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

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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; de 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 (X0) 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 (ci) and the pair-rule gene odd-paired of Drosophila and the vertebrate Gli genes, which together form a subfamily within the family of Krüppel-like zinc finger DNA-binding proteins and transcription factors (Zarkower and Hodgkin, 1992; Benedykt 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]; Hodgkin, 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

Figure 1. n1084 and n3082 Map within 0.04 Map Units of Each Other
(Top) Genetic map of a portion of the right arm of chromosome V and (Middle) corresponding physical map showing the predicted transcription units of the BH3 gene and an adjacent gene, F23B12.1. Genes and mutations used for the four-factor-mapping experiment are indicated. Numbers below the genetic map represent the fraction of the recombination events that occurred between particular loci found in the 70 non-Rol and 70 non-Unc recombinants identified between rol-4 and unc-76. (Bottom) 5' and 3' regulatory regions of the BH3 gene that were used to construct the Pegl-1::gfp reporter construct pBC99 and the similar pegl-1 (gf)::gfp construct pBC104 are indicated.
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 egl-1(e911). Data shown are averages ± standard deviations of results obtained from six 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 egl-1(e911); unc-76(e911) transgenic lines, and analyzed for their abilities to cause killing as described in (B). The data are averages ± standard deviations of results obtained from six (N2), eight (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) 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 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 (+/+ ) 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) n3082 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 (gf) mutation in the egl-1 gene, egl-1(n1084 n3082gf).

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>
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<td>TRA-1-binding site</td>
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<tr>
<td>C. briggsae egl-1 locus</td>
<td>n3801 bp</td>
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<tr>
<td>C. elegans egl-1 locus</td>
<td>+5645 bp</td>
</tr>
<tr>
<td>n487, n1084, n1796</td>
<td></td>
</tr>
<tr>
<td>n986, n987, n2164</td>
<td></td>
</tr>
<tr>
<td>n2248</td>
<td></td>
</tr>
<tr>
<td>TRA-1-binding site</td>
<td></td>
</tr>
<tr>
<td>C. briggsae egl-1 locus</td>
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</tr>
<tr>
<td>C. elegans egl-1 locus</td>
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<tr>
<td>n487, n1084, n1796</td>
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</tr>
<tr>
<td>n986, n987, n2164</td>
<td></td>
</tr>
<tr>
<td>n2248</td>
<td></td>
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</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:gf) (Figure 1). This reporter construct was introduced into animals carrying the ced-3(9f) mutation n717. ced-3 acts downstream of egl-1 genetically, and ced-3(9f) 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)
TRA-1A Binds to the Putative TRA-1A-Binding Site In Vitro

To determine whether TRA-1A can bind to the conserved putative TRA-1A-binding site downstream of the egl-1 transcription unit, we performed gel mobility shift assays. We found that in vitro translated, full-length TRA-1A protein could bind to and shift a probe consisting of a 297 bp DNA fragment that we amplified from sequences 5526 to 5823 bp downstream of the egl-1 stop codon. TRA-1A was able to bind to the probe containing the putative TRA-1A-binding site amplified from wild-type animals but failed to bind to the corresponding probes amplified from animals carrying the egl-1(gf) mutation n1084, n2164, or n2248, which represent the three different G-to-A transitions (Figure 4A).

To examine the binding of TRA-1A to the mutant TRA-1A-binding sites in more detail, we performed competition experiments. In these experiments, we used in vitro translated full-length TRA-1A protein, labeled wild-type 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.

tra-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 if or gf mutations in the tra-1 gene. tra-1(If) 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(If) 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(e1099If) 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 XO animals, 82% of the HSNs survived in XO animals heterozygous for the tra-(gf) mutation e1575 (tra-e1575gf/+) (Table 3). That 18% of the HSNs in tra-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-1(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 destination of the HSNs was scored as described by Nomarski optics (Sulston and Horvitz, 1977). The presence of HSNs was scored as follows: +/unc-76(e911), egl-1(n1084) +/egl-1(n1084) unc-76(e911), egl-1(n1084) /+ unc-76(e911), egl-1(n1084) /+ egl-1(n1084) unc-76(e911), egl-1(n1084) /+ unc-76(e911), egl-1(n2164)/+ /egl-1(n2248) unc-76(e911), egl-1(n2248) /+/unc-76(e911), n, number of HSNs.

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

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<th>Genotype</th>
<th>Percent of HSNs Present (n)</th>
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<td>+/+</td>
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<tr>
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<tr>
<td>egl-1(n1084gf)/+</td>
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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(e911), egl-1(n1084) +/egl-1(n1084) unc-76(e911), egl-1(n1084)/+/unc-76(e911), egl-1(n2164)/+/egl-1(n2248) unc-76(e911), egl-1(n2248)+/+/unc-76(e911), 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>
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<td>96 (52)</td>
</tr>
<tr>
<td>egl-1(n12164gf)+/</td>
<td>11 (80)</td>
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<tr>
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<tr>
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<tr>
<td>egl-1(n1084 n3082gf) +</td>
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<tr>
<td>egl-1(n1084 n3082gf) +/+</td>
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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(n1084) +/egl-1(n1084) unc-76(e911), egl-1(n1084)+/+/unc-76(e911), egl-1(n2164)/+/egl-1(n2248) unc-76(e911), egl-1(n2248)+/+/unc-76(e911), n, number of HSNs.

