The *Caenorhabditis elegans* gene *ham-2* links Hox patterning to migration of the HSN motor neuron

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The *Caenorhabditis elegans* HSN motor neurons permit genetic analysis of neuronal development at single-cell resolution. The *egl-5* Hox gene, which patterns the posterior of the embryo, is required for both early (embryonic) and late (larval) development of the HSN. Here we show that *ham-2* encodes a zinc finger protein that acts downstream of *egl-5* to direct HSN cell migration, an early differentiation event. We also demonstrate that the EGL-43 zinc finger protein, also required for HSN migration, is expressed in the HSN specifically during its migration. In an *egl-5* mutant background, the HSN still expresses EGL-43, but expression is no longer down-regulated at the end of the cell's migration. Finally, we find a new role in early HSN differentiation for UNC-86, a POU homeodomain transcription factor shown previously to act downstream of *egl-5* in the regulation of late HSN differentiation. In an *unc-86; ham-2* double mutant the HSNs are defective in EGL-43 down-regulation, an *egl-5*-like phenotype that is absent in either single mutant. Thus, in the HSN, a Hox gene, *egl-5*, regulates cell fate by activating the transcription of genes encoding the transcription factors HAM-2 and UNC-86 that in turn individually control some differentiation events and combinatorially affect others.

[*Key Words*: *ham-2; egl-5; unc-86*; Hox; neuronal migration; zinc finger]

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Nervous systems are made of many different types of neurons that differ in position, morphology, connectivity, and neurotransmitter expression. This diversity is generated through transcription factors that direct each neuron's program of differentiation. Transcription factors required for neuronal development have been identified in vertebrates (Bang and Goulding 1996; DeLapeyriere and Henderson 1997) as well as in *Caenorhabditis elegans* (Sengupta and Bargmann 1996; Ruvkun 1997), but ordering these transcription factors into regulatory pathways and defining the aspects of cell fate that they control has been difficult.

The establishment of an anteroposterior pattern in the embryo is an early step in the differentiation of neurons, ensuring that neurons in each part of the nervous system will adopt the proper fates. This anteroposterior patterning is established by the regional expression of each member of a cluster of homeobox genes known as Hox genes (Lewis 1978; McGinnis et al. 1984). Hox genes pattern structures as diverse as the segments of *Drosophila* embryos and the segments of the mammalian hindbrain (Lewis 1978; Keynes and Krumlauf 1994). Mutations in *C. elegans* and mouse Hox genes both cause transformations in cell fates and defects in neuronal migrations (Salser and Kenyon 1994; Studer et al. 1996).

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Although the means by which an initial anteroposterior morphogenetic gradient generates the normal pattern of Hox gene expression have been described in exquisite detail in *Drosophila* (for review, see St Johnston and Nüsslein-Volhard 1992), how Hox gene products and other transcription factors work together subsequently to specify cellular identity is much less understood. The mechanisms by which the Hox transcription factors activate downstream genes and the identities of these target genes are still being elucidated (for review, see Graba et al. 1997). Some researchers have used the DNA-binding property of Hox homeodomains to identify candidate downstream genes that might determine cell fates. Hox proteins bind the promoters of genes encoding adhesion molecules such as connectin and NCAM, for example, but whether Hox proteins activate these genes directly in vivo has not been fully established (Gould et al. 1990; Gould and White 1992; Edelman and Jones 1998).

Genetic analysis offers another approach to understanding how Hox transcription factors influence cell differentiation. For example, several long-distance cell migrations in *C. elegans* are under the control of Hox genes. The Hox genes *lin-39* and *mab-5* have been shown to function cell-autonomously in guiding the migrations of the QR and QL neuroblasts, respectively (Kenyon 1986; Clark et al. 1993). The anteriorly migrating QR neuroblast can be redirected posteriorly by pulses of *mab-5* administered by heat shock during its migration

(Salser and Kenyon 1992; Harris et al. 1996). The fact that QR responds quickly to these *mab-5* pulses suggests that the regulation of cell migrations by these Hox genes is relatively direct and not through a multi-tiered transcriptional cascade. Despite the Hox genes' importance in directing Q-neuroblast migrations, all known mutations that affect the directional guidance of the Q-cell migrations appear to act upstream of *mab-5* (Harris et al. 1996).

Another Hox gene, *egl-5*, is required for proper migration of the hermaphrodite-specific neurons, or HSNs (Desai et al. 1988). *egl-5* is a key regulator of HSN cell identity because *egl-5* mutant HSNs are defective both in embryonic differentiation steps, such as cell death and cell migration, as well as in larval differentiation steps, such as neurotransmitter expression (Desai et al. 1988). In this paper, we construct a pathway of transcription factors that act along with *egl-5* in directing these steps in HSN differentiation. We show that a zinc finger protein encoded by the gene *ham-2* acts downstream of *egl-5* to promote HSN migration. *ham-2* is the first cell-migration gene found to act downstream of a Hox gene. In contrast, we find that another zinc finger protein required for HSN migration, EGL-43, is activated in the HSN in a Hox-independent manner. The gene *unc-86*, which encodes a POU homeodomain protein that promotes larval HSN fates (Desai et al. 1988; Finney et al. 1988), has been shown to act downstream of *egl-5* (Baumeister et al. 1996). We find here that *unc-86* also plays a redundant role with *ham-2* in EGL-43 down-regulation in the embryo. Finally, we extend previous results and find that *egl-5* is required for proper execution of the sex-specific cell death decision of the HSN, and that this function of *egl-5* occurs independently of the *ham-2*, *egl-43*, and *unc-86* transcription factors.

Results

ham-2 *encodes a zinc finger protein required for migration of the HSN motor neurons*

The HSNs, a bilateral pair of serotonergic motor neurons, are born in the tail of the embryo and migrate anteriorly to flank the gonad primordium near the middle of the body. The HSNs undergo apoptosis in males but survive in hermaphrodites. Later during larval development the HSNs terminally differentiate. They extend axons that innervate the egg-laying muscles and express the neurotransmitter serotonin (Desai et al. 1988; Garriga et al. 1993a). The HSNs are required for normal egg laying. Laser-operated animals lacking HSNs and mutant animals with defects in HSN development or function are egg-laying defective, or Egl (Trent et al. 1983; Desai et al. 1988). Systematic screens for *C. elegans* mutants with defective HSNs have identified many genes required for the development of these neurons (Trent et al. 1983; Desai et al. 1988; Desai and Horvitz 1989; Garriga and Stern 1994; Dianne Parry, P.D. Baum, and G. Garriga, unpubl.).

