Lineage-specific regulators couple cell lineage asymmetry to the transcription of the *Caenorhabditis elegans* POU gene *unc-86* during neurogenesis

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The POU homeo box gene *unc-86* specifies neuroblast and neural identities in the developing *Caenorhabditis elegans* nervous system. After an asymmetric neuroblast division, *unc-86* is expressed in one of two daughter cells in 27 lineage classes that are not obviously related by function or position. We show here that *unc-86* transcriptional regulatory regions detect cell lineage asymmetry to activate *unc-86* expression in one of two neuroblast daughter cells. Distinct regulatory regions activate *unc-86* expression in particular sets of sublineages. Therefore the *unc-86* regulatory region integrates distinct cell lineage asymmetry cues to activate *unc-86* expression in the many classes of neuroblast cell lineages. In agreement with such lineage-specific regulation of *unc-86* asymmetric activation, mutations in *lin-11* (LIM homeo box), *ham-1*, and *lin-17* affect the asymmetry of *unc-86* expression in particular cell lineages, and mutations in *lin-32* (*achaete/scute* family), *vab-3* (Pax-6 homolog) and *egl-5* (*Abd-B* homolog) affect the establishment of *unc-86* expression in other cell lineages. Homologs of *unc-86* and many of these *unc-86* regulators have been implicated in control of neurogenesis in vertebrates and invertebrates. These data suggest that *unc-86* acts in a phylogenetically conserved pathway that couples neuroblast cell lineage asymmetry to the generation of diverse neural types.

[Key Words: POU homeo domain protein; *C. elegans*; neurogenesis; *unc-86*; asymmetric cell division; transcriptional regulation]

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Transcription factors that are expressed in complex patterns have been shown to control key events in neural development (Jan and Jan 1994). These transcription factors regulate the pattern of neurogenesis in spatial domains (Doe et al. 1988; Skeath et al. 1992; Salser et al. 1993) or in stereotyped cell lineages (Finney et al. 1988; Miller et al. 1992; Jarman et al. 1993). They also regulate the detailed features of neural type such as patterns of synaptic connectivity (Miller et al. 1992) and the expression of signaling pathways in particular neural types (Desai et al. 1988; Johnson and Hirsh 1990; McIntire et al. 1993; Jin et al. 1994). They regulate the expression of many downstream effector genes (Xue et al. 1992) that actually mediate the complex recognition and signaling events during neurogenesis and neural function. The expression pattern of these transcription factor genes define where and when particular neural types are generated, or where and when specific events in neurogenesis occur. The control of their activation in complex pat-

terns that presage key neurogenic events is a fundamental problem in neurogenesis.

unc-86 has such a central role in *Caenorhabditis elegans* neurogenesis. *unc-86* couples asymmetry in neuroblast cell lineages to the generation of particular neuroblast and neural types during neural development (Finney and Ruvkun 1990). *unc-86* mutations perturb neuronal cell lineages by disrupting the generation of mother/daughter asymmetry. In wild type, asymmetric neuroblast divisions generate two daughter cells that differ from one another and from their mother. In *unc-86* mutants, one neuroblast daughter of particular asymmetric divisions retains the cell identity normally associated with its mother, whereas the other daughter cell is not affected. This leads to cell lineage reiterations that generate extra copies of some neurons and fail to generate other neurons. *unc-86* mutations also cause defects in other neurons that are not associated with cell lineage aberrations (Desai et al. 1988).

unc-86 encodes a POU-domain transcription factor (Finney et al. 1988; Ruvkun and Finney 1991). UNC-86 protein is expressed in 57 out of the 302 neurons of the *C. elegans* nervous system, including particular sensory

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low) (Finney et al. 1988; R. Baumeister and G. Ruvkun, unpubl.). Rescue of egg-laying defects was not scored because of a similar defect associated with the *rol-6* transformation marker. Transgenic *unc-86(n846)* animals bearing this *unc-86* DNA fragment express Unc-86 protein in the normal set of 57 neurons, whereas no expression of UNC-86 is detectable in *unc-86(n846)* animals (Fig. 2).