Figure 4. TRA-1A Binds to the Putative TRA-1A-Binding Site Downstream of the egl-1 Transcription Unit

(A) TRA-1A binds to the wild-type but not the mutant putative TRA-1A-binding sites. The arrow points to the specific complexes formed by full-length TRA-1A protein and the DNA fragment used as probe. TRA-1-containing lysate (0.5 μl [lanes 1, 7, 11, and 15], 0.15 μl [lanes 3, 9, 13, and 17], 0.38 μl [lane 4], 0.75 μl [lane 5], or 1.5 μl [lanes 6, 10, 14, and 18]) or control lysate (1.5 μl [lanes 2, 8, 12, and 16]) was incubated with 4 ng of labeled wild-type (lanes 1-6) or unlabeled (lanes 7-10) egl-1(n1084) (lanes 11-14), or egl-1(n2248) (lanes 15-18) containing DNA fragments and analyzed for gel mobility shifts as described in Experimental Procedures.

(B) DNA fragments containing the egl-1(gf) mutations functioned as weak competitors. The arrow points to the specific complexes formed by full-length TRA-1A protein and the DNA fragment used as probe. TRA-1-containing reticulocyte lysate (1.5 μl) was incubated with 2 ng of labeled wild-type DNA fragments and with 0 ng (0; lanes 1, 6, 11, and 16), 2 ng (1; lanes 2, 7, 12, and 17), 20 ng (2×; lanes 3, 8, 13, and 18), 200 ng (20×; lanes 4, 9, 14, and 19), or 1 μg (500×; lanes 5, 10, 15, and 20) of unlabeled competitor DNA, amplified from wild-type (lanes 1-5), egl-1(n1084gf) (lanes 6-10), egl-1(n2164gf) (lanes 11-15), or from egl-1(n2248gf) (lanes 16-20) animals. Results were analyzed as described in Experimental Procedures.

(C) Comparison of the efficiencies of the various competitors. The data shown were generated as described in (B) and analyzed using a Phosphomager. The amount of TRA-1A-DNA complex formed in the absence of competitor and the DNA fragments and analyzed for gel mobility shifts as described in Experimental Procedures.
were present in X0 animals heterozygous for the egl-1(gf) mutation n2164, whereas 82% survived in X0 animals heterozygous for the tra-1(gf) mutation e1575. We found that only 8% of the HSNs were present in X0 animals heterozygous for both tra-1(e1575gf) and egl-1(n2164gf), tra-1(e1575gf)/++; egl-1(n2164gf)/++, suggesting that the egl-1(gf) mutation is able to suppress the tra-1(gf) mutation. These results indicate that with respect to the HSN cell death fate egl-1 is epistatic to tra-1 and that egl-1 therefore acts downstream of or in parallel to tra-1 in the HSNs. tra-1 therefore acts as a negative regulator of egl-1 in these neurons (Figure 5A).

**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.

Figure 5B illustrates how the transcription of the egl-1 gene might be regulated in the sexually dimorphic HSNs. In wild-type hermaphrodites (XX), TRA-1A activity is high, and the binding of TRA-1A to the TRA-1A-binding 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 the 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 n3082f) hermaphrodites (egl-1(fff)), the egl-1 gene is also inappropriately activated, but because the egl-1 coding region carries a mutation, the gf mutation n3082, an inactive gene product is made which is unable to induce programmed cell death.

The inability of TRA-1A to repress egl-1, as occurs in wild-type males as a consequence of low TRA-1A activity or in egl-1(gf) hermaphrodites as a consequence of a disrupted TRA-1A-binding site, causes the HSNs but not other cells in the animal to undergo programmed cell death. Furthermore, active TRA-1A protein blocks only the deaths of the HSNs in hermaphrodites but not the deaths of other cells destined to die. We therefore propose that the regulation of egl-1 in the HSNs involves another factor, such as an HSN-specific activator of egl-1 expression, and that TRA-1 specifically blocks this activator.

The egl-1 gene represents the first identified direct target of TRA-1A in the soma, where TRA-1A functions as the terminal regulator of sexual phenotype. It has been proposed that TRA-1A implements sexual fate by promoting female-specific and by suppressing male-specific programs required for sexual differentiation (Zarkower and Hodgkin, 1993). The deaths of the HSNs can be regarded as part of the male-specific program, and by repressing egl-1 transcription in the HSNs in hermaphrodites, TRA-1A suppresses this program. The programmed cell death pathway is therefore used in the sexually dimorphic HSNs to execute sexual differentiation.