Two *ham-2* mutants, *n1332* and *mu1*, were isolated from mutator strains with high rates of transposable element activity. Both mutants have HSN migration defects, but lack obvious pleiotropies (Fig. 1). The original *ham-2* mutant, *n1332*, was reported to have defects in HSN serotonin expression (Desai et al. 1988), but we find 90% of the HSNs of *n1332* and *mu1* hermaphrodites express detectable levels of serotonin, a percentage much higher than that described originally for *n1332* hermaphrodites. Because low-penetrance defects in HSN serotonin expression are displayed by many HSN migration mutants, the *ham-2* HSN serotonin defects may result from abnormal cell-body positioning (Garriga et al. 1993a). Two EMS-induced *ham-2* mutations, *gm16*, and *gm48*, in addition to causing HSN migration defects, also cause first larval stage arrest with a Pun (pharynx unattached to the nose) phenotype (Fig. 2). Larval arrest is complete for *gm16* animals, but only partially penetrant for *gm48* animals. *gm48* hermaphrodites also display the weakest HSN migration defect (Fig. 1).

ham-2 was cloned (see Materials and Methods) and found to encode a protein with zinc finger motifs (Fig. 3). A subclone containing the *ham-2* gene rescued the Ham and Pun defects of *ham-2* mutants. The alleles *n1332* and *mu1* are transposon insertions in the first intron of the gene. The alleles *gm16* and *gm48* are missense mutations. *gm16* changes a conserved amino acid in the first zinc finger. This mutation is predicted to alter or destroy the DNA-binding specificity of the protein (Berg and Shi 1996). *gm48* alters a conserved histidine that is predicted to coordinate zinc in the first zinc finger. Taken together, the phenotypic and molecular analyses indicate that *gm48* reduces *ham-2* function, and *gm16* may eliminate it. In contrast, *n1332* and *mu1* appear to be regulatory mutations that reduce severely or eliminate *ham-2* function in the HSNs, but not in other cells. Antibody-staining experiments with anti-HAM-2 antiserum are consistent with this hypothesis (see below).

HAM-2 and EGL-43 proteins are expressed in the HSN nucleus during migration

Like *ham-2*, the *egl-43* gene encodes a zinc finger protein required for HSN migration (Garriga et al. 1993b). Polyclonal antisera raised against the HAM-2 and EGL-43 proteins were used to stain wild-type embryos (Figs. 4 and 5). Experiments to confirm that the staining observed with these antisera reflected the distribution of HAM-2 and EGL-43 are described in Materials and Methods. HAM-2 and EGL-43 proteins localized to cell nuclei, consistent with their proposed function as transcription factors. The HSNs were identified in these embryos by double labeling with an anti-UNC-86 antiserum (Finney and Ruvkun 1990). Both HAM-2 and EGL-43 are expressed in the HSN before and during the cell's migration from the tail to the gonad primordium of the embryo (Figs. 4 and 5, A–F). Whereas EGL-43 expression is down-regulated after HSN migration, HAM-2 expression continues in larval HSNs (not shown). Both transcription factors are expressed in other cells besides the HSN. In cases in which these cells have been identified, as a

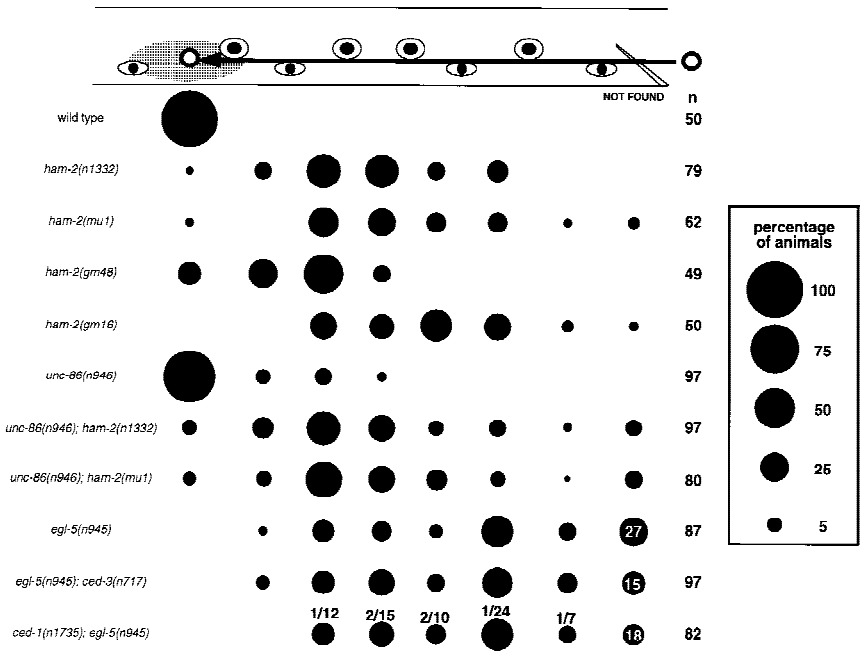


Figure 1. HSN migration and cell-death defects of transcription factor mutants. The HSN normally migrates from the tail to the side of the gonad primordium in embryos. This figure shows the final positions of HSNs in L1 larvae from various strains, as scored by Nomarski optics. At the *top*, the positions of landmark hypodermal cells (eye-shaped symbols) and the gonad (gray oval) are depicted. The arrow indicates the HSN migration route. The area of each circle in the chart is proportional to the percentage of HSNs in that position along the anteroposterior axis of the worm (see key at *right*). In some strains, some HSNs could not be found in a percentage of the sides scored; these are represented at the *right* of the figure in the not found column. HSNs that could not be found along the migratory route either failed to migrate from their birthplace in the tail, where we cannot distinguish them from other neurons of the lumbar ganglia, or were missing. (n) The number of HSNs scored. We scored approximately equal numbers of left and right HSNs for each strain. The *ced-3* mutation was used to reveal HSNs missing because of programmed cell deaths. The distribution of the HSNs in *ced-1* and *ced-3* mutants was the same as in wild type and is not shown. The percentage of HSNs that could not be found in *egl-5*, *egl-5; ced-3*, and *ced-1; egl-5* mutants are indicated. The fractions above the circles for *ced-1; egl-5* indicate the number of cell corpses/total number of cell corpses and surviving HSNs at each position. No cell corpses were seen along the HSN migratory route in *ced-1* mutants. The *unc-86* alleles *e1416*, *n306*, *n843*, and *n844* had effects similar to *unc-86(n946)* on HSN cell-body position, both as single mutants and as double-mutant combinations with the *ham-2(mu1)* mutation (not shown). 12% of the HSNs in *egl-43(n997)* mutants migrated out of the tail between V5 (the fourth marker cell from the right) and P11/12 (the most right marker cell) ($n = 50$). 4% of the HSNs in *egl-43(n997); egl-5(n945)* migrated out of the tail between P9/10 (the third marker cell from the right) and P11/12 ($n = 50$). None of the HSNs in *egl-43(n997) unc-4(e120); ham-2(n1332)* migrated out of the tail ($n = 40$).

group they share no obvious developmental or functional characteristics with the HSN.

Hypodermal nuclei also express HAM-2 briefly. We examined embryos double stained with a mouse anti-HAM-2 antiserum and a rabbit antiserum recognizing the hypodermal transcription factor LIN-26. Initially, hypodermal cells expressed both proteins, but HAM-2 expression ceased when embryos reached comma stage, whereas LIN-26 expression persisted (Labouesse et al. 1996; data not shown). Because *lin-26* mutants arrest as embryos, and *ham-2(gm16)* mutants arrest as L1 larvae, we tested whether *lin-26* might regulate *ham-2* hypodermal expression. We found that HAM-2 staining in the hypodermal cells was not affected in *lin-26* null mutants (M. Labouesse, pers. comm.), suggesting that *ham-2* does not function downstream of *lin-26*. The pattern of anti-HAM-2 staining was similar to the expression of GFP from a *ham-2–gfp* transgene.