Fusion genes bearing a segment from 5.1 kb upstream of the *unc-86* transcription start to +52 of the *unc-86* mRNA (fusion gene ABC₁₂₃₄, see Fig. 3) are expressed in wild-type animals in the same 57 neurons that normally express *unc-86* (Figs. 3A and 4A). Because *unc-86* encodes a transcription factor that could regulate its own expression, this fusion gene expression could either reflect an autoregulatory response to endogenous chromosomal *unc-86* gene activity, or a response to regulatory factors that normally initiate asymmetric *unc-86* expression. To disable any autoregulation, we determined the pattern of fusion gene expression in an *unc-86* null mutant that expresses no endogenous UNC-86 protein (Figs. 3 and 4).

We found that *unc-86* does autoregulate and that only in absence of autoregulation could the activity of enhancers for the establishment of *unc-86* expression be detected. The same fusion gene extrachromosomal arrays analyzed in wild type were transferred by mating to *unc-86* null mutants. The determination of patterns of *unc-86* reporter gene activation in an *unc-86* mutant is complicated by the neurogenesis defects in this mutant. In those cell lineages where *unc-86* is first expressed in a nondividing differentiating neuron, reporter gene expression in these neurons was recognized by the position of

the cell bodies and processes (Fig. 3A). In those cell lineages where *unc-86* is first expressed in neuroblasts, these neuroblasts generate reiterated sets of recognizable neurons in an *unc-86* mutant that express particular fusion genes (Fig. 3A). The -5.1-kb to +52-bp *unc-86* fusion (ABC₁₂₃₄, Fig. 3A) is initially expressed in *unc-86* mutant animals in the complete set of neuroblasts and neurons that initiate *unc-86* expression in wild type (Fig. 4C). Derivatives of this fusion gene that delete overlapping 41 and 45 nucleotide segments of the 52 nucleotides from the *unc-86* mRNA (ABC_{1234Δ1} ABC_{1234Δ2}, Fig. 3B) are also expressed in all neurons that normally express *unc-86*. The expression levels in *unc-86* mutant embryos are similar to those in wild-type embryos. However, both β -galactosidase and GFP expression levels from fusion genes decrease dramatically at post-L1 stages in *unc-86* null mutants, in contrast to wild type.

Therefore, the 5.1 kb upstream of the *unc-86* transcript is sufficient to establish *unc-86* expression in the correct neuroblasts and neurons but cannot maintain that expression in the absence of *unc-86* gene activity. These data show that asymmetric expression of *unc-86* in these neural cell lineages is based on asymmetric regulation of *unc-86* transcription rather than, for example, asymmetric segregation or activation of the *unc-86* mRNA, because no segments in the *unc-86* mRNA are essential to recapitulate normal *unc-86* regulation.

Lineage-specific establishment of the asymmetric *unc-86* expression pattern

Dissection of the *unc-86* transcriptional regulatory region revealed that distinct regulatory segments are acti-

Figure 2. A 10,390-bp transgenic fragment containing the *unc-86* gene rescues the *unc-86(n846)* null phenotype. (Top) Immunostaining with Unc-86-specific antibody. (Bottom) Nuclear DAPI staining. (A) *unc-86(n846)*; Ex(ABC₁₂₃₄WXYZ) animal (for nomenclature, see legend of Fig. 3) containing the 10,390-bp *unc-86* transgene expresses UNC-86 protein in a normal manner and is wild type for *unc-86*-mediated behaviors. Shown is normal expression of *unc-86* in the anterior ganglion and deirid neurons. (B) An adult *unc-86(n846)*; Ex(ABC₁₂₃₄WXYZ) animal showing normal HSN up-regulation of *unc-86* expression. The expression of *unc-86* in the HSN(L/R) is much brighter than that of the postdeirid neurons PVD and PVM; all these neurons express similar levels of Unc-86 at earlier stages (R. Baumeister and G. Ruvkun, unpubl.). (C) No expression of *unc-86* can be detected in the *unc-86(n846)* null mutant animal.

