Multiple HOM-C gene interactions specify cell fates in the nematode central nervous system

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Intricate patterns of overlapping HOM-C gene expression along the A/P axis have been observed in many organisms; however, the significance of these patterns in establishing the ultimate fates of individual cells is not well understood. We have examined the expression of the *Caenorhabditis elegans* *Antennapedia* homolog *mab-5* and its role in specifying cell fates in the posterior of the ventral nerve cord. We find that the pattern of fates specified by *mab-5* not only depends on *mab-5* expression but also on post-translational interactions with the neighboring HOM-C gene *lin-39* and a second, inferred gene activity. Where *mab-5* expression overlaps with *lin-39* activity, they can interact in two different ways depending on the cell type: They can either effectively neutralize one another where they are both expressed or *lin-39* can predominate over *mab-5*. As observed for *Antennapedia* in *Drosophila*, expression of *mab-5* itself is repressed by the next most posterior HOM-C gene, *egl-5*. Thus, a surprising diversity in HOM-C regulatory mechanisms exists within a small set of cells even in a simple organism.

[Key Words: *Caenorhabditis elegans*; anterior-posterior axis; homeotic cluster; *mab-5*; combinatorial interactions; homeo box]

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Organisms as diverse as insects, vertebrates, and nematodes use a conserved system of *Antennapedia*-class homeo box genes to pattern the anterior-posterior (A/P) axis [for review, see McGinnis and Krumlauf 1992; also see Clark et al. 1993; Wang et al. 1993]. These homeotic complex (HOM-C) genes are expressed in restricted domains along the A/P axis and are thought to act as transcriptional regulators to give different regions of the body their identities.

In insects, where the metameric organization of the body is especially striking, HOM-C genes are often thought of as acting to specify parasegment identities. Consistent with this view, abrupt changes in HOM-C gene expression correspond to a first approximation with parasegment boundaries (Duncan 1986; Ingham and Martinez-Arias 1986; Carroll et al. 1988). Moreover, several HOM-C genes cause near complete specification of particular segmental identities when expressed ectopically under the control of heat shock promoters (Gibson and Gehring 1988; Kuziora and McGinnis 1988; González-Reyes and Morata 1990; Mann and Hogness 1990). Thus, despite the fact that domains of HOM-C gene expression overlap along the A/P axis, in many cases, a single gene is largely responsible for the identity of a particular parasegment (see, however, Struhl 1982).

This basic model (one gene, one parasegment identity) helps to describe the role of HOM-C genes in patterning whole body parts. At the level of single cells, however,

expression of these genes is surprisingly complicated. Within parasegments, cells expressing a given HOM-C gene are interspersed with nonexpressing cells and with cells expressing other HOM-C members (Beachy et al. 1985; White and Wilcox 1985; Carroll et al. 1986; Wirtz et al. 1986). In some cases, particularly in the central nervous system (CNS), specific neurons maintain strong coexpression of multiple HOM-C genes (White and Wilcox 1985; Carroll et al. 1986, 1988; Celniker et al. 1990; Karch et al. 1990). These expression patterns raise several questions. To what extent does HOM-C gene expression directly specify the domain of gene function? Moreover, in cells expressing multiple HOM-C genes, what type of functional interactions can take place? We have taken advantage of the reproducible cell lineage and simplicity of *Caenorhabditis elegans* to explore these questions with single-cell resolution.

A cluster of four HOM-C genes has been identified in *C. elegans* (Schaller et al. 1990; Bürglin et al. 1991; Kenyon and Wang 1991). Mutations in three of the genes, *lin-39*, *mab-5*, and *egl-5*, affect development in sequential regions of the body along the A/P axis (Kenyon 1986; Chisholm 1991; Clark et al. 1993; Wang et al. 1993). These genes resemble the *Deformed*/*Sex-combs-reduced*, *Antennapedia*/*Ultrabithorax* (*Ubx*)/*abdominal-A*, and *Abdominal-B* (*Abd-B*) genes in *Drosophila* and are arranged similarly within the cluster. In contrast to previous reports (Bürglin et al. 1991; Kenyon and Wang

1991), the *C. elegans* genome sequencing project has recently found *ceh-13*, a *labial* homolog, to lie between *lin-39* and *mab-5* suggesting that part of the *C. elegans* cluster has been inverted (R. Waterston, pers. comm.).

The *C. elegans* gene *mab-5* is most closely related to *Drosophila Antennapedia* and is needed to specify the fates of many neuronal, epidermal, and mesodermal cells in a posterior body domain (Kenyon 1986; Costa et al. 1988). We have examined the role of *mab-5* in patterning the ventral ectoderm, which derives from 12 precursor cells (P1–P12; see Fig. 1A). During the first larval stage, these cells divide in a stereotypical pattern to contribute neurons to the ventral nerve cord. Each P cell gives rise to one epidermal cell and five neuronal cells with distinct fates (Sulston and Horvitz 1976). The HOM-C genes create variations in this basic pattern of fates, causing cells in specific body regions to adopt specialized fates. In the posterior of the male, *mab-5* is required to specify the proper fates of three sets of P-cell descendants: the Pn.p cells, which adopt epidermal fates, the Pn.aap cells, which produce sex-specific neurons, and the Pn.aap cells, which become motoneurons (Fig. 1B).

If *mab-5* activity were simply restricted to the posterior P-cell lineages and acted as a switch to turn on *mab-5*-dependent specializations, one might expect to see a

contiguous block of specialized cells in the posterior; however, this is not the observed pattern. The boundary between specialized cells and more anterior cells occurs in different positions along the A/P axis for each of the three decisions controlled by *mab-5* (Fig. 1C). Five Pn.p cells are affected by *mab-5*, but only three Pn.aap cells and only two Pn.aap cells are affected.