Regulatory mutations in ham-2 *and* egl-43 *block protein expression in the HSN*

The *ham-2* alleles *n1332* and *mu1*, which contain transposon insertions in the first intron of the gene (Fig. 3), and the *egl-43* alleles, *n997*, and *n1079*, which contain an identical 790-bp deletion 3′ to the coding region (Materials and Methods), are likely to be regulatory mutations. Consistent with this hypothesis, staining of these mutants showed reduced protein expression in the HSN,

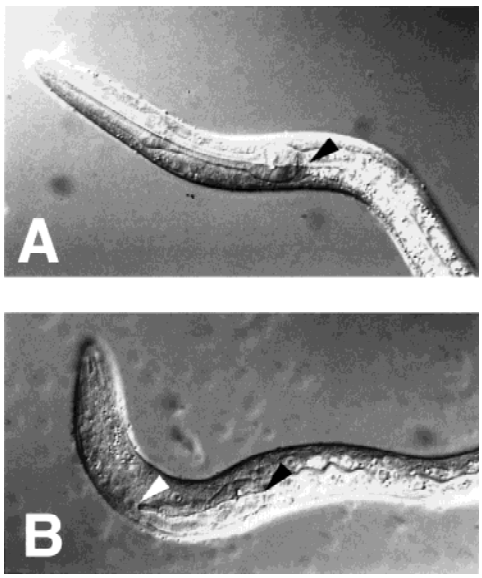


Figure 2. The Pun phenotype. Nomarski photomicrographs of L1 (first stage) larvae. The anterior (white arrowhead) and posterior (black arrowhead) ends of the pharynx are indicated. (*A*) wild type; (*B*) *ham-2(gm16)* mutant.

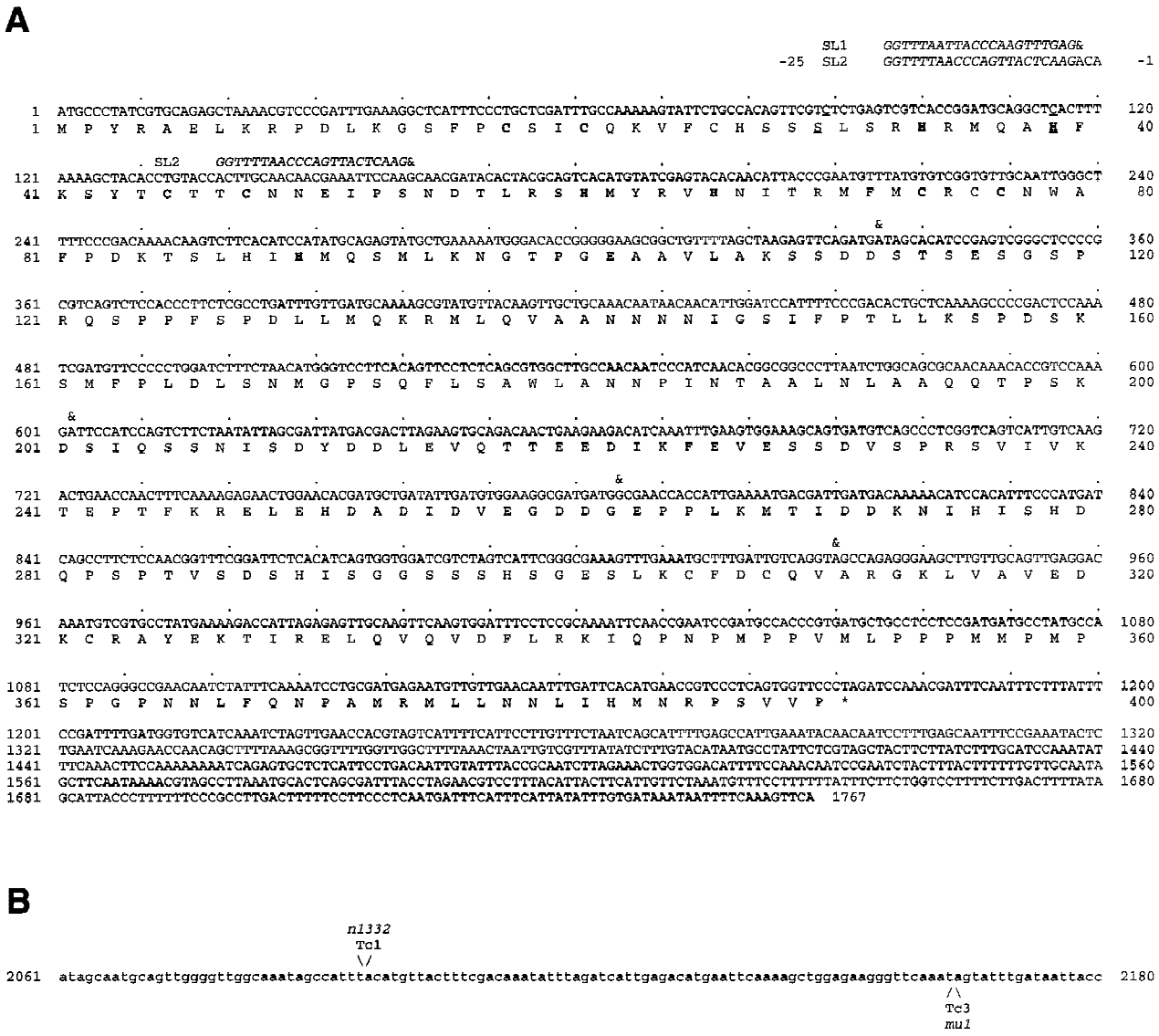


Figure 3. Two *ham-2* transcripts encode zinc finger proteins. (*A*) Sequence and predicted product of two *ham-2* cDNA products are shown. The longer cDNA is trans-spliced to SL1 or SL2 (italics). A shorter transcript is trans-spliced to SL2 at the beginning of the second exon (nucleotide 161). (&) The beginning of each exon. Cysteines and histidines predicted to form zinc fingers are in bold. An acidic region (amino acids 248–263) or a proline-rich region (amino acids 344–364) could potentially act as activation domains. In *ham-2(gm16)*, nucleotide 89 is changed from c to t (underlined). This mutation changes amino acid 30 from S to F. The amino acid in this position of the finger motif is predicted to determine DNA-binding specificity (Berg and Shi 1996). In *ham-2(gm48)*, nucleotide 115 is changed from c to t (underlined). This mutation changes amino acid 39, a histidine predicted to coordinate the zinc atom in the first zinc finger, to a tyrosine. (*B*) Transposon insertions in the first intron of *ham-2*. Only a portion of the 2260 nucleotide first intron is shown. Nucleotides are numbered starting with the first nucleotide of the intron. The *ham-2(n1332)* and *ham-2(mu1)* transposon insertion sites are indicated.

with protein levels in most other cells unaffected. Staining of *n1332* and *mu1* mutants with an anti-HAM-2 antiserum showed no detectable HAM-2 expression in the HSNs during their migrations, despite normal expression in some other neurons and in the hypodermis (Fig. 4G–I; Table 1; data not shown). Faint staining was seen occasionally in the postmigratory HSNs of older *ham-2(mu1)* animals (not shown). Likewise, staining of *egl-43(n1079)* animals with an anti-EGL-43 antiserum revealed that most cells still expressed normal protein levels, but that the HSNs expressed no detectable EGL-43 (Fig. 5G–I; data not shown). HSN expression of UNC-86 was normal in the *ham-2* and *egl-43* mutants (Figs. 4 and 5, H). We also found that *ham-2* mutants had normal HSN EGL-43 expression, and *egl-43* mutants had normal HSN HAM-2 expression, suggesting that these genes act in parallel to regulate HSN migration (Table 1). The *egl-43* deletion and the *ham-2* transposon insertions likely disrupt enhancers required for HSN expression, but neither the *egl-43* 3′ region nor the *ham-2* first intron are