vated in specific cell lineages to assemble the complex *unc-86* expression pattern. In an *unc-86* null mutant, *unc-86* fusion genes bearing distinct segments of the regulatory region (Fig. 3A) were activated in subsets of the *unc-86* expression pattern. For example, the BC₁₂₃₄ fusion gene (Fig. 3A) that bears the -2.8-kb to +52-bp *unc-86* region is expressed in L1 stage animals in a subset of the normal *unc-86* expression pattern (Fig. 4E). It is expressed in RIH, RIR, but not in the IL, NSM, HSN, or in the neuroblasts Q.p, V5.paap, and T.ppp. In contrast, the AC₁₂₃₄ fusion gene (Fig. 3A) that bears -5.1- to -2.7-kb and -0.8 kb to +52 bp is expressed in the neurons IL, NSM, HSN, and in the neuroblasts Q.p, V5.paap, and T.ppp, but is not expressed in RIH or RIR (Fig. 4F). Both fusion genes are expressed in other cell lineages (Fig. 3A).

These *unc-86* regulatory segments are also sufficient to confer expression on reporter genes bearing an unrelated promoter. *mec-7*/GFP fusion genes bearing *unc-86* DNA segments from the -5.1- to -2.8-kb region (segment A') or the -2.7- to -0.8-kb region (segment B') cloned upstream of the probable *mec-7* proximal promoter element were expressed in sets of neurons in an *unc-86* mutant that are nearly identical to the analogous promoter deletion constructs bearing the corresponding *unc-86* segments (Fig. 3A and 4J-N). The -2.7- to -0.8-kb segment functioned in both orientations; only the normally oriented -5.1- to -2.8-kb segment was tested for regulatory activity (data not shown). The basic *mec-7*/GFP reporter gene into which *unc-86* DNA segments were placed is weakly expressed at late stages in two tail neurons and AVK in an *unc-86(n846)* mutant and in

Figure 3. (See facing page for legend.)

these neurons plus the six touch neurons in wild type (Fig. 3A). Therefore *unc-86* DNA segments specifically confer GFP expression in other neurons.

These data show that the -5.1 - to -2.8 -kb *unc-86* region bears enhancers that are necessary and sufficient to activate *unc-86* expression in the II, NSM, HSN, Q.p, V5.paap and T.ppp neuroblasts and neurons, whereas enhancers in the -2.6 - to -0.8 -kb segment are necessary and sufficient to activate *unc-86* expression in the RIR and RIH neurons. Enhancers for the activation of *unc-86* expression in other cell lineages are located in both segments (Fig. 3A). No *unc-86* regulatory activity was detected in the -754 - to $+52$ -bp DNA segment (Fig. 3A), that is likely to bear the proximal *unc-86* promoter elements. This particular proximal region is not required for asymmetric *unc-86* activation because *unc-86* regulatory segments can activate expression of the *mec-7* reporter gene bearing other proximal elements (Fig. 4).

The region 3' to the *unc-86* start site also contains segments that can activate asymmetric expression of *unc-86* in particular neural cell lineages in *unc-86* null mutants. Addition of the $+53$ - to $+5.2$ -kb segment to the inactive -422 - to $+52$ -bp fusion gene (fusion gene C₃₄WXYZ, Fig. 3A) activates expression of this reporter gene in particular neural lineages but not in others. Expression was absent in the cell lineages that generate anterior sensory neurons but present in many cell lineages located more posteriorly (Figs. 3A and 4H,I). Derivatives of this fusion gene missing various segments mapped the major enhancer activity for these cell lineages to the $+2.8$ - to $+5.3$ -kb segment (Z in Fig. 3A). The $+2.8$ - to $+5.3$ -kb *unc-86* segment in either orienta-

tion was also sufficient to confer expression in a similar set of neurons on the heterologous *mec-7*/GFP fusion gene in an *unc-86* null mutant (Figs. 3A and 4N).

None of the fusion genes bearing fragments of the *unc-86* regulatory domain express ectopically when transformed at low-copy number. However, high-copy transgenes revealed an example of negative regulation of asymmetric *unc-86* expression in the ADL neurons that is not dependent on functional UNC-86 protein (Fig. 4G). *unc-86* expression in ADL may be under negative regulation by a repressor that can be titrated.