How could *mab-5* be regulated to produce such a complex pattern? One possibility is that the expression of *mab-5* itself is complex. *mab-5* could be expressed specifically in those cells that adopt *mab-5*-dependent specializations and not in more anterior cells. In this case, *mab-5*-expressing and -nonexpressing cells would necessarily be interspersed with one another (Fig. 1D). Alternatively, *mab-5* could be expressed in a graded fashion, and cells could decide whether to adopt specialized fates based on the level of *mab-5*. Finally, it is possible that *mab-5* is expressed in a simple block of cells in the posterior. The boundary of *mab-5* expression could then determine one of the boundaries between specialized and more anterior cell fates and the other boundaries could arise through interactions with other genes.

To distinguish between these models we have examined the *mab-5* expression pattern, as well as the effects of expressing *mab-5* ectopically. We find that *Mab-5* pro-

Figure 1. Models to explain the role of *mab-5* in patterning the ventral nerve cord. (A) Location of the 12 P cells in the ventral nerve cord before they divide to generate neural and epidermal descendants. Ventral is down, and anterior is to the left. P1–P12 are named according to their relative positions along the A/P axis. (B) Individual P-cell lineage, showing the three types of descendants influenced by *mab-5* in the male. The Pn.p cell is epidermal and requires *mab-5* to avoid fusion with the surrounding epidermal syncytium. The Pn.aap cell is a sex-specific neuronal cell and requires *mab-5* to divide. The Pn.aap cell is a VB type motoneuron, except in P(11,12) lineages, where it requires *mab-5* to undergo programmed cell death. (C) Schematic diagram showing the overall pattern of P descendants that require *mab-5* (in bold). Five Pn.p, three Pn.aap, and two Pn.aap cells require *mab-5* to adopt their normal fates. The cells are represented by circles, diamonds, and squares, respectively, and are derived from the P lineages indicated above (1–12). (D) Three models for how *mab-5* activity could be regulated to produce this pattern: (1) Expression of *mab-5* itself is regulated so as to influence only

those cells that require *mab-5*. Expressing cells (filled) must be interspersed with nonexpressing cells (open). (2) Graded expression of *mab-5* is interpreted using multiple thresholds to create multiple boundaries in cell fate. (3) *mab-5* expression only determines one of the boundaries in cell fate. Other genes (X and Y) act to create other boundaries in cell fate. Dotted lines mark the locations of the three anterior boundaries in cell fate.

tein is present in a contiguous block of cells in the posterior ventral nerve cord, where it serves to define only one of the boundaries in cell fate. The other boundaries arise through interactions with the more anterior HOM-C gene *lin-39* and an inferred gene activity in the posterior. The interactions between these genes vary depending upon the cell type: In the Pn.p cells, *mab-5* and *lin-39* effectively neutralize one another where they are both expressed; in contrast, in the Pn.aap cells, *lin-39* activity is dominant over *mab-5* activity; finally, in the Pn.aaap cells, *mab-5* is not sufficient on its own but may instead cooperate with another gene activity. These interactions are sufficient to explain the complex anterior boundary of *mab-5* activity.

Previous work demonstrates that the posterior boundary of *mab-5* function is restricted by the HOM-C gene *egl-5* (Chisholm 1991): Normally, *mab-5* mutations alter the P[7–11].p fates, whereas in an *egl-5* background *mab-5* mutations affect P[7–12].p. Consistent with this genetic interaction, we have found that *egl-5* represses *mab-5* expression in the P12 lineage.

Results

mab-5 is expressed in a contiguous region of the ventral cord

To get a basic understanding of how *mab-5* is regulated to create differences along the A/P axis, we first examined the pattern of *mab-5* expression at different times in development using polyclonal antibodies (Fig. 2A–D). Mab-5 protein was localized to the nuclei of many cells in the posterior, consistent with its role as a region-specific transcription regulator. We found detectable levels of Mab-5 in the posterior P lineages (P7–P12), but not in more anterior cells. Within the P7–P12 region, Mab-5 expression was graded with the highest levels in the P10 descendants. The descendants of any one P cell expressed comparable levels of Mab-5.

In newly hatched larvae, the P cells are not yet positioned ventrally but, instead, are located in two rows of six cells: one row on each side of the animal. In both sexes, Mab-5 protein was seen consistently in nuclei of P9/10 and P11/12 (Fig. 2B) but not in more anterior P cells. Mab-5 was also detected at high levels in the posterior juvenile motoneurons located ventrally (Fig. 2A), although its role in these cells is unknown.

Approximately 8 hr after the time of hatching, the P cells descend into the ventral nerve cord and interdigitate to form a single row of 12 cells, P1–P12. In both sexes, we detected Mab-5 in P7 and P8, as well as in P9–P12 (not shown). In slightly older larvae, in which some P cells had divided, Mab-5 was found consistently in P7–P12 descendants but not in more anterior P descendants (Fig. 2C).

The completion of these divisions establishes a row of P-derived neuronal cells interspersed with juvenile motoneurons and epidermal Pn.p cells. In the male, descendants of P10 consistently expressed high levels of Mab-5, those of P9 and P11 expressed lower levels, and those of

P7 and P8 expressed barely detectable levels (Fig. 2D). P12 descendants were found to express Mab-5 only until the division of P12.a.

The descendants of any one P cell expressed comparable levels of Mab-5, the only exceptions being the Pn.p cells, which have larger and more diffuse nuclei, and the Pn.aap cells, which consistently expressed lower levels than their neighbors. Expression levels in the P descendants were similar to those seen in the neighboring juvenile motoneurons: Both sets of cells expressed *mab-5* in a graded pattern, tapering off toward the anterior.

The expression pattern in hermaphrodites was identical to that just described in males, with two exceptions: the P[9–11].aap cells, which undergo programmed cell death, and the P[9–11].p cells, which fuse with the epidermal syncytium. Both of these cell types no longer expressed *mab-5* by L2 (Fig. 2E).