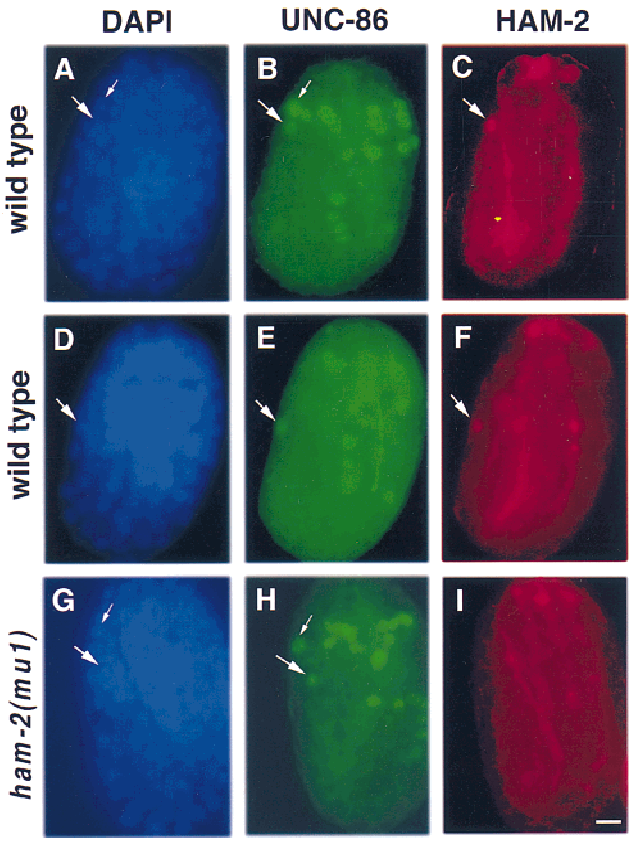


Figure 4. HAM-2 protein is expressed in the HSN nucleus during migration. Fluorescence photomicrographs of wild-type (*A–F*) and *ham-2(mu1)* mutant (*G–I*) embryos that have been stained with DAPI (blue), an UNC-86 antiserum (green) and a HAM-2 antiserum (red). Each panel presents a left lateral view of the embryo, with the developing tail to the left, the head to the right and the end of the tail and the top of the head both oriented upwards. The right sides are out of focus. (*A–C*) A wild-type embryo at ~410 min after first cleavage, in which the HSN has not migrated out of the tail. (*A*) DAPI staining. The nuclei of the HSN (large arrow) and the ALN/PLM precursor (small arrow) are shown. (*B*) Anti-UNC-86 staining. Both the HSN (large arrow) and the ALN/PLM precursor (small arrow) express UNC-86. (*C*) Anti-HAM-2 staining. The HSN nucleus (large arrow) expresses HAM-2; several HAM-2 expressing cells in the head are out of focus. (*D–F*) A wild-type embryo at ~430 min after first cleavage. (*D*) DAPI staining reveals the position of the HSN nucleus (arrow). The ALN/PLM precursor nucleus is out of the plane of focus. (*E*) Anti-UNC-86 staining. Shortly after commencing its anteriorly directed migration, the HSN begins expressing UNC-86 (arrow; Finney and Ruvkun 1990). (*F*) Anti-HAM-2 staining. During its migration, the HSN also expresses HAM-2 (arrow). (*G–I*) A *ham-2(mu1)* mutant embryo at ~430 min after first cleavage. (*G*) DAPI staining reveals the positions of the HSN (large arrow) and the ALN/PLM precursor (small arrow) nuclei. (*H*) Anti-UNC-86 staining. The HSN (large arrow) and ALN/PLM precursor (small arrow) show normal expression of UNC-86. (*I*) Anti-HAM-2 staining. Although expression of HAM-2 in the head (mostly out of focus) and in the hypodermis (at an earlier stage not shown here) are normal, the HSN does not express HAM-2 in *ham-2(mu1)* embryos. The HSNs also lack HAM-2 expression in *ham2(n1332)* animals (not shown). Scale bar, 5 μm.

sufficient to drive HSN expression in GFP reporter constructs (data not shown).

In addition to eliminating EGL-43 expression in the HSN, the *egl-43(n1079)* mutation also affects EGL-43 expression in the ADE neuron and in the phasmid neurons PHA and PHB. Reduced EGL-43 expression in the phasmid neurons correlates with the phasmid dye-loading defect also observed in *egl-43* mutants (Garriga et al. 1993b). Because the HSNs and PHBs are sister cells, current *C. elegans* mosaic analysis techniques cannot distinguish between *egl-43* acting in the HSN or the PHB to promote HSN migration (Herman 1989; Sulston et al. 1983). It is likely, however, that *egl-43* acts cell-autonomously to promote HSN migration, because in individual animals the severity of the HSN migration defects does not correlate with the presence or absence of EGL-43 in the phasmid neurons (Table 2). We have been unable to detect any defects in the development of the ADE neurons of *egl-43* mutants (data not shown).

egl-5 *regulates* ham-2 *and* egl-43 *expression in the HSN*

In most HSN migration mutants, the HSNs either fail to migrate or migrate partially out of the tail (Desai et al. 1988; Forrester and Garriga 1997; Garriga and Stern 1994). Because HSN migration mutants all have a similar phenotype, it has been impossible to perform genetic epistasis experiments to order them into a pathway. However, as some of the genes required for HSN migration have now been cloned, antibody staining experiments make it possible to order these genes based on molecular epistasis (Table 1). We found that HSN expression of HAM-2 and EGL-43 was normal in most HSN migration mutant backgrounds. However, we found striking effects on the expression of HAM-2 and EGL-43 in *egl-5* mutants. *egl-5* encodes an *Abdominal-B* homolog that plays a critical role in specifying most steps in HSN differentiation, including cell migration, cell death, and neurotransmitter expression (Desai et al. 1988; Wang et al. 1993). First, *egl-5* mutants showed no HSN expression of HAM-2, suggesting that *egl-5* acts upstream of *ham-2*. Consistent with this hypothesis, *egl-5; ham-2* double mutants did not display a more severe HSN migration defect than *egl-5* single mutants (not shown). Second, *egl-5* mutants failed to down-regulate HSN expression of EGL-43 normally after HSN migration was complete (Fig. 6). This deregulation of *egl-43* expression is probably not a result of the *egl-5* migration defect, because EGL-43 is turned off at the normal time in other mutants with misplaced HSNs (data not shown). The lineal sisters of the HSNs, the PHB sensory neurons, also express EGL-43 from when they are born during embryogenesis, but they continue to express EGL-43 though adulthood. Thus, one interpretation of the continued EGL-43 expression is that *egl-5* mutant HSNs are transformed partially into their sister cell. To test this hypothesis, we determined whether *egl-5* HSNs express an *srb-6–gfp* transgene. *srb-6* encodes a seven-transmembrane molecule that may function as chemoreceptor for