Multiple *unc-86* autoregulatory regions

Unlike the distinctive expression patterns in an *unc-86* null mutant exhibited by fusion genes bearing different segments from the *unc-86* promoter, the pattern of expression in *unc-86*(+) animals bearing these fusion genes are identical. Each fusion gene is expressed in all 57 neurons that normally express *unc-86* and expression continues at all developmental stages (Figs. 3A, 4A,D,H,K). *unc-86* DNA segments from these regions are also sufficient to confer to reporter genes bearing unrelated proximal elements expression in the 57 neurons that normally express *unc-86*: addition of the -5.1 - to -2.8 -kb (A'), the -2.7 - to -0.8 -kb (B'), or the $+2.8$ - to $+5.3$ -kb (Z) *unc-86* segments to either *mec-7*/GFP or *pes-10*/*lacZ* fusion genes activates expression of these reporter genes in the entire *unc-86* expression pattern (Figs. 3A and 4K). Therefore, there are multiple *unc-86* autoregulation sites distributed over regions that also bear cell-lineage-specific enhancers for the establishment of *unc-86* expres-

Figure 3. *unc-86* regulatory segments bear cell lineage-specific and autogenous regulatory activities. (A) Promoter fragments used in the GFP and *lacZ* fusions are shown schematically to the left and their patterns of expression in wild-type (top right) and in an *unc-86* null mutant (bottom right) are indicated. The 10.4-kb *unc-86* DNA fragment that complements an *unc-86*(n846) null allele is shown at left, in the center. The genomic organization of *unc-86* is depicted. Transcription (Tc) start and putative poly(A) site (pA) are indicated; (shaded boxes) exons. Segments used in the promoter analysis are labeled A (-5098 :*SpeI* to -2737 :*BglII*), A' (-5098 :*SpeI* to -2809 :*HindIII*), B (-2737 :*BglII* to -635 :*BamHI*), B' (-2669 :*HindIII* to -754 :*HindIII*), C₁-C₄ (-754 :*HindIII* to $+52$ ATG), W ($+127$:*XbaI* to $+836$:*HindIII*), X ($+836$:*HindIII* to $+1784$:*StyI*), Y ($+1784$:*StyI* to $+2748$:*SpeI*), Z ($+2784$:*SpeI* to $+5293$:*EcoRI*). Segment labels were used to name the *unc-86* fusion genes. Also shown are the *unc-86* segments fused to *mec-7*/GFP reporter genes, indicated by large arrowheads. Expression of *unc-86* reporter genes at the left is indicated on the right by a solid circle under each of the sublineages from Fig. 1 that activate expression of *unc-86*. These sublineages are displayed from anterior to posterior. Thick lines indicate expression of UNC-86 in those sublineages. (●) Expression of the particular fusion gene in the particular sublineage. (○) Weak expression. For wild type (top right panel), the sublineages are exactly as shown in Fig. 1. For *unc-86* mutant animals (bottom right panel), lack of *unc-86* gene activity alters many neuroblast cell lineages, as indicated. (1) AB.p(l/r)apaaa "deirid" lineage. (2) AB.arpp(a/p)app "ALM/BDU" lineage. (3) Q(L/R) lineage. (4) V5(L/R).paa "postdeirid" lineage. (5) AB.p(l/r)apapp "PLM/ALN" lineage. (6) T(L/R).pp lineage. (X) Programmed cell death. (c) Compact nucleus indicative of neural fate [lineage aberrations are described in Chalfie et al. (1981)]. The basic *mec-7*/GFP reporter gene without added *unc-86* DNA segments is expressed in the mechanosensory neurons ALM, AVM/PVM, and PLM in wild-type (▲), but because expression of *mec-7* is dependent on *unc-86*, it is not expressed in these cell lineages in an *unc-86* mutant. Therefore the regulatory activities of *unc-86* DNA segments could not be scored in the three sublineages that generate mechanosensory neurons in wild type but could be scored in these altered lineages in an *unc-86* mutant. Potential *unc-86*-binding sites indicated by inverted triangles in the center diagram (consensus sequence: CATnnnA/TAAT) (Xue et al. 1992; Li et al. 1993) are located at position -3950 (segment A), -1668 , and -1214 (B), -231 (C), $+1085$ (X), $+2391$ (Y), and $+4386$ (Z). Segments A, B, and Z in *unc-86* are each sufficient to respond to endogenous UNC-86 protein in all 57 neurons, suggesting that the potential binding sites on these segments may be functional. (B) Deletion of sequences in the transcribed region do not affect the expression pattern of *unc-86* promoter-GFP reporter genes. The sequence flanking the *unc-86* transcription start is shown. The mRNA start is indicated by an arrow, the two possible in-frame translational start codons are shown with a shaded background. Inverted triangles indicate the reading frame; the bracket at right indicates the splice site of the first exon. Deletion of these sequences results in weaker expression levels, which could reflect a less than optimal translational initiation signal caused by changes in the sequence flanking the ATG.

