family of transcription factors. Ci acts as a transcriptional activator of the patched and wingless genes in vivo through three Ci-binding sites about 600 to 750 bp upstream of the transcriptional start site in the case of the patched gene (Alexandre et al., 1996) and through four Ci-binding sites 5.7 to 4.7 kb upstream of the transcriptional start site in the case of the wingless gene (von Ohlen et al., 1997). A single binding site for the mammalian Gli protein located 6 kb downstream of the transcription unit encoding hepatic nuclear factor-l (HNF-3β) has been found to be required for correct HNF-3β expression (Sasaki et al., 1997).

Ci and Gli proteins have been shown to act as repressors and activators of transcription in vivo (reviewed by J ohnson and Scott, 1998). It remains to be determined whether TRA-1A can act not only as a repressor but also as an activator of transcription in C. elegans.

The Dominant egl-1(gf) Phenotype Is a Result of the Loss of Binding of a Repressor

The egl-1(gf) mutations, which are single-base changes in a TRA-1A-binding site, strongly reduce the ability of TRA-1A to bind to this site in vitro. The loss or reduction of TRA-1A binding results in the inappropriate activation of egl-1, which accounts for the dominant nature of the egl-1(gf) mutations. There are only a few examples in multicellular organisms in which the loss of binding of a transcriptional repressor results in a dominant phenotype. The Abd-A (Abd-A, abdominal A) gene of the Drosophila bithorax complex is required for the specification of the identity of the second through fourth abdominal segments (A2-A4). Mutations that disrupt the binding site for the transcriptional repressor Krüppel in an intron of the Abd-A transcription unit (Hab mutations, hyperabdominal) cause ectopic expression of Abd-A in the third thoracic segment (T3), which results in the dominant transformation of T3 toward A2 (Shimell et al., 1994). Similarly, dominant gf mutations in the promoter 1 (P1) of the C. elegans gene ccd-1 (her-1, hermaphroditization), an upstream gene in the regulatory pathway for sex determination, are thought to disrupt a binding site for the gene product of the add-1 gene (add, sex and dosage compensation), a protein containing seven zinc fingers (Nonet and Meyer, 1991) and that acts as a negative regulator of her-1 (Trent et al., 1991; Perry et al., 1994).

Transcriptional Regulation of egl-1 Might Integrate Cell Death Regulatory Signals

It is possible that the activity of egl-1 is regulated at a transcriptional level not only in the HSNs but also in all other somatic cells. Our preliminary data suggest that the egl-1 gene is transcriptionally active specifically in cells that are destined to die during development (our unpublished observations). The transcriptional regulation of egl-1 might therefore be complex. Consistent with this hypothesis is our observation that extensive regions of identity, suggestive of extensive cis regulatory regions, exist between the C. elegans and the C. briggsae sequences downstream of the egl-1 transcription unit (our unpublished observations).

Regulation at the transcriptional level might also be the mechanism by which the activity of the mammalian protein DP-5 (Imaizumi et al., 1997), another member of the subfamily of BH3 domain±only cell death activators, is controlled. The transcriptional upregulation of the DP-5 gene in cultured sympathetic neurons after the removal of nerve growth factor coincides with the programmed deaths of these cells (Imaizumi et al., 1997). Other mammalian BH3 domain±only cell death activators, Bad and Bid for example, appear to be regulated at the posttranslational level. The ability of Bad to bind to and thereby negatively regulate Bcl2-like cell death inhibitors depends on its state of phosphorylation, which is regulated by the presence or absence of extracellular survival factors via the phosphoinositide 3-kinase/Akt signal transduction cascade (reviewed by Franke and Cantley, 1997). Bid appears to be activated through specific Caspase 8-dependent cleavage following cell death signaling through the tumor necrosis factor receptor or by Fas (Li et al., 1998; Luo et al., 1998).

egl-1 Acts at an Interface between Pathways that Specify Cell Fate and that Execute a Specific Cell Fate