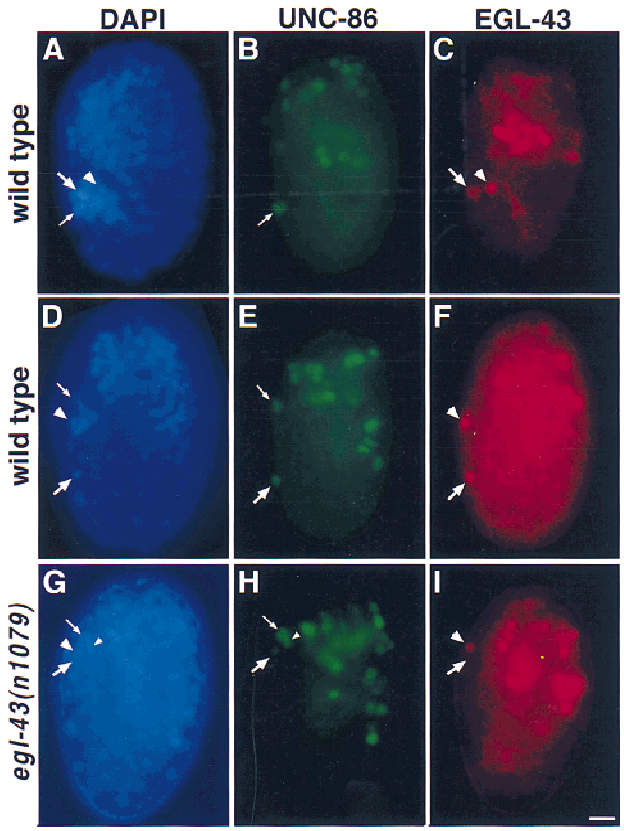


Figure 5. EGL-43 protein is expressed in the HSN nucleus during migration. Fluorescence photomicrographs of wild-type (*A–F*) and *egl-43(n1079)* mutant (*G–I*) embryos that have been stained with DAPI (blue), an UNC-86 antiserum (green), and an EGL-43 antiserum (red). Each panel presents a left lateral view of the embryo, with the developing tail to the left, the head to the right and the end of the tail and the top of the head both oriented upwards. The right sides are out of focus. (*A–C*) A wild-type embryo at ~400 min after first cleavage. (*A*) DAPI staining was used to visualize cell nuclei. The HSN/PHB precursor (large arrow), PHA sensory neuron (arrowhead) and ALN/PLM precursor (small arrow) nuclei are located in the tail. (*B*) Anti-UNC-86 staining. At this time, only the ALN/PLM precursor nucleus (small arrow) expresses UNC-86 in the tail (Finney and Ruvkun 1990). (*C*) Anti-EGL-43 staining. At this time, only the HSN/PHB precursor (large arrow), which will divide to generate an HSN and PHB neuron, and the PHA sensory neuron (arrowhead) express EGL-43 in the tail. (*D–F*) Wild-type embryo at ~430 min after first cleavage. (*D*) DAPI staining reveals the positions of the HSN nucleus (large arrow) which has migrated out of the tail, as well as a phasmid neuron nucleus (arrowhead) and an ALN/PLM precursor nucleus (small arrow) in the tail. (*E*) Anti-UNC-86 staining. Shortly after commencing its anteriorly directed migration, the HSN begins expressing UNC-86 (large arrow; Finney and Ruvkun 1990). (*F*) Anti-EGL-43 staining. During its migration, the HSN also expresses EGL-43-(large arrow). One of the EGL-43-expressing phasmid neuron nuclei (arrowhead) is also visible. (*G–I*) *egl-43(n1079)* mutant embryo at ~430 min after first cleavage. (*G*) DAPI staining of this *egl-43(n1079)* mutant embryo shows the position of the HSN nucleus in the tail (large arrow). The phasmid neuron (large arrowhead), ALN/PLM precursor (small arrow) and the sister cell of the ALN/PLM precursor (small arrowhead) nuclei are indicated. (*H*) UNC-86 expression. The HSN neuron expressing UNC-86 (large arrow) has not migrated from its birthplace in the tail. The nuclei of the ALN/PLM precursor (small arrow) and its sister cell (small arrow) are indicated. (*I*) Anti-EGL-43 staining. One of the phasmid neurons (large arrowhead) but not the HSN neuron (large arrow) is expressing EGL-43. Scale bar, 5 μm.

the detection of volatile repellents (Troemel et al. 1995). The phasmid neurons PHA and PHB express *srb-6–gfp*, whereas the HSNs do not. Consistent with the hypothesis that HSNs are transformed partially into PHBs, *egl-5* HSNs often express *srb-6–gfp* (Fig. 6).

unc-86; ham-2 *double mutants phenocopy* egl-5 *mutants in their failure to down-regulate HSN EGL-43 expression*

Expression of the UNC-86 POU homeodomain transcription factor in the HSN requires *egl-5* (Baumeister et al. 1996), similar to the *egl-5* requirement for HAM-2 expression in the HSN. UNC-86 expression in the HSN begins when the neuron initiates its migration, and we found low penetrance HSN migration defects in *unc-86* mutants (Fig. 1). In contrast to the weak early HSN migration defect, *unc-86* mutants display severe defects in HSN traits expressed later during larval development: hood formation (a morphological marker of neuronal maturation) and serotonin expression fail to occur (Desai et al. 1988). From the analysis of single mutants, it appeared that *ham-2* and *unc-86* play distinct roles in early and late HSN development, respectively. Nonetheless, because UNC-86 expression in the HSN begins early, it remained possible that *unc-86* played a redundant role in early HSN development that was masked by *ham-2* function. To test this possibility, we examined the HSN phenotype of an *unc-86; ham-2* double mutant. We did not uncover an additional role for *unc-86* in HSN migration: *unc-86; ham-2* double mutants had no more severe an HSN migration defect than *ham-2* mutants (Fig. 1). To our surprise, we found that the *unc-86; ham-2* double mutant displayed synthetic phenotypes not seen in either single mutant alone: EGL-43 expression was often not properly down-regulated and *srb-6–gfp* was expressed ectopically in the HSNs of *unc-86; ham-2* mutants (Fig. 6). As in wild-type animals, the HSNs of *unc-86* or *ham-2* single mutants down-regulated EGL-43 expression after they finished migrating and did not express *srb-6–gfp* (Fig. 6).

egl-5 *regulates the HSN's decision to live or die*

In addition to their other differentiation defects, the HSNs of *egl-5* mutants also display defects in sex-specific programmed cell death. During male embryonic development, the HSNs normally die (Sulston and Horvitz 1977). Desai et al. (1988) reported that HSNs sometimes survived in *egl-5* males. In addition, they found that *egl-5*

Table 1. *Expression of HSN transcription factors in transcription factor mutant backgrounds*

Strain	L.G.	Encodes	EGL-43 expression	HAM-2 expression	UNC-86 expression
egl-5(n945)	III	Hox	persists in HSNs	absent in HSNs	absent in HSNs
egl-43(n1079)	II	Zn finger	absent in HSNs, ADEs and phasmid neurons	w.t.	w.t.
ham-2(n1332)	X	Zn finger	w.t.	absent in HSNs	w.t.
unc-86(n946)	III	POU	w.t.	w.t.	absent in all UNC-86-expressing cells

L1 larvae carrying loss-of-function mutations in genes encoding HSN transcription factors were stained with antisera to EGL-43, HAM-2 (Materials and Methods),and UNC-86 proteins (Finney and Ruvkun 1990). Over 50 animals of each mutant background were stained with each antiserum. (L.G.) Linkage group for each gene. The cloning of *egl-5, egl-43,* and *unc-86* were described in Wang et al. (1993), Garriga et al. (1993b), and Finney and Ruvkun (1990), respectively. The absence of UNC-86 expression in the HSNs of *egl-5* mutants was first noted in Baumeister et al. (1996).

was able to suppress the inappropriate cell deaths of HSNs in hermaphrodites carrying dominant alleles of *egl-1*. The gene *egl-1* encodes a negative regulator of the Bcl-2 homolog CED-9 that is necessary for all programmed cell deaths in *C. elegans*, suggesting that dominant *egl-1* alleles are regulatory mutations that cause the HSNs to die in hermaphrodites, the male HSN fate (Trent et al. 1983; Conradt and Horvitz 1998).