This simple pattern argues against the first model (Fig. 1D) in which multiple boundaries of *mab-5* expression directly determine the pattern of cell fates. We do not see multiple boundaries of expression; instead, the spatial profile of *mab-5* expression is similar in all of the P-cell descendants. The anterior boundary of expression, which lies between the P6 and P7 descendants, coincides with only one of the cell-fate decisions: that occurring in the Pn.p cells. To learn whether *mab-5* expression actually determines the location of this boundary, we examined the effects of ectopic *mab-5* expression using both a *mab-5* gain-of-function promoter mutant, *e1751(gf)*, and a heat shock promoter fusion.

mab-5 expression determines which Pn.p cells fuse with the epidermal syncytium

In Pn.p cells, *mab-5* determines whether cells will remain mononucleate or fuse with the epidermal syncytium (Kenyon 1986). Pn.p cells 7–11 in the normal male are affected by *mab-5* activity, whereas 1–6 are not (Fig. 1C). To alter the pattern of *mab-5* expression, we first used the promoter mutation *mab-5(e1751gf)* described previously (Hedgecock et al. 1987; Salser and Kenyon 1992). In this background the domain of *mab-5* expression extended anteriorly to include every P-cell descendant (Fig. 2G). Moreover, the fusion decisions of Pn.p cells 1–6 as well as 7–11 were affected (see below; Fig. 3E–F). Thus, the boundary between affected and unaffected Pn.p cells appears to be determined by the boundary of *mab-5* expression.

mab-5 interacts combinatorially with *lin-39* in Pn.p cells

Remarkably, within the domain of *mab-5* expression in the experiment just described, its effect on cell fusion depended on interactions with another HOM-C gene, *lin-39*. Previous work (Clark et al. 1993; Wang et al. 1993) has shown that when neither gene is active, P[1–11].p cells all fuse with the epidermal syncytium (Fig. 3A). When either *mab-5* or *lin-39* acts on its own, it can prevent fusion of the Pn.p cells in its domain of activity (Fig. 3B,C). Finally, where the domains of *mab-5* and

Figure 2. Mab-5 protein distribution in larvae. (A–B) Early L1 male. On the right side (B), P9/10 and P11/12 stain, as do their bilaterally symmetric homologs on the left (not shown). The more anterior P1/2, P3/4, P5/6, and P7/8 cells are unstained. The epidermal cell V6, M myoblast, and several posterior body wall muscles also stain. In the ventral nerve cord (A), seven embryonically produced juvenile motoneurons, DB6–DA8, stain. This pattern is reproducible ($n = 61$ sides, ≥ 25 male, ≥ 17 hermaphrodite, $n = 17$ ventral, 10 male, 7 hermaphrodite). Nonspecific germ-line staining is indicated (^) (see Materials and methods). (C) Older L1 male (~ 12 hr after hatching). P1–P10 have divided, P11–P12 have not. Mab-5 is detected in P7–P12 or descendants but not in P1–P6 descendants. Juvenile motoneurons continue to stain ($n = 14$ male, 3 hermaphrodite). (D) Early L2 male ($n = 38$). Pn.p nuclei are indicated. Other P descendants are mixed with juvenile motoneurons and cannot be distinguished unequivocally on the basis of morphology or staining. P12 descendants do not stain at this stage. (E) Early L2 hermaphrodite. P(9–11).p cells have fused with the epidermal syncytium and no longer express *mab-5* ($\uparrow$). P(9–11).aap cells have undergone programmed cell death. Aside from these differences, expression is identical to that in the male ($n = 18$). (F) *lin-39(n1760)* early L1 male. As in wild type, P7/8 does not stain at this stage ($n = 16$). Expression first appears when P7–8 cells enter the ventral cord ($n = 6$) and after they divide ($n = 3$). The pattern is indistinguishable from wild type, although fainter. (G) *mab-5(e1751gf)* L1 male ~ 12 hr post hatching. All P descendants stain ($n = 10$). (H) *egl-5(-)* early L2 male. Mab-5 is not only detected in P9–P11 descendants, as in wild type (D), but also in P12.p and in four additional cells that appear to be the P12.a descendants ($n = 6$). In hermaphrodites, only three additional cells stain, presumably because of the death of P12.aap in this background ($n = 13$). Precursors of these cells can be identified unambiguously and consistently exhibit Mab-5 labeling ($n = 8$).

lin-39 activity overlap in the wild-type male, something unexpected happens: *lin-39* and *mab-5* effectively neutralize one another, leading to fusion of Pn.p cells 7–8 (Fig. 3D).

Several models could account for this interaction. One possibility is that *lin-39* and *mab-5* simply repress each other transcriptionally in the region of overlap (cells 7–8). Thus, neither gene would achieve a high enough level of activity to prevent cell fusion. Consistent with the model, we did find that *mab-5* expression was lower in the P(7–8) lineages than in other P lineages: As already described, it is undetectable in P7/8 at the time of hatch-

ing and only barely detectable in the final P(7–8) descendants.

Further observations, however, argue against the mutual repression model. First, no increase in Mab-5 levels was seen in P7/8 or P(7–8).p in a *lin-39(-)* background (Fig. 2F). Conversely, a *lin-39-lacZ* fusion was expressed strongly in both P5/6 (83%) and P7/8 (96%, $n = 24$), and expression was not increased in a *mab-5(-)* background (57%, 82%, $n = 28$). Finally, according to the mutual repression model, the level of Mab-5 protein in the zone of overlap (where cells fuse) should be lower than the level outside this region (where cells remain unfused). In