sion (Fig. 3A). The distinction between the expression pattern of these reporter genes in an *unc-86* mutant vs. wild type suggest that these autoregulatory sites can respond to asymmetrically activated endogenous Unc-86 protein regardless of whether the fusion gene bears regulatory regions necessary for the initial activation of *unc-86* expression in a particular cell lineage. For example, the fusion gene *Z'mec-7/GFP* (Fig. 3A) can only establish *unc-86* expression in a small number of neurons but can respond to endogenous *unc-86* activity in all 57 neurons that express the gene.

Regulators of asymmetric *unc-86* expression

Because our analysis showed that asymmetric expres-

sion of *unc-86* is transcriptionally based and that distinct segments in the *unc-86* promoter regulate its expression in particular cells, we surveyed candidate mutants for perturbations in the expression of *unc-86* in any or all of the sublineages that express the gene, as determined by immunofluorescent detection of UNC-86 protein (Finney and Ruvkun 1990). This analysis revealed genes that regulate the symmetry or activation of *unc-86* expression in particular subdomains of its expression pattern.

lin-11 controls the asymmetry of *unc-86* expression in the cell lineages that generate RIR

lin-11 controls cell lineage asymmetry in the ectoderm

Figure 4. (See facing page for J–N and legend.)

(Freyd et al. 1990). We find ectopic expression of *unc-86* in the interneuron AVG in animals carrying a strong *lin-11(n389)* allele (Figs. 5A and 6). *unc-86* expression was detected in the AVG interneuron at all stages in

more than 50% of the animals surveyed ($n = 90$). AVG is the sister of the *unc-86*-expressing interneuron RIR. Therefore, normal *lin-11* activity prevents the symmetric expression of *unc-86* in both AVG and RIR, presum-