We identified a new cell-death phenotype for *egl-5* mutants: the HSNs occasionally died in *egl-5* hermaphrodites (Fig. 1). When we scored *egl-5* mutants by Nomarski optics, only 76% of the HSNs were located along their migratory route. The missing HSNs might have failed to migrate from their birthplace in the tail, where they would be indistinguishable from other neurons of the lumbar ganglia; alternatively, these HSNs might have died. To distinguish between these possibilities, we determined the positions of the HSNs in *egl-5; ced-3* hermaphrodites (Fig. 1). The gene *ced-3* is necessary for all programmed cell deaths in *C. elegans*; in *ced-3* males; for example, the HSNs of males survive (Ellis and Horvitz 1986). We found that the percentage of HSNs detected along the migratory route increased to 85% in *egl-5; ced-3* double-mutant hermaphrodites, consistent with the hypothesis that HSNs undergo apoptosis occasionally in *egl-5* hermaphrodites. Presumably, the 15% of HSNs still unaccounted for in these double mutants were hidden among the neurons of the tail. To confirm that HSN apoptosis was taking place, we made use of the *ced-1* mutation, which disrupts the cell corpse engulfment process and allows corpses to persist (Hedgecock et al. 1983). When we could not identify an HSN on a given side of a *ced-1; egl-5* hermaphrodite, an ectopic cell-death corpse was often present along the HSN's migratory route on that side (Fig. 1). This phenotype was not observed in *ced-1* hermaphrodites (not shown).

Because the *ham-2* and *unc-86* genes act redundantly downstream of *egl-5* to down-regulate EGL-43 HSN expression, it seemed plausible that *ham-2* and *unc-86* could also coordinately control HSN sex-specific cell death. However, no HSNs were missing in *unc-86; ham-2* hermaphrodites—all HSNs were detected along their migratory route (Fig. 1). In addition, no HSNs survived in *unc-86; egl-1; ham-2* hermaphrodites (not shown). In contrast to the *egl-5* mutants, the HSN cell death decision was implemented properly in *unc-86; ham-2* animals.

Table 2. *EGL-43 expression in the phasmid neurons does not affect the extent of HSN migration in* egl-43 *mutants*

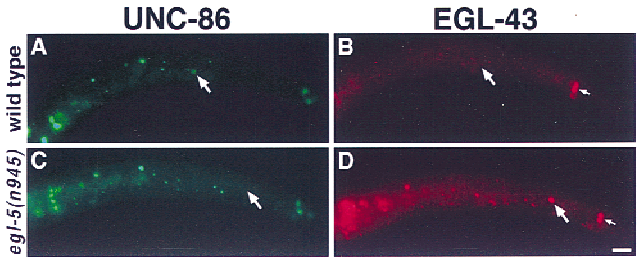
No. of phasmid neurons expressing EGL-43	HSN position			
	>20%	1–20%	0%	*n*
2	0	66	33	6
1	15	58	27	26
0	13	56	31	16

egl-43(n1079) L1 larvae were double strained with an anti-UNC-86 antiserum to detect the HSNs (Finney and Ruvkun 1990) and an anti-EGL-43 antiserum to detect the phasmid neurons. HSN positions were measured relative to the phasmid neurons and gonad, which mark the beginning (0%) and end (100%) of the HSN migration route, respectively. Animals were then scored for expression of EGL-43 in both, one; or neither of the PHA and PHB phasmid neurons on that side of the animal.

Discussion

Throughout the animal kingdom, Hox genes play a fundamental role in anteroposterior patterning, but the mechanisms by which they specify cell fates have not been examined in detail at single-cell resolution. Here, we have identified *ham-2*, a gene that encodes a probable zinc-finger transcription factor required for HSN migration. We have shown that HAM-2 acts downstream of the Hox protein EGL-5, and examined its regulatory interactions with the Hox-dependent transcription factor UNC-86 and the Hox-independent transcription factor EGL-43, two other proteins that also promote HSN differentiation. Until now, it has been difficult to determine unambiguously the order of actions of genes regulating HSN differentiation (Desai et al. 1988). Using analysis of single and multiple mutants, as well as molecular analysis of protein expression in different mutant backgrounds, we have been able to propose a regulatory pathway for HSN development (Fig. 7).

The *Abdominal-B* homolog *egl-5* is a general regulator of HSN identity. Unlike mutations in other transcription factors, which only affect certain aspects of HSN differentiation, *egl-5* mutations disrupt HSN sex-specific



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Strain	%HSNs staining*	(n)	% HSNs expressing†	(n)
wild type	0%	78	0%	100
unc-86(n946)	0%	50	0%	100
ham-2(n1332)	4%	50	3%	100
unc-86(n946); ham-2(n1332)	35%	69	66%	100
egl-5(n945)	72%	75	40%	100

* L1 larvae were stained with an anti-EGL-43 antiserum
† larvae contained the *srb-6-gfp* transgene

Figure 6. Some HSN migration mutants fail to down-regulate EGL-43 and repress *srb-6*. (*A–D*) Fluorescence photomicrographs of wild-type (*A*,*B*) and *egl-5(n945)* mutant (*C*,*D*) L1 larvae that have been fixed and stained with UNC-86 and EGL-43 antisera. All four panels present left lateral views of the animals with anterior to the left. The right sides are out of focus. (*A*) In wild-type animals, the HSN neuron is located in the midbody and expresses UNC-86 (arrow). (*B*) EGL-43 expression in the same larva as in *A*. The PHA and PHB phasmid neuron nuclei (small arrow) express EGL-43 but the HSN neuron (arrow) does not. (*C*) In *egl-5(n945)* L1 larvae, posteriorly displaced HSNs (arrow) often fail to express UNC-86 (Baumeister et al. 1996). (*D*) EGL-43 expression in the same larva pictured in *C*. In contrast to wild-type, the posteriorly displaced HSN does express EGL-43 (arrow). The phasmid neurons also express EGL-43 (small arrow). Scale bar, 15 μm. (*E*) Table quantifying persistence of EGL-43 expression and presence of *srb-6–gfp* in the HSNs of transcription factor mutants.

cell death, migration, and later phenotypes, such as neurotransmitter expression. *egl-5* is also required for proper expression of two downstream transcription factors, UNC-86 and HAM-2, in the HSNs, and may act through these genes to implement HSN cell fate. Because HSN expression of EGL-43 and *srb-6–gfp* in *egl-5* mutants resembles the normal expression of these markers in the lineal sisters of the HSNs, the PHB sensory neurons, many of these defects may result from a partial transformation of HSN into PHB. Although *egl-5* plays an important role in determining HSN identity, it is not the sole determinant of HSN identity. The various HSN phenotypes of *egl-5* null mutants are not fully penetrant, and UNC-86 and HAM-2 are occasionally expressed in HSNs lacking *egl-5* function. The HSNs, although misplaced and morphologically abnormal, can still migrate partially in *egl-5* null mutants. Furthermore, *egl-5* alone cannot confer HSN identity on a given cell: EGL-5 is expressed in other tail neurons that differentiate into non-HSN fates (Wang et al. 1993).