Figure 3. *mab-5* and *lin-39* interact combinatorially to influence the fusion of Pn.p cells. (A–E) Schematic diagram of Pn.p cell fates in various mutant backgrounds. Ovals represent unfused Pn.p cells; dashes represent Pn.p cells that have fused with the surrounding syncytium. Domains of *mab-5* and *lin-39* activity are hatched, and the overlap is darkly shaded. The indicated *mab-5(gf)* phenotype applies to both *e1751gf* and to *mab-5(-)* animals expressing *mab-5* under heat shock control. (F) Representative pattern of fusion in *mab-5(e1751gf)* male visualized by staining with mAb MH27. The outlines of P[1–2 and 9–11].p are still visible, whereas P[3–8].p cells have fused with the syncytium and are no longer detectable. In a population of *e1751* males, the frequencies with which P[1–11].p remained unfused were, respectively, 100%, 100%, 12%, 0%, 2%, 2%, 0%, 0%, 100%, 100%, and 100% ($n = 52$). (G) Fusion pattern in a *mab-5(-)* male expressing *mab-5* under heat shock control. The outlines of P[1–2 and 9–11].p remain visible, whereas P[3–8].p cells have fused with the syncytium. The frequencies with which P[1–11].p cells remained unfused were, respectively, 45%, 58%, 27%, 3%, 12%, 6%, 9%, 3%, 91%, 78%, and 87% ($n = 32$). For comparison, see control results below. Hermaphrodites were relatively unaffected: The frequencies with which hermaphrodite Pn.p cells remained unfused were, respectively, 2%, 5%, 93%, 98%, 100%, 100%, 100%, 98%, 15%, 20%, and 32% ($n = 60$). (H) Representative *mab-5(-)* control male that was heat-shocked in the same manner as that shown in G but does not carry the heat shock promoter–*mab-5* fusion construct. P[3–8].p always remain unfused, whereas P[1–2 and 9–11].p always fuse ($n = 25$) animals. Similarly, in animals carrying the construct but not exposed to high temperatures, P[3–8].p cells almost always remain unfused and P[1–2 and 9–11].p cells almost always fuse. The frequencies with which P[1–11].p remain unfused are: 0, 0, 100%, 98%, 100%, 100%, 100%, 100%, 2%, 4%, 4% ($n = 53$).

mab-5(e1751gf) larvae, however, *mab-5* was expressed at comparable levels in P2.p and P3.p (Fig. 2G) despite the fact that it has opposite effects on these two cells. In

P2.p, *mab-5(e1751gf)* prevents fusion, whereas in P3.p, *mab-5(e1751gf)* induces fusion (Fig. 3E–F).

These observations suggest that the antagonism between *mab-5* and *lin-39* does not occur at the level of transcription. To eliminate this possibility more convincingly, we expressed *mab-5* under the control of a heterologous promoter immune to the effects of *lin-39*. We placed *Mab-5* under heat shock control in a *mab-5(-)* background. In control animals (not heat-shocked or lacking the fusion construct), the pattern of fates was identical to that seen in *mab-5(-)* larvae (Fig. 3H). In males induced to express *mab-5* ubiquitously, however, the pattern was completely inverted (Fig. 3G). Pn.p cells in the *lin-39* domain (3–8) fused with the syncytium, whereas those outside this domain (1–2 and 9–11) remained unfused. Because a heterologous promoter was used to drive *mab-5* expression in these experiments, transcriptional repression of *mab-5* by *lin-39* is not required for the two genes to neutralize one another. Instead, these data and the observed expression patterns argue that post-translational interactions cause effective cancellation where both genes are expressed.

The effects of *mab-5* on cell fusion are known to be specific to the male (Kenyon 1986). Because *mab-5* expression is observed in both male and hermaphrodite Pn.p cells, *mab-5* must somehow be prevented from functioning in the hermaphrodite. Consistent with this, we found that ectopic *mab-5* expression under heat shock control, or in *e1751gf* larvae, had little or no effect on the hermaphrodite Pn.p fusion pattern [see legend to Fig. 3(G)]. *mab-5* expression both failed to prevent fusion of Pn.p cells outside the *lin-39* domain and failed to antagonize *lin-39* in the region of overlap. Thus, although HOM-C gene expression along the A/P axis appears to be regulated similarly in males and hermaphrodites, the downstream effects on cell fates can be modulated. In *C. elegans*, sex determination has been shown to proceed through a cascade of gene interactions culminating in hermaphrodite-specific expression of the zinc finger transcription factor *tra-1* (Zarkower and Hodgkin 1992). In hermaphrodites, where *tra-1* is active, *mab-5* has no effect. One possibility is that *tra-1* interferes directly with the binding or activity of *mab-5* on the promoters of target genes.

lin-39 activity, not *mab-5* expression, positions the boundary between cell fates in Pn.aap cells

In Pn.aap cells of the male, *mab-5* controls a different decision: whether to produce a single neuron or to divide again to produce two neurons. *mab-5* is required for Pn.aap cells 9–11 to divide (Fig. 4A), and without it they behave like P2.aap cells, becoming single neurons with uniquely positioned nuclei (Fig. 4B; Kenyon 1986). When we expressed *mab-5* ectopically using the promoter mutation *e1751gf*, Pn.aap cells 1 and 2 divided consistently, showing that *mab-5* activity is sufficient to produce this division (Fig. 4C). In the wild type, then, *mab-5* activity must be off in cells 1–2 and on in 9–11, consistent with the expression pattern that we have seen.

Figure 4. *lin-39* obscures the underlying boundary of *mab-5* activity in Pn.aap cells. (A–E) Schematic diagram of Pn.aap cell fates in various mutant backgrounds. (X) Programmed cell death; (N) single neurons with compact nuclei positioned dorsally in the cord. Pairs of motoneurons are represented with white circles; the posterior of each pair contains the neurotransmitter serotonin (Loer and Kenyon 1993). Pairs of nonserotonergic cells are represented with black circles; EM reconstructions of wild type suggest that one of each pair is an interneuron; the other makes no known electrical or chemical junctions (J. White, pers. comm.). Domains of *mab-5* activity are shaded; *lin-39* activity in promoting production of motoneurons is shown in black. *lin-39* is also active outside this domain in preventing cell death. (C) Four *mab-5(e1751gf)* lineages were followed in males. P(1–12).aap cells always divided. Analysis of serotonin immunoreactivity demonstrates that only the descendants of P(3–8).aap cells express serotonin ($n = 97$). (D) Four lineages were followed in males: one in a *lin-39(mu26)* background, and three in a *lin-39(n1760)* background. In all four lineages, P(1–4).aap cells underwent programmed cell death, whereas P(7–11).aap cells survived. The fate of P(5–6).aap cells was variable: in *mu26* they both died, in one *n1760* lineage, both died, in another, only P5.aap died, and in the third, neither cell died. Surviving cells generally went on to divide in L3, the only exceptions being a single P7.aap cell in *mu26*, and a single P8.aap cell in *n1760*. Although these cells swelled up during L3, they never divided. (E) A single lineage was followed in a *lin-39(n1760) mab-5(e1239)* male. P(1–11).aap all underwent programmed cell death. P12.aap survived, presumably because of *egl-5* activity.