Figure 4. Expression of *lacZ* and GFP reporter constructs in wild-type and *unc-86* mutant animals. (A, B) Expression of a GFP fusion gene bearing the -5.1 -kb to $+52$ -bp region of *unc-86* (fusion gene ABC_{1234} GFP) in wild-type L1 animals in all neurons. Both cell bodies and neural processes are fluorescent. The animal in A shows expression of GFP from the *unc-86* promoter in the anterior ganglion neurons, in a mismigrated (*) ALM neuron, the BDU, and HSN neurons. Shown in B is expression of this fusion gene in the deirid neurons, BDU, ALM, HSN, and Q.p. (C) The same -5.1 -kb to $+52$ -bp GFP fusion gene (ABC_{1234} GFP) in an *unc-86(n846)* mutant. (Top) Ventral view, showing expression of GFP in the sensory neurons of the anterior ganglion, I1, RIH, AIM, Q.p, and AB.p(l/r)apaaap. Because of the *rol-6(su1006)* injection marker, the animal is twisted along its longitudinal axis. (vc) ventral cord. (Bottom) Expression of GFP in Q.p, T.ppp, and AB.plapappp cell lineages that generate the tail neurons. (Left lateral view) Late L1 animal. (D) A -5.1 -kb to $+52$ -bp *lacZ* fusion gene (ABC_{1234} *lacZ* fusion gene) in a wild-type animal is expressed in all neurons that normally express *unc-86*. (Top) Anti- β -galactosidase antibody staining. (Bottom) DAPI. (E, F) Comparison of the -2.7 -kb to $+52$ -bp GFP fusion gene (BC_{1234}) and the -5.1 -kb to -2.7 -bp, -0.8 -kb to $+52$ -bp GFP fusion gene (AC_{1234}) expression patterns in *unc-86(n846)* animals. (E) BC_{1234} activates GFP expression in the ventral ganglion neurons RIR and RIH and in other neurons. (F) AC_{1234} is expressed in the Q, V5.paap, and T.ppp (tail) lineages and head neurons, but not in RIR and RIH. (G) Expression of a -0.4 - to $+2.8$ -kb *lacZ* fusion gene (C_{34} WXY-*lacZ*) at high copy in adult *unc-86(n846)* animals, dorsal view. The only two neurons that express *lacZ* are ADL(L/R). No transgene expression was detected in transgenic wild-type animals bearing C_{34} WXY at low-copy number or C_{1234} at low- or high-copy number. (H, I) Establishment and maintenance of *unc-86* expression function independently. Comparison of *lacZ* expression pattern of C_{34} WXYZ in L2 wild-type with *unc-86(n846)* animals. (H) C_{34} WXYZ is expressed in all 57 neurons in wild-type background. Three focal planes of the same animal and DAPI staining. (I) (Top) In the particular *unc-86(n846)* animal, C_{34} WXYZ is expressed in PVR, a neuron at the AIZ position [derived from AB.p(L/R)apaaa], and, ectopically in ADL head neurons. (Bottom) DAPI staining. (J) Expression of the B'*mec-7*/GFP fusion gene bearing the -2.7 to -0.8 -kb *unc-86* segment in the RIH neuron in an *unc-86(n846)* animal. (K) The same -2.7 - to -0.8 -kb B'*mec-7*/GFP reporter gene in wild type is expressed in all 57 neurons that express *unc-86*. Shown are the subset of these neurons that express in this particular animal. (L) Expression of the A'*mec-7*/GFP fusion gene bearing the -5.1 - to -2.8 -kb *unc-86* segment in the abnormal Q lineage in *unc-86(n846)* mutant animals is evinced by the expression of GFP in reiterated PQR neurons. (M) The B' *mec-7*/GFP fusion gene is also expressed in the reiterated Q lineages that generate the AQR and PQR neurons of an *unc-86(n846)* animal. (N) A Z'*mec-7*/GFP fusion gene bearing the $+2.8$ - to $+5.3$ -kb *unc-86* segment is expressed in the URX, RIH, and reiterated PQR neurons in an *unc-86(n846)* animal. The expression in RIH and RIR by fusion genes bearing this segment differs from the expression of the C_{34} WXYZ fusion gene that also bears this *unc-86* segment. Either orientation of this segment upstream of *mec-7*/GFP confers expression in these neurons. It is possible that either the location of this segment 3' to a proximal element in C_{34} WXYZ, or the distinct proximal element in this reporter gene, accounts for this difference in expression.

Figure 5. Regulators of *unc-86* expression. (Top) Anti-UNC-86 antibody staining in various mutants. (Bottom) DAPI. (A) *lin-11(n389)*. (Top and center) Two focal planes of head region; ectopic *unc-86* expression in AVG and the putative AVL. (B) Left lateral view of *ham-1(n1810)* tail (left focal plane). Two additional *unc-86*-expressing cells are found between PVM/PVDL (postdeirid) and tail neurons (right). The position of HSNL is indicated. (C) Dorsal view of *lin-17(n671)* tail. Only six cells express UNC-86, PHC(L/R) and PLN(L/R) expression is missing. (Left) ventral plane; (Right) dorsal plane. (D,E) *egl-5(n486)* animals do not activate *unc-86* expression in the HSN neurons. In D, the HSN shows a migration defect as well as a failure to activate *unc-86* expression. In E, the HSN migrates normally but does not activate *unc-86* expression. (F) *vab-3(e1062)* show disordered anterior ganglion and missing neurons that normally express *unc-86*. *unc-86* expression in the URX, RIH, RIR, and deirid neurons is normal. (G) *lin-32(u282)*. Few neurons in the anterior ganglion express *unc-86*. Deirid neurons are absent. The six IL2 neurons are either missing or do not express Unc-86. Although additional cells are probably also affected, they could not be identified unambiguously because of a general displacement of neurons in the anterior ganglion. There is normal *unc-86* expression in RIH, RIR, and AIM neurons. (H) ALM migration defect in *lin-32(u282)* animals; dorsal view. (I) For comparison, the normal pattern of *unc-86* expression in the head of a wild-type animal, right focal plane.