We have shown that *ham-2* is required for HSN migration. Our analysis of two regulatory mutations in the *ham-2* gene, which cause HSN migration defects and which reduce or eliminate HAM-2 expression in the HSN without affecting other cells, suggests that *ham-2* functions in the HSN. By contrast, mutations that alter the *ham-2* coding region, and thus presumably disrupt *ham-2* function in all the cells in which it is normally expressed, do not cause additional neuronal migration defects, but do cause defects in the attachment of the pharynx to the front of the worm. Based on this additional phenotype, it is possible that adhesion molecules might be among HAM-2's targets.

ham-2 mutants do not have as severe an HSN migration defect as *egl-5* mutants do. Either none of our *ham-2* alleles is null, or *egl-5* has additional targets that promote HSN migration. Finally, although the *ham-2* alleles *n1332* and *mu1* do not appear to produce defects in later stages of HSN development, such as serotonin expression, we cannot rule out the possibility that these alleles provide some *ham-2* function in the HSN during those later stages. It is possible that *ham-2* is not completely specific for the early migratory stage of HSN development.

unc-86 has been shown previously to be required for late HSN fates, such as hood formation and serotonin expression (Desai et al. 1988). Although UNC-86 is ex-

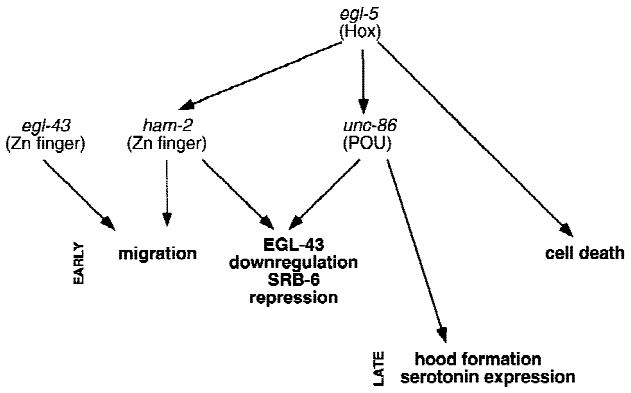

Figure 7. A transcriptional network regulates HSN development. This figure shows transcription factors expressed in the HSN and required for HSN differentiation. The arrows designate functional relationships determined by genetic criteria; these relationships may be direct or indirect. The zinc finger protein encoded by the gene *sem-4*, which acts in late HSN differentiation (Basson and Horvitz 1996), is not depicted in this chart because its relationships to *egl-5* and *unc-86* have not been determined. Consistent with *egl-43* and *egl-5/ham-2* acting independently to regulate HSN migration, HSN migration in *egl-43; egl-5* and *egl-43; ham-2* double mutants is more severely defective than in any of the single mutants.

pressed in the HSN during migration, null *unc-86* mutants have weak migration defects, and do not enhance the migration defects of *ham-2* mutants. *unc-86* plays a second role in early HSN differentiation, but this role is only revealed in a *ham-2* double mutant.

egl-43 has been described previously as a transcription factor required for HSN migration (Garriga et al. 1993b). Here we show that the identical 3′ deletion found in the *egl-43* alleles *n997* and *n1079* is likely to be a regulatory mutation that removes EGL-43 from only a few cells: the HSNs, the ADE neurons, and the phasmid neurons. *egl-43* appears to act in the HSNs to promote HSN migration and in the phasmid neurons to promote their differentiation. Unlike HAM-2 and UNC-86, the onset of EGL-43 expression in the HSN is independent of *egl-5*. On the other hand, *egl-5* appears to act via *ham-2* and *unc-86* to down-regulate EGL-43 expression and repress *srb-6* expression in the HSN.

We have also demonstrated a role for *egl-5* in proper execution of the sex-specific cell-death decision that the HSN makes during embryonic development. *egl-5* function is required to prevent both the inappropriate cell death of the HSN in hermaphrodites and the aberrant survival of the HSN in males. Although we have identified roles for *unc-86* and *ham-2* in mediating many *egl-5* functions in the HSN, we were unable to uncover any role for these genes in regulating HSN cell death. One possible mediator of *egl-5*'s role in the HSN cell-death decision is the gene *tra-1*, which encodes a zinc-finger protein that acts autonomously as the terminal regulatory gene of the sex-determination pathway and controls the HSN sexual fate in hermaphrodites by preventing its death (Hunter and Wood 1990; Zarkower and Hodgkin 1992). As a regulator of HSN sex-specific survival, EGL-5 could act by altering expression of *egl-1* in the HSN, either directly by binding to *egl-1* regulatory sequences or indirectly though TRA-1.

Why have Hox targets been difficult to identify and analyze through genetic means? Our results suggest three reasons that Hox target genes may have mutant phenotypes that are unrecognizably different from the mutant phenotypes of the Hox genes themselves. First, Hox target genes may play general roles in development, with their roles downstream of individual Hox genes comprising only a minor part of their function. Specific regulatory mutations that reduced *ham-2* function in the HSN were isolated in screens for HSN migration defects. The widespread expression pattern of *ham-2*, and the lethal phenotype of *ham-2(gm16)* suggest that *ham-2*'s role downstream of *egl-5* in promoting HSN migration is only one part of its biological function. Second, our results show that Hox target genes may each only carry out a small subset of the roles played by the Hox gene itself, and that these target genes may have redundant roles. Although certain HSN differentiation events regulated by *egl-5*, such as cell migration and neurotransmitter synthesis, are carried out by individual downstream regulators, others, such as the down-regulation of EGL-43, may be carried out by multiple downstream targets in a redundant fashion. Still other steps in HSN differentiation regulated by *egl-5*, such as the sex-specific cell-death decision, may be carried out by additional downstream genes that still remain to be identified. Third, Hox genes are not fully responsible for cell fate determination. The HSNs migrate partially even in *egl-5* null mutants, suggesting that HSN migration is determined only partly by Hox-directed cell-fate determination and partly regulated by Hox-independent regulators, such as *egl-43*. Taken together, the complexity of *egl-5* function suggests that it will be difficult to identify Hox targets by genetic phenotypes alone.

Often the expression of one or two transcription factors can confer a specific cell fate. For example, expression of both *unc-86* and *mec-3* is required for mechanosensory neuron cell fate (Way and Chalfie 1989; Xue et al. 1993). Yet a few transcription factors are known that are only responsible for one portion of a neuron's differentiation program. These transcription factors may act after more general fate determinants in the process of neuronal differentiation (Lewin 1994). For example, the Hox genes *mab-5* and *lin-39* are not master regulators of Q-neuroblast identity in the way that *egl-5* is a master regulator of HSN identity. Mutations in these genes affect the direction and extent of Q-neuroblast migrations, but other aspects of these cells are not affected (Wang et al. 1993). As another example, the ODR-7 transcription factor appears to be required for regulating the odorant specificity of the AWA sensory neuron, but is not required for proper morphological development of that neuron (Sengupta et al. 1994). Here, in the case of the HSN, we have described a collection of transcription factors, *unc-86*, *egl-43*, and *ham-2*, that appear to regulate different subsets of HSN differentiation.

A challenge for the future will be to identify the targets of *egl-43* and *ham-2* that act directly in HSN migration. The relevant effectors may be represented among the large collection of mutants required for HSN migration (Garriga and Stern 1994).