Pn.aap cells 3–8, in the central body of the male, adopt a different fate: They divide to produce two motoneurons, one of which contains the neurotransmitter serotonin (Loer and Kenyon 1993). None of the neurons produced by cells 9–11 is a motoneuron or contains serotonin. Loss of *mab-5* ($n = 44$), *egl-5* ($n = 36$), or both activities ($n = 39$) did not affect the production of these serotonergic neurons. Even in *mab-5(e1751gf)* larvae, P(3–8).aap consistently divided and produced six serotonergic neurons ($n = 97$; Fig. 4C). We wanted to know why Pn.aap cells in the middle of the body adopt this different fate and why they fail to respond to *mab-5*.

Previous work has shown that the HOM-C gene *lin-39* is required for production of these serotonergic motoneurons in the mid-body region (Loer and Kenyon 1993). To

see whether *lin-39* might obscure the underlying activity of *mab-5* in P(3–8).aap cells, we examined the pattern of cell divisions in a *lin-39(-)* background. Indeed, in *lin-39(-)* larvae a new functional boundary was seen, which coincided with the border of *mab-5* expression: Posterior Pn.aap cells 7–11 divided to produce nonserotonergic neurons, whereas cells 1–6 underwent programmed cell death (Fig. 4D). The boundary was somewhat variable: Often P6.aap cells, and sometimes even P5.aap cells, divided like 7–11. To see whether these cell divisions reflect *mab-5* activity, we examined the pattern of cell fates in a *lin-39 mab-5* double mutant. In these animals, cells 1–11 all did undergo programmed cell death (Fig. 4E), showing that *mab-5* activity is required for the survival and division of Pn.aap cells 7–11 (and sometimes 5 and 6) in a *lin-39(-)* background. This spatial pattern of *mab-5* activity corresponds with the observed expression pattern of *mab-5* and the pattern of activity seen in Pn.p cells. In the wild-type, however, *lin-39* activity overlaps with the domain of *mab-5* expression and predominates, causing cells 7–8 to produce motoneurons.

Both *mab-5* and *lin-39*, then, suffice to promote the division of Pn.aap cells, but they specify different roles and neurotransmitter phenotypes for the resulting neurons. Unexpectedly, *lin-39* not only was required for division of Pn.aap cells 3–8 to produce motoneurons but also for survival of P2.aap cells in the wild-type and cells 9–11 in a *mab-5(-)* background. We do not yet understand why *lin-39* activity promotes the division of cells in the 3–8 region but merely prevents the death of cells 2 and 9–11. The *lin-39-lacZ* fusion was expressed at detectable levels in P(3–8) lineages but not outside this domain (data not shown; see also Wang et al. 1993). It therefore seems likely that the level or timing of *lin-39* expression is important in creating these differences in the final effect, although we cannot rule out the involvement of other regionally expressed genes.

mab-5 is necessary but not sufficient to promote cell death in Pn.aap cells

In Pn.aap cells 11–12, *mab-5* is required to specify programmed cell death (Fig. 5A). Without *mab-5*, these cells instead appear to adopt the fate of cells 1–10, which are VB motoneurons (Kenyon 1986; Fig. 5B). In sharp contrast to this pattern of fates, high levels of *mab-5* expression were observed in cells 9–10 in the wild type, which do not undergo programmed cell death (Fig. 2D). This suggested that Pn.aap cells 11–12 might be the only homologs competent to respond to *mab-5* in this manner. To test this, we expressed *Mab-5* using the heat shock construct in a *mab-5(-)* background. Only cells 11 and 12 responded consistently by undergoing programmed cell death (Fig. 5C). From this, we infer the presence of an unidentified gene activity in the posterior that cooperates with *mab-5* or, conversely, some activity in the anterior that antagonizes *mab-5*. We have ruled out the involvement of other known HOM-C members; neither *lin-39* nor *egl-5* mutations affected the pattern of VB cell deaths ($n = 22$, $n = 11$, respectively).

Figure 5. *mab-5* is necessary but not sufficient to induce programmed cell death of Pn.aap cells. (A–C) Diagram of Pn.aap fates. (X) Programmed cell death; (B) VB motoneurons. Shading represents *mab-5* activity rather than expression. (C) Four lineages were performed in *mab-5*(–) males expressing *mab-5* under heat shock control. In 4/4 animals P11.aap cells underwent programmed cell death; in 1/4 animals, P12.aap cells died; P(6–10).aap cells always survived; no cell deaths were observed anterior to P6.aap. A time course was done in a *ced-1*(–);*mab-5*(–) background, to determine the best time to induce *mab-5* expression (see Materials and methods). The optimal time to elicit death of P(11–12).aap cells was broad: 8–14 hr post-hatching. P12.aap deaths were observed in 50%, P11.aap deaths in 27%, and rarely were there deaths in the position of P10.aap (3%, $n = 70$). No more anterior cell deaths were seen.

mab-5 expression in P12 descendants is repressed by *egl-5*

Although Mab-5 was detected at high levels in P12 itself, expression declined during the P12 divisions and was not observed in the final descendants (Fig. 2, cf. B and D). Previous work has shown that mutations in the more posterior HOM-C gene *egl-5* cause homeotic transformations in which P12 adopts a P11-like fate. This suggested that in *egl-5*(–) larvae, *mab-5* might be expressed in descendants of both P11 and P12. In *egl-5*(–) larvae, we found strong *mab-5* expression in P12 descendants at all stages of division, including the final pattern in the second larval stage (Fig. 2H). We infer that in the wild type, *egl-5* activity leads to the repression of *mab-5* expression in the P12 lineage.