We have shown that the egl-1 gene is under the direct control of the sex determination pathway. egl-1 may also be the target of the ces-1 and ces-2 (ces, cell death specification) pathway, which is involved in the cell death specification of the sister cells of the NSM neurosecretory motor neurons (Ellis and Horvitz, 1991; Metzstein et al., 1996) and which appears to act upstream of egl-1 genetically (Conradt and Horvitz, 1998). Thus, egl-1 may generally act at the interface between pathways that specify cell fate during C. elegans development and a pathway that is involved in the execution of a cell fate, programmed cell death. Such a role is similar to that of the Drosophila gene string, which encodes a CDC25-type tyrosine protein phosphatase required for cell cycle progression (reviewed by Follette and O‘Farrell, 1997). The pattern of embryonic cell division cycles during Drosophila embryogenesis is determined by pattern-forming genes that transmit developmental cues, such as positional information, to the string locus. This pattern is then executed through the transcriptional activation of the string gene, the product of which triggers mitosis (Edgar et al., 1994). Like the string locus of Drosophila, the egl-1 locus of C. elegans might integrate various developmental signals and translate these signals into action, that is, the execution of another cell division cycle in the case of string and the execution of programmed cell death in the case of egl-1.

An analysis of the regulation of the egl-1 gene might identify the pathways that determine the fate of programmed cell death in all somatic cells that are destined to die during C. elegans development. Such studies should help reveal how multiple developmental signals are integrated and how the process of programmed cell death is used during development not only to create sexual dimorphism within the nervous system but more generally to form a functional organism.
Experimental Procedures

General Methods and Strains
C. elegans strains were cultured at 20 °C unless otherwise noted as described by Brenner (1974). The strain N2 (Bristol) was used as wild-type strain. Mutations used in this study are listed below and are described by Riddle et al. (1997), except where noted otherwise. LGI: ced-1(e17355). LGII: tra-1(e1575); tra-1(e1099), unc-69(e587). LGIV: ced-3(n717). LGV: rol-4(sc8), him-5(e1490), egf-1(n487), egf-1(n896), egf-1(n897), egf-1(n1084), egf-1(n1796), egf-1(n2164), egf-1(n2248) (J. Yuan et al., unpublished data), egf-1(n1084) n3082 (Conradt and Horvitz, 1998), unc-76(e911). LGX: lin-15(n765); axs36 (Ppesc-10::gfp; M. Wallenfang et al., personal communication).

Four-Factor Mapping
Standard genetic techniques were used to separate the egfl-1 mutations n1084 and n3082 on LGV (Brenner, 1974). Briefly, recombinants between rol-4 and unc-76 were obtained by screening progeny of rol-4(sc8) + × unc-76(e911) + × egf-1(n1084) n3082 + or rol-4(sc8) egf-1(n1084) n3082 unc-76(e911) + × + × for Rol Non-Unc and heta Non-Rol animals. Homozygous recombinants were analyzed for the presence of n1084 by scoring adult hemaphrodites for egg-laying defects (Egl phenotype) and for the presence of n3082 by analyzing the anterior pharynx of larvae at the fourth larval stage for defects in programmed cell death ( Ced phenotype), as described previously (Conradt and Horvitz, 1998).

Transgenic Animals
Germline transformation was performed as described by Mello and Fire (1995). For transformation with DNA fragments amplified by PCR, ced-1(e17355); egf-1(n1084) n3082) unc-76(e911) animals were injected with gel-purified PCR products (2 ng/µl), and the coinjection marker p76-16B (50 ng/µl), which rescues the unc-76(e911) uncoordinated phenotype (Bloom and Horvitz, 1997). Non-Unc transgenic F1 animals were picked and used to establish lines. The rescue of the Ced phenotype and the presence of the HSNs were determined in transgenic animals at the first larval stage as described (Conradt and Horvitz, 1998). The DNA fragments were amplified by PCR using a polymerase mix suitable for long-range PCR (Clontech) and appropriate primers. Chimeric fragments were generated by digesting fragments with PsI and subsequent ligation of appropriate fragments. For transformation with GFP reporter constructs, ced-3(n717); lin-15(n765) animals were injected with plasmids pBC99 (Peg1-1::gfp) or pBC104 (Peg1-1::gfp) (2 ng/µl) and the coinjection marker pl15-EK (50 ng/µl), which rescues the lin-15(n765) multivulva phenotype (Clark et al., 1994). Injected animals were shifted to 24 °C, and non-Muv transgenic F1 animals were picked to establish lines, which were maintained at 24 °C. To construct pBC99, bases +174 to +5820 (5′-3′) downstream of the stop codon of the egf-1 gene and bases −1914 to −837 (5′-3′) upstream of the stop codon were amplified with appropriate primers and cloned into the SpeI-Apal (5′-3′) and PstI-BamHI (5′-3′) sites of vector pPD95.69, respectively (A. Fire, et al., personal communication). pBC104 was generated from pPC99 by introducing a G to A transition at bp +5635 downstream of the stop codon of the egf-1 gene using PCR-mediated mutagenesis and appropriate primers. The nucleotide sequences of all cloned fragments that were amplified by PCR were determined.