bearing the strong *egl-5(n486)* allele, the presumptive HSNs are generated normally but are defective in migration as well as serotonin synthesis, and the animals are egg-laying defective (Desai et al. 1988). More than 95% of the *egl-5(n486)* animals ($n = 111$ of 115) do not express *unc-86* in the HSN neuron, indicating that *egl-5* acts upstream of *unc-86* and is a potential regulator of *unc-86* expression in the HSNs (Figs. 5D, E).

unc-86 expression in cells other than the HSNs was not affected in the *egl-5* mutant. However, in some cases the migration of neurons generated from the Q lineage is disturbed (Fig. 6). Mutations in other HOM-C genes (*mab-5*, *lin-39*) do not result in *unc-86* expression defects but lead to migration defects of the Q lineage (Kenyon 1986; Chisholm 1991; Salser and Kenyon 1992; Clark et al. 1993; Wang et al. 1993) (Fig. 6).

vab-3, the *C. elegans* homolog of Pax-6, is necessary for *unc-86* expression in sensory head neurons

vab-3 mutants are defective in several sensory functions including chemosensation and thermotaxis (Lewis and Hodgkin 1977). *vab-3* is the *C. elegans* gene homolog of Pax-6/eyeless, which specifies eye development in vertebrates and invertebrates (Chisholm and Horvitz 1995; Zhang and Emmons 1995). The strong *vab-3* alleles *e648* and *e1062* result in missing *unc-86* expression in several neurons in the head. On average, 10 cells (10.3 ± 1.6 ; 60 animals) in *vab-3(e1062)* and 12 (12.1 ± 1.7 ; 59 animals) in *vab-3(e648)* express *unc-86* in the anterior ganglion, compared with 18.0 ± 0.04 (150 animals) in wild type (Figs. 5F and 6). The identity of the neurons that do not express *unc-86* could not be determined, as the overall anatomy of the anterior ganglion is severely disturbed in these mutants. However, all missing neurons are normally generated in the anterior sensory regions of the head, suggesting that *vab-3* acts in a particular anterior spatial domain. *vab-3* function in tail development has been described (Chow and Emmons 1994; Zhang and Emmons 1995), but expression of *unc-86* in the *vab-3* mutant tail was normal. Therefore *vab-3* acts upstream of *unc-86* expression in the generation or differentiation of neurons of the anterior ganglion.

lin-32 acts upstream of *unc-86* in sensory neural cell lineages

lin-32 encodes a basic-helix-loop-helix protein similar to *Drosophila atonal* and is necessary for the specification of neuroblast fate in several lineages (Zhao and Emmons 1995). In animals bearing the strong *lin-32(u282)* allele (Zhao and Emmons 1995), the Q and V5 neuroblasts are transformed to ectodermal cells (Chalfie and Au 1989; Zhao and Emmons 1995). Consistent with a *lin-32* function in the generation of the neuroblasts that express *unc-86*, no *unc-86* expression was observed in these transformed ectoblast cell lineages (data not shown). We observe other defects in the *unc-86* expression pattern in the neurons of the anterior ganglion, the deirid, and the tail (Fig. 5G). *unc-86* expression in other

cells, for example, HSN, BDU, and ALM was not affected by the *lin-32(282)* mutation, although the latter are frequently displaced (Fig. 5H). Therefore, *lin-32* acts upstream of *unc-86* in many but not all of the neuroblast cell lineages that express *unc-86*.

Discussion

All of the 302 neurons of the *C. elegans* nervous system are generated from neuroblast cell lineages that are asymmetric (Sulston and Horvitz 1977; Sulston et al. 1983). The POU homeo box gene *unc-86* couples cell lineage asymmetry to the generation of neuron and neuroblast identity in 27 of the 118 classes of neurons (Finney and Ruvkun 1990). Asymmetric activation of *unc-86* expression may be an initial step in a cascade of transcriptional regulation during neuron and neuroblast differentiation (Way and Chalfie 1989; Hamelin et al. 1992).