We were surprised to find that *mab-5* expression disappears in the P12 lineage long before the P12.aap cell is born. This cell requires *mab-5* activity to undergo programmed cell death. To resolve the question of when the Pn.aap fate is specified, we carried out a time course using the heat shock–*mab-5* construct to induce *mab-5* expression at different times in development (see legend to Fig. 5). We found that the most effective time to induce *mab-5* expression to specify Pn.aap cell death is between 8 and 14 hr post-hatching, 2–8 hr before the birth of these cells. Thus, specification of the cell death appears to occur before the cell is born, but after the division of the P cells, consistent with the observed *mab-5* expression pattern in the P12 lineage.

Discussion

In the ventral nerve cord of *C. elegans*, the stereotyped divisions of 12 precursor cells lead to the production of a

repeating pattern of neuronal cell types. Position-specific variation in the fates of these repeated cell types is established through the localized activity of the HOM-C genes, much as segmental diversity is produced in *Drosophila*. The reproducible lineage and fate of these cells has provided us with an opportunity to correlate patterns of HOM-C gene expression and function at the level of single cells. We have examined the role of one HOM-C gene, *mab-5*, in patterning the posterior ventral nerve cord. We find that interactions between this gene and other HOM-C members play a significant role in establishing the final pattern of cell fates.

mab-5 affects the fates of three types of P-cell descendants in the male: the Pn.p cell, which is epidermal, the Pn.aap cell, which is a sex-specific neuronal cell, and the Pn.aap cell, which is a type-VB motoneuron. Each of these cell types is repeated 12 times along the length of the body; and, in each case, *mab-5* causes a contiguous group of cells in the posterior to adopt specialized fates. However, the boundary between specialized fates and more anterior fates occurs in a different A/P location for each of these decisions. We have found that *mab-5* activity is regulated in three different ways to produce these boundaries (Fig. 6A).

In Pn.p cells, the level of Mab-5 protein itself serves to position a boundary between cells that fuse with the syncytium, and those that remain mononucleate. Mab-5 is expressed in a simple contiguous block of cells in the posterior nerve cord. The anterior boundary of this expression, between P6.p and P7.p, coincides with a boundary between fused and unfused cells. When the domain of expression is expanded anteriorly, the domain of *mab-5* function also expands.

In contrast, in Pn.aap cells, *lin-39* activity, rather than *mab-5* expression, produces the boundary between different cell fates. Previous work has shown that *lin-39* promotes the production of motoneurons by P(3–8).aap. We find that in these cells *lin-39* activity is dominant over *mab-5* and that this obscures the underlying pattern of *mab-5* activity. In the absence of *lin-39*, an underlying border is revealed: *mab-5* activity extends to P(7–8).aap, in agreement with the observed expression pattern and pattern of activity in Pn.p cells. Analogous interactions occur between the corresponding genes in *Drosophila*: Mutations in *Scr* lead to transformations in segmental identity that reveal the underlying activity of *Antp* (Struhl 1982; Kaufman et al. 1990).

In Pn.aap cells too, the boundary between cell fates does not result from differences in *mab-5* expression. Mab-5 is not only expressed strongly in P11.aap cells, where it induces programmed cell death, but also in P10.aap, which becomes a VB motoneuron. Moreover, if Mab-5 is expressed ubiquitously, under the control of a heat shock promoter, only P(11–12).aap cells respond efficiently. This suggests the presence of a cooperative gene activity in the posterior, or alternatively, an antagonistic activity in the anterior. Other known HOM-C members (*lin-39* and *egl-5*) do not affect the decision.

Not only do the three distinct anterior boundaries of *mab-5* activity arise from the localized action of three

Figure 6. Genes that regulate the pattern of cell fates in the posterior nerve cord. (A) Localized activities of three different genes position boundaries in cell fate. In Pn.p cells (circles), the pattern of responding cells is determined by *mab-5* expression. In Pn.aap cells (diamonds), overlapping *lin-39* activity obscures effects of *mab-5* in P(7-8).aap. In Pn.aaap cells (squares), *mab-5* can induce only P(11-12).aap to die. This may reflect localized activity of a coactivator in the posterior (as shown) or an antagonistic activity in the anterior. (B) Interactions between these genes vary depending on the cell type. In Pn.p cells, *mab-5* and *lin-39* each prevent cell fusion; but where both are expressed, they effectively cancel. In Pn.aap cells, *lin-39* is dominant over *mab-5* and promotes motoneuron production. In Pn.aaap cells, *mab-5* and a postulated gene activity may cooperate to induce programmed cell death.

different genes, but where these gene activities overlap, they interact in different ways depending on the cell type (Fig. 6B). They can neutralize one another, one gene can predominate over the other, and *mab-5* may cooperate with an unidentified gene in the posterior.

In Pn.p cells, both *lin-39* and *mab-5* prevent cell fusion (see Fig. 6B); however, they effectively cancel each other out with respect to this decision in the zone of overlap. We have shown that this cancellation does not result from mutual transcriptional repression. Instead, whenever both genes are expressed, they antagonize one another's activity. This occurs whether *Mab-5* is expressed under its own promoter, a rearranged promoter with ectopic activity, or a heat shock promoter. The ratio of *mab-5* to *lin-39* does not seem to be crucial for cancellation, as each of these methods of expression leads to somewhat different levels of *Mab-5*. Therefore, it seems unlikely that the two proteins cancel through formation of inactive heterodimers; instead, they may interact on the promoters of downstream genes involved in cell fusion. For example, *mab-5* and *lin-39* might each serve to repress a gene involved in cell fusion; but in combination, the genes could either fail to repress or might even activate expression of the fusion program.

In Pn.aap cells, *lin-39* and *mab-5* do not cancel each other out; instead *lin-39* overrides *mab-5* function. Thus, the same pair of genes can interact differently depending on the cell type or the decision that they are controlling. Previous work in *Drosophila*, although controversial, has suggested the presence of a functional hierarchy between HOM-C genes, termed posterior prevalence (González-Reyes et al. 1990). Our work suggests that prevalence of one gene over another is not necessarily

a property of the gene products themselves but, instead, reflects the type of decision they are controlling and/or the cellular environment.