Molecular Analysis
Standard molecular biological procedures were employed (Sambrook et al., 1989), unless otherwise noted. Primers used throughout this study were based on the sequence of the egf-1 locus as determined by the C. elegans Sequencing Consortium (1998). The sequences of the egf-1(gf) mutant alleles were determined from PCR products amplified from genomic DNA isolated from the various gfp mutants. DNA sequences were determined using an automated ABI 373 DNA sequencer (Applied Biosystems). The C. briggsae egf-1 locus was identified by probing a C. briggsae fosmid grid (Genome Sciences) with the egf-1 cDNA. The sequence of the positive clone (fosmid G27D19) was determined by the C. elegans Sequencing Consortium.

Analysis of gfp Expression in HSNs
The HSNs were identified in transgenic larvae at the first larval stage using Nomarski optics as described (Sulston and Horvitz, 1977) and analyzed for gfp expression using epifluorescence. The sex of the larvae was determined by the positions of the coelomocytes relative to the gonadal primordium and by the appearance of the nuclei of the B and C cephalic cells in the tail (Sulston and Horvitz, 1977). Data presented in the text were from one representative line each (Pegl-1:gfp and Pegl-1:lgf:::gfp). Similar results were obtained with three (Pegl-1:gfp) and four (Pegl-1:lgf:::gf) additional independent lines, respectively.

Gel Mobility Shift and Competition Experiments
For gel mobility shift experiments, probes were generated by PCR amplification at low dATP concentration (20 µM) and in the presence of 20 µCi ([32P]dATP, using primers #15 (5′-CTGGTCTGAAGCTCTAAATTT-3′) and #46 (5′-GTGCTGAACTGACATGCGG-3′). Labeled PCR products were purified on 6% acrylamide/TBE gel. TRA-1A protein was generated by in vitro transcription and translation of a full-length tra-1 cDNA (pDA128; D. Zarkower, personal communication) and a T7-based coupled reticuloocyte lysate (NTT Coupled Reti-culocyte System). Gel mobility shift assays were performed as described (Pollock and Treisman, 1990; Zarkower and Hodgkin, 1993) and were incubated for 20 min at room temperature before electrophoresis on 4% acrylamide gels/0.5× TBE. Unless otherwise noted, the assays contained 1.5 µl of reticulocyte lysate (source of TRA-1A) and 4 ng of labeled PCR fragments (probe). Competitor DNA was generated by PCR amplification using primers #15 and #46 and appropriate templates. PCR products were gel purified. Competition experiments were performed with 2 ng of labeled PCR fragments as probe and 0, 2, 20, 200, or 1000 ng of unlabeled PCR fragments as competitor.

Analysis of HSN Survival in tra-1 Mutants
tra-1(e1099f) XX animals were obtained and analyzed as follows. Non-Muv and C. briggsae fosmid clones for the egfl-1(gf) mutation e1099f) unc-69(e567) were picked and analyzed for the presence of HSNs as described (Conradt and Horvitz, 1998) and recovered and allowed to mature. Homozygous tra-1(e1099f) XX animals were recognized by their male somatic phenotype. tra-1(e1099f); egfl-1(n1084) n3082(f) XX animals were obtained from tra-1(e1099f) unc-69(e567); egfl-1(n1084) n3082(f) mothers. tra-1(e1575gf) X0 animals were obtained by mating fertile spermless females of genotype tra-1(e1095); tra-1(e1575gf) X0 animals were obtained by mating fertile spermless females of genotype tra-1(e1095); him-5(e1490) with males of genotype axts36/0 (axts36 is the construct Ppesc-10::gfp integrated on the X chromosome. Paternally inherited, axts36-derived GFP is therefore a marker for XX cross-progeny; M. Wallenfang et al., personal communication). L1 progeny were analyzed for the presence of HSNs and axts36 (GFP), recovered, and allowed to mature. tra-1(e1575gf) X0 animals were recognized among GFP+ animals by their female somatic phenotype. To obtain tra-1(e1575gf) X0 animals, we crossed tra-2(e1095); tra-1(e1575gf) +; egfl-1(n1264gf) X0 animals, we crossed tra-2(e1095); tra-1(e1575gf) +; him-5(e1490) hermaphrodites with egfl-1(n1264gf); axts36/0 males.

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References