**TRA-1A Represses egl-1 at a Distance**

The TRA-1A-binding site is located 5.6 kb downstream of the egl-1 transcription unit, beyond another transcription unit, F23B12.1 (Figure 2A). F23B12.1 is encoded on the opposite strand to egl-1, and its gene product is predicted to be similar to serine/threonine protein phosphatases (C. elegans Sequencing Consortium, 1998). It is possible that the TRA-1A-binding site also affects the transcriptional regulation of F23B12.1. However, the F23B12.1 transcription unit does not appear to be present at the corresponding location with respect to the egl-1 locus of C. briggsae (our unpublished observations), supporting the hypothesis that the TRA-1A-binding site acts to regulate egl-1.
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 3.7 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-3 (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 Johnson 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 her-1 (her, hermaphroditization), an upstream gene in the regulatory pathway for sex determination, are thought to disrupt a binding site for the gene product of the sd-1 gene (sdc, 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 DP5 (Imai and Horvitz, 1997), another member of the subfamily of BH3 domain-only cell death activators, is controlled. The transcriptional upregulation of the DP5 gene in cultured sympathetic neurons after the removal of nerve growth factor coincides with the programmed deaths of these cells (Imai and Horvitz, 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 through 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 neuroendocrine motor neurons (Elias 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 grown 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. LGL: ced-1(e1735); LGIII: tra-1(e1575), tra-1(e1099), unc-69(e587). LGIV: ced-3(n717). LGV: rol-4(s58), him-5(e1490), egl-1(n487), egl-1(n986), egl-1(n987), egl-1(n1084), egl-1(n1796), egl-1(n2164), egl-1(n2248) J. Yuan et al., unpublished data), egl-1(n1084 n3082) (Conradt and Horvitz, 1998), unc-76(e911). LGX: lin-15(n765); axis36 (Ppex-10::gfpl; M. Wallenfang et al., personal communication).

Four-Factor Mapping
Standard genetic techniques were used to separate the egl-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(e58) + x unc-76(e911) ± egl-1(n1084 n3082) + or rol-4(e58) egl-1(n1084 n3082) unc-76(e911) + + + + + + + for Rol non- and Unc non-phenotypes. Homozygous recombinants were analyzed for the presence of n1084 by scoring adult hermaphrodites 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
 Germine transformation was performed as described by Mello and Fire (1995). For transformation with DNA fragments amplified by PCR, ced-1(e1735); egl-1(n1084 n3082) unc-76(e911) animals were injected with gel-purified PCR fragments (2 ng/µl), and the injection marker p76-16B (50 ng/µl), which rescues the unc-76(e911) uncordinated 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 PstI and subsequent ligation of appropriate fragments. For transformation with GFP reporter constructs, ced-3(n717); lin-15(n765) animals were injected with plasmids pBCC9 (Pegl-1::gfp) or pBC104 (Pegl-1::gfp-3) (2 ng/µl) and the coinjection marker pl15-5K (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 pBCC9, bases 174 to 5820 (5' - 3') downstream of the stop codon of the egl-1 gene and bases 1914 to 837 (5' - 3') upstream of the stop codon were amplified with appropriate primers and cloned into the Spell-Apal (5' - 3') and PsiI-BamHI (5' - 3') sites of vector pPD95.69, respectively (A. Fire et al., personal communication). pBC104 was generated from pPC99 by introducing a 5'- to 3'- transition at bp - 5635 downstream of the stop codon of the egl-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 egl-1 locus as determined by the C. elegans Sequencing Consortium (1998). The sequences of the egl-1(gf) mutant alleles were determined from PCR products amplified from genomic DNA isolated from the various gf mutants. DNA sequences were determined using an automated ABI 373 DNA sequence (Applied Biosystems). The C. briggsae egl-1 locus was identified by probing a C. briggsae fosmid grid (Genome Systems) with the egl-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 ectodermal cells in the tail (Sulston and Horvitz, 1977). Data from the two transgenes were analyzed in the same experiment. The coinjection marker pL15-EK (50 ng/l#15 and #46 and appropriate templates. PCR products were gel purified. Competition experiments were performed with 2 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 hermaphrodites of genotype tra-1(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); egl-1(n1084 n3082) XX animals were obtained from tra-1(e1099f) unc-69(e567); egl-1(n1084 n3082) females. Homozygous tra-1(e1575gf) / X0 animals were obtained by mating fertile spermless females of genotype tra-1(e1095); tra-1(e1575gf) / +; him-5(e1490) with males of genotype axis36/0 (axis36 is the construct Ppex-10::gfp integrated on the X chromosome. Paternally inherited, axis36-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 axis36 (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) / +; egl-1(n2164gf) X0 animals, we crossed tra-2(e1095); tra-1(e1575gf) / +; him-5(e1490) hermaphrodites with egl-1(n2164gf); axis36/0 males.

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