In Pn.aaap cells, *mab-5* appears to interact with an unidentified gene activity outside the HOM-C. *Abd-B* function in *Drosophila* appears to be similarly regulated in parasegments 14 and 15 by the localized activity of the *spalt* gene, which is also not a member of the HOM-C (Jürgens 1987, 1988).

These three interactions are sufficient to explain the staggered anterior border of *mab-5* activity. In the posterior, we have found that the HOM-C gene *egl-5* represses *mab-5* expression in the P12 lineage. Similar interaction is seen at the transcriptional level between the corresponding *Drosophila* genes *Ubx* and *Abd-B* (Struhl and White 1985; Casanova et al. 1986; Casanova and White 1987; Karch et al. 1990; Macías et al. 1990). Although the functional significance of this interaction has been questioned previously (González-Reyes et al. 1990), more recent work suggests that it plays a significant role in restricting *Ubx* activity (Lamka 1992). It may be that in *C. elegans*, repression of *mab-5* by *egl-5* helps to limit its domain of activity.

Previous analysis of the *mab-5* phenotype suggested that the P2 lineage might be representative of the ground state, that is, the underlying pattern that is modified through the action of the HOM-C genes (Kenyon 1986). In *mab-5* mutants, cells P9-P12 all do adopt a P2-like fate. Our further analysis of the *lin-39 mab-5* double mutant, however, suggests that the ground state may instead be P1-like. In the double mutant background, P(2-11).aap cells all undergo programmed cell death like P1.aap. Moreover, the other P(2-11) descendants behave

similarly to P1 descendants as far as their pattern of division and/or fusion. Complete resolution of the issue of the ground state may depend on the isolation of mutations in the *C. elegans labial* homolog *ceh-13*.

Currently, it is not known what mechanism generates the graded pattern of *mab-5* expression seen in both the P descendants and juvenile motoneurons. One possibility is that similar, perhaps positional, mechanisms regulate *mab-5* expression levels in the two cell types. Certain aspects of the *mab-5* expression pattern imply that cell-extrinsic mechanisms are used to regulate the level of Mab-5. The P9/10 cells arise from lineally identical positions on the left and right sides of the animal and interdigitate at random when they move into the ventral cord (Sulston and Horvitz 1976). When they first enter the cord they express apparently equal levels of Mab-5. Nevertheless, P10 descendants consistently come to express higher levels of Mab-5 than P9 descendants. This may reflect signaling between the P cells themselves or other positional cues.

Interestingly, we see *mab-5* expression not only in the posterior motoneurons, where its function is unknown, but also in the posterior body muscles. This raises the possibility that HOM-C genes play a role in neuromuscular junction specificity. We also find, however, that these genes may play a more basic role in muscle attachment. Mutations in both *mab-5* and *lin-39* lead to defects in ventral body-wall muscle attachment in their domains of expression (see Materials and methods).

In summary, we have examined the role of one HOM-C gene in patterning the posterior ventral nerve cord. We find that diverse interactions between this gene and other HOM-C members play a significant role in regulating the final pattern of cell fates. Both cross-regulation of expression and several types of post-translational interactions are important. Some of the interactions that we have observed have been described previously in *Drosophila* at the level of whole body segments. We find that these interactions can also occur at the level of single cells and that they vary depending on the cell type. The flexibility of these interactions suggests that overlapping domains of HOM-C expression serve simply as raw material for patterning cell fates: The basic information can be logically integrated and interpreted in many different ways, depending on the cell type and the requirements of the organism.

Materials and methods

Nematode strains

Nematode strains were maintained as in Brenner (1974) and Wood et al. (1988). Analysis was done at 20°C except for heat shock inductions. Males were generated using *him-5(e1490)*, which boosts incidence of male self-progeny. Other mutations used were *mab-5(e2088)* and *mab-5(e1239)*, which do not express detectable levels of Mab-5 (see below) and appear to be null by genetic criteria (Kenyon 1986); *mab-5(e1751gf)* (Hedgecock et al. 1987), a large tandem duplication of the gene that alters promoter activity (Salser and Kenyon 1992) leading to increased and ectopic expression; *lin-39(n1760)* (Clark et al.

1993) and *lin-39(mu26)* (Wang et al. 1993), both strong alleles, one of which (*mu26*) disrupts the homeo domain; *egl-5(n945)*, an amber mutation that appears null by genetic criteria (Chisholm 1991); and *ced-1(e1735)*, which prevents engulfment of dying cells (Hedgecock et al. 1983).

Antiserum preparation

mab-5-coding sequences were cloned in *Escherichia coli* vectors pGEX-3X and pAR3040 to produce Mab-5–glutathione-S-transferase (GST) and Mab-5– β -galactosidase (β -gal) fusions. Both were insoluble and purified from inclusion bodies by SDS-PAGE. Electroeluted proteins were administered to six rabbits. Immunizations were 250 μ g in complete Freund's adjuvant, followed by 150- μ g boosts in incomplete Freund's adjuvant at 4-week intervals. A rabbit immunized with β -gal fusion and boosted with GST fusion gave the best response. Sera were tested on larvae expressing Mab-5 under heat shock control. Preimmune sera did not react in this assay.

Following the fourth boost, sera were affinity purified against Mab-5–GST on nitrocellulose and eluted in 0.2 M NaCl/0.2 M glycine (pH 2.5). Greater than 80% of reactivity was recovered. This preparation cross-reacts with germ-line granules, a common problem with rabbit and mouse sera. Germ-line reactivity is not attributable to Mab-5, as it is present in *mab-5(-)* strains. Nuclear staining appears specific, as it is absent in *mab-5(e2088)* and *mab-5(e1239)*. Before use, affinity-purified antibodies were preadsorbed to *mab-5(e2088)* larvae to reduce background. Larvae were prepared as described below but equilibrated in 5 mM EDTA/5 mM EGTA/0.01% Na₃TBSTw (137 mM NaCl, 2.7 mM KCl, 25 mM Tris at pH 7.7, 0.5% Tween 20). Sixty-five microliters of sonicated larvae was blocked with 1% BSA and 5% goat serum in 90 μ l. Ten microliters of purified antibody was added and preadsorbed overnight at 37°C. Debris was removed with a 30-min microcentrifuge spin.