Materials and methods

C. elegans *genetics*

Strains were grown at 20°C unless otherwise stated, and were maintained as described by Brenner (1974). In addition to the wild-type strain N2, strains with the following mutations were used in this work: *L.G. I: ced-1(e1735)* (Hedgecock et al. 1983). *L.G. II: egl-27(n170)* (Trent et al. 1983); *egl-43(n997)*, *egl-43(n1079)* (Desai et al. 1988); *unc-4(e120)* (Brenner 1974). *L.G. III: unc-86(e1416)*, *unc-86(n306)*, *unc-86(n843)*, *unc-86(n844)*, *unc-86(n946)* (Chalfie et al. 1981; Desai and Horvitz 1989); *egl-5(n945)* (Desai and Horvitz 1989); *gmIs12 (srb-6–gfp) + rol-6 (su1006)* (Troemel et al. 1995; N. Hawkins, pers. comm.). *L.G. IV: egl-18(n162)* (Trent et al. 1983); *egl-20(n585)* (Trent et al. 1983); *ced-3(n717)* (Ellis and Horvitz 1986). *L.G. V: him-5(e1490)* (Hodgkin et al. 1979). *L.G. X: lin-32(u282)* (Chalfie and Au 1989); *unc-20(e112ts)* (Brenner 1974); *lin-18(e620)* (Ferguson and Horvitz 1985); *dpy-23(e840)* (Hodgkin 1983); *ham-2(n1332)* (Desai et al. 1988); *ham-2(mu1)* (Cynthia Kenyon, pers. comm.); *ham-2(gm16)* and *ham-2(gm48)* (this study); *lon-2(e678)* (Brenner 1974).

Isolation of new ham-2 *alleles*

ham-2(gm16) was isolated in a *ham-2* noncomplementation screen. Briefly, *unc-20(e112ts) lin-18(e620)* hermaphrodites were mutagenized with EMS and crossed to *ham-2(n1332) lon-2(e678)* males. 16,200 non-Unc cross progeny were screened for Egl defects, yielding one new allele, *gm16*. *ham-2(gm48)* was isolated in a direct screen for mutants with misplaced HSNs (Dianne Parry, P.D. Baum, and G. Garriga, unpubl.).

Cloning of ham-2

ham-2 was genetically mapped to the interval between *dpy-23* and *lon-2* on the X chromosome, and then RFLP mapping was used to locate *ham-2* on the physical map. Three RFLPs were identified by probing genomic Southern blots of polymorphic *C. elegans* strains with cosmid clones from the region: *gmP1* (a *Hin*DIII digest of the strain RW7000 probed with the cosmid W01H2), *gmP2* (a *Xho*I digest of the strain N62 with the cosmid F02E8, and *muP1* (an *Eco*RI digest of N62 with the cosmid K02G10; Mary Sym, pers. comm.). Transheterozygous strains were constructed that had a *dpy-23(e840) ham-2(n1332) lon-2(e678)* X chromosome and either a RW7000 or an N62 X chromosome. Lon non-Dpy recombinants were selected and progeny of these recombinants that were homozygous for the Lon non-Dpy recombinant chromosome were scored for the Ham phenotype and assayed for polymorphisms by Southern blot. Of 19 Lon non-Dpy recombinants, all picked up *gmP1*. Of 20 Lon non-Dpy recombinants, 4 out of 5 Ham recombinants, and all 15 non-Ham recombinants picked up *gmP2*. In addition, none of the 5 Ham recombinants, and 4 out of 15 non-Ham recombinants picked up *muP1*. Taken together, these data placed *ham-2* between the *gmP2* and *muP1* RFLPs.

Two overlapping cosmids from this region, C04F8 and F42G12, rescued the HSN migration defect of *ham-2(n1332)* mutants. In addition, a Southern blot of *ham-2(n1332)* and *ham-2(mu1)* genomic DNA probed with the cosmid C04F8 revealed DNA rearrangements relative to wild type (not shown). Finally, a 7-kb *Avr*II–*Kpn*I genomic subclone predicted to contain only the *ham-2* gene (pGM134) rescued the HSN migration defect of *ham-2(n1332)* mutants. A GFP construct, pGM138, was constructed by cloning the GFP gene from pPD95.69 (Andrew Fire, pers. comm.) as a *Bam*HI–*Fsp*I fragment into pGM134 cut with *Bam*HI and *Kpn*I (blunted).

Analysis of ham-2 *transcripts*

The *ham-2* genomic region had been sequenced by the *C. elegans* Genome Sequencing Project (cosmid C07A12; accession no.U41542). A cDNA for the long transcript of *ham-2*, yk82h11, was a gift of Yuji Kohara (National Institute of Genetics, Mishima, Japan). The cDNA was sequenced using the method of Strathmann et al. (1991). We also searched for alternative transcripts using the 5′ RACE technique, as well as RT–PCR with gene-specific primers and primers to the SL-1 and SL-2 splice leaders. The long SL-1 transcript was amplified consistently; the SL-2 transcripts were only detected in a minority of PCR reactions. The genomic organization of the *ham-2* gene does not conform to the usual pattern for SL2 trans-splicing of polycistronic mRNAs (Blumenthal 1995).

Allele sequencing

All mutant alleles were sequenced using multiple independent PCR amplifications of genomic DNA. The *egl-43* alleles were determined previously to have deletions 3′ to the coding region of the gene based on Southern blotting (Garriga et al. 1993b). Sequencing of the *egl-43(n997)* and *egl-43(n1079)* alleles revealed identical 790-bp deletions corresponding to nucleotides 21,803–22,592 of the genomic sequence derived by the *C. elegans* Genome Sequencing Project from the cosmid R53 (accession no. Z66515).

anti-HAM-2 and anti-EGL-43 antisera

The *Bam*HI–*Kpn*I carboxy-terminal fragment of the yk82h11 *ham-2* cDNA was cloned into the pRSET B 6×His vector (Invitrogen). This carboxy-terminal portion was chosen because the amino terminus showed extensive homology to other predicted *C. elegans* proteins. Antibodies to this carboxy-terminal fusion protein would be expected to react with both the long and short *ham-2* gene products. The protein was expressed in *E. coli*, purified with nickel-agarose resin and injected into mice. Ascites fluid was induced and collected. Antisera from mice were able to recognize specifically bacterially expressed HAM-2 on a Western blot (not shown). Nematodes were stained by the method of Finney and Ruvkun (1990). The staining pattern for anti-HAM–2 antisera is likely to be correct because the expression pattern obtained with the antisera was identical to that seen with a genomic *ham-2–GFP* construct (pGM138). In addition, the antisera showed an identical staining pattern after affinity purification against bacterial HAM-2 immobilized on nitrocellulose strips as described (Harlow and Lane 1988).

To generate EGL-43 protein, three cDNAs corresponding to different regions of EGL-43 were amplified by RT–PCR and cloned into the *Pst*I site of the pRSET B 6×His vector (Invitrogen); protein was made and antisera raised as described above. Antisera were affinity purified using columns of protein coupled to Reacti-Gel 6× (Pierce). Antisera raised against bacterial fusion proteins containing two distinct nonoverlapping regions of EGL-43 produced identical staining patterns. In addition, animals homozygous for the *mnDf24* deficiency, which completely removes the *egl-43* gene (Sigurdson et al. 1984), did not show any nuclear staining, suggesting that the staining pattern observed was specific for EGL-43. The genotypes of putative *mnDf24* L1 larvae were confirmed by PCR.

EGL-43-expressing cells in embryos and larvae were identified by position, and, where possible, these identifications were confirmed by double-staining experiments using other markers. Besides the HSN/PHB precursor, the HSNs, and the PHA and PHB phasmid neurons (Fig. 4; Tables 1 and 2), EGL-43 was also expressed in the FLPL/R, PVDL/R, ADFL/R, ADLL/R, LUAL/R (only during embryogenesis), ADEL/R, RIGL/R, ASHL/R, ASJL/R, ASIL/R, CEPDL/R, CEPVL/R, OLQDL/R, OLQVL/R, IL1VL/R, IL1DL/R, URADL/R, the T-blast cells, and the somatic gonad precursors Z1 and Z4 as well as some of their descendants.

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