Antibody staining of whole-mount larvae

For Mab-5 staining, larvae were prepared as described (Finney and Ruvkun 1990), with changes: 10–100 μ l of larvae was frozen in 1 ml of ice-cold 1% paraformaldehyde in MRWB (80 mM KCl, 20 mM NaCl, 10 mM EGTA, 5 mM spermidine HCl, 15 mM Na PIPES at pH 7.4, 25% MeOH). Tubes were thawed and fixed overnight at 4°C. Subsequent washes and incubations were 1 ml at 25°C unless indicated. Larvae were rinsed twice in ice-cold Tris–Triton (100 mM Tris at pH 7.4, 1% Triton X-100, 1 mM EDTA), incubated for 2 hr at 37°C in 2% BME/Tris–Triton, rinsed in borate (25 mM NaBO₃ at pH 9.2), incubated for 15 min in 30 mM DTT/borate, rinsed twice in borate, incubated for 15 min in 1% H₂O₂/borate, and rinsed again in borate. Steps in borate were repeated to further permeabilize larvae. The larvae were then rinsed twice in 0.001% Triton X-100 and settled onto polylysine slides, 22-mm² coverslips were applied, excess liquid was removed, slides were frozen on dry ice, and coverslips were pried off. Slides were thawed in 30% EtOH in TBSTw, rinsed in TBSTw, blocked in 1% BSA/5% goat serum/TBSTw for 30 min at 37°C, incubated with 15 μ l of preadsorbed primary antibody for 2 hr at 37°C, rinsed 4 $\times$ 1 hr in 37°C TBSTw, reblocked, incubated for 2 hr with rhodamine goat anti-rabbit (1 : 200) in 1% BSA/5% goat serum/ TBSTw, and rinsed 3 $\times$ 15 min at 37°C. Larvae were mounted in 80% glycerol, 2% *n*-propyl galate, and 5 μ g/ml of DAPI and photographed with Kodak Tech Pan. Costaining with anti-histone monoclonal antibody (Chemicon MAB052) was used to gauge tissue accessibility.

Pn.p cell fusion was visualized as described (Kenyon 1986), using the mouse mAb MH27, which stains a component of

septate junctions (Francis and Waterston 1991). Serotonin immunoreactivity was detected as described (Desai et al. 1988), with slight modifications (Loer and Kenyon 1993).

Expression of *Mab-5* under heat shock control

Integrants of the extrachromosomal array *muEx14* (Salser and Kenyon 1992) were obtained by γ -irradiation (C. Kari, A. Fire, and R. Herman, pers. comm.). The array carries the marker *unc-31(+)* as well as the fusion placing *mab-5* under the control of the *hsp16.1* heat shock promoter (Stringham et al. 1992). Two of the integrants, *mul59* and *mul10*, gave reliable induction of *mab-5*. These were outcrossed three times to *unc-31(e169)*; *him-5(e1490)* to remove γ -induced mutations, and crossed into an *mab-5(e2088)*; *unc-31(e169)*; *him-5(e1490)* background. Both insertions are X-linked, one close to *lon-2*.

To affect Pn.p fusion, larvae were given 31°C pulses, 15–30 min in length, with 90-min recovery periods at 20°C. Pulses started 6–11 hr after hatching and continued for 12 hr, at which time larvae were prepared for MH27 staining. (Pn.p cells are born at 8–10 hr.) *e2088*; *e169*; *e1490*; *mul59* and *e2088*; *e169*; *e1490*; *mul10* gave similar results, and data were pooled. *e2088*; *e1490* larvae given heat shocks in parallel were unaffected. *e2088*; *e169*; *e1490*; *mul59* and *e2088*; *e169*; *e1490*; *mul10* larvae grown at 20°C were also unaffected.

To determine the optimal time for *mab-5* induction to specify programmed cell death in Pn.aap cells, *e1735*; *e2088*; *e169*; *e1490*; *mul59* larvae were given 30-min heat shocks at 6–8, 8–10, 10–12, 12–14, or 14–16 hr post-hatch. Cell deaths were scored at 17–19 hr. Frequencies of cell death during these periods were 3/30, 11/22, 11/28, 13/20, and 3/20 (for P12.aap), 2/30, 4/22, 11/28, 4/20, and 4/20 (for P11.aap), and 0/30, 2/22, 0/28, 0/20, and 0/20 (for P10.aap). Deaths were identified on the basis of position and scored in males to avoid confusion with Pn.aap deaths. To definitively identify dying cells, four lineages were performed in *e2088*; *e169*; *e1490*; *mul59* males. Fifteen-minute heat shocks were given at 2-hr intervals starting after the division of P12.a (~11 hr post-hatch).

Analysis of mutant lineages

Larvae were mounted as described (Wood et al. 1988) and observed using Nomarski optics. Pn.aap divisions in *e1751* were observed to the end of L3. *n1760*, *mu26*, and *n1760 e1239* L1 lineages leading to Pn.aap cells were followed. Occasional polarity reversals in P-cell divisions were observed in *n1760* and *mu26*. Cell deaths were noted, and larvae were allowed to develop on petri plates until L3. After remounting, divisions of surviving Pn.aap cells were observed to the end of L3. Detached ventral body-wall muscles were seen in *mab-5* (8/26 *e2088* late L1s between V5.a and anus), *lin-39* (12/25 *n1760* late L1s between V1.a and V4.p), as well as the double mutant (from V2.p back to the anus). Defects in P-descendant migrations were also seen: P1 descendants in wild type migrate anteriorly. In *lin-39* larvae, P(2–5).a descendants migrated anteriorly like P1.a descendants in wild type. In *lin-39 mab-5*, P(1–11).a descendants migrated anteriorly. Finally, *mab-5(1751gf)* blocked anterior migrations of P1.p and P1.a.

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