Establishment of unc-86 expression is transcriptional and modular

We show here that the transcriptional regulatory region of *unc-86* detects asymmetries in cell lineages to activate *unc-86* expression in particular daughter neuroblasts and neurons. Segments from both the 5' and 3' *unc-86* regulatory region are necessary and sufficient for the activation of asymmetric *unc-86* expression (Fig. 3). Furthermore, distinct segments of the *unc-86* regulatory region respond to asymmetric cues only in particular neural lineages (Fig. 3). This analysis suggests that the distinct transcriptional regulators of *unc-86* expression in these neural lineages are either asymmetrically segregated or differentially activated (or inactivated) in these asymmetric cell divisions. For example, asymmetrically activated transcription factors in the RIH and RIR cell lineages act through enhancers located between -2.7 to -0.8 kb and $+2.8$ to $+5.3$ kb in the *unc-86* promoter, whereas transcription factors that are activated in the I1, NSM, V5.paa, T.ppp, and Q.p cell lineages act through enhancers located between -5.1 to -2.8 kb (Fig. 3). In other cell lineages, our analysis suggests that distinct transcription factors bind to sites located in multiple *unc-86* regulatory regions (Fig. 3). Our analysis does not distinguish whether distinct factors act in each of the cell lineages or whether distinct combinations of factors interact on the *unc-86* promoter in particular cell lineages. Even if there are regulators common to these cell lineages, the observation that *unc-86* regulatory regions respond to distinct cell lineage-specific cues for the asymmetric activation of the gene shows that there must be key regulators whose activation is specific to the particular cell lineages. This logic is consistent with the analysis of transregulators, which demonstrates regional and cell lineage specificity.

Redundant regulatory regions for maintenance of unc-86 expression

Expression of all *unc-86* promoter-reporter genes is tran-

sient in an *unc-86* null mutant and fades soon after the expression is initiated. In contrast, the expression of the same genes is maintained when transformed into wild-type animals that express a functional UNC-86 protein. These results indicate that *unc-86* is necessary, either directly or indirectly, for its own maintained expression. Stronger evidence for *unc-86* autoregulatory function comes from the distinct patterns of expression that most reporter genes show in wild type compared with *unc-86* null mutants, where autoregulation is disabled (Figs. 3 and 4H,I). Autoregulatory segments are located both 5' and 3' of the transcriptional start and are redundant, because any one of these segments is sufficient to confer *unc-86*-regulated fusion gene expression in all neurons that normally express the gene. *unc-86* autoregulation functions independently from *unc-86* establishment. For example, the fusion gene C_{34} WXYZ can initiate *unc-86* expression in only 19 neurons but responds to *unc-86* autoregulation in all 57 neurons (Fig. 4H,I).

unc-86 encodes a transcription factor and therefore is a likely candidate to directly autoregulate its own expression (Finney et al. 1988; Xue et al. 1992). Analogous autoregulation of the POU gene Pit-1 has been observed (Chen et al. 1990). By sequence analysis, there are seven consensus UNC-86 binding sites in the 10.4-kb *unc-86* gene fragment, based on those detected biochemically in *mec-3* and *brn-3* (Xue et al. 1992; Li et al. 1993) (Fig. 3A). *unc-86* autoregulation could also be achieved by an indirect mechanism (Bienz 1994).

The *unc-86* autoregulation we observe is inconsistent with the previous observation of normal UNC-86 expression level in strains carrying strong *unc-86* alleles that make immunodetectable protein (Finney and Ruvkun 1990). There are several possible explanations for this discrepancy. For example, if in addition to positive autoregulation, *unc-86* also negatively autoregulates its expression, as has been found for Pit-1 (Chen et al. 1990), *unc-86* mutants may compensate for a decrease in *unc-86* activity with an increase in expression of a nonfunctional protein. On the other hand, the particular mutant UNC-86 proteins tested to date might function normally for autoregulation, but not in the specification of neuron and neuroblast identity. Alternatively, there may be other positive regulatory sites not present on the fusion genes tested that activate *unc-86* expression in a non-*unc-86*-dependent manner. A less likely alternative explanation is that either wild-type or mutant UNC-86 is a stable protein.

The analysis of the *unc-86* promoter in both wild-type and *unc-86* mutants reveal two aspects to *unc-86* asymmetric activation. Initially, the complex *unc-86* regulatory region builds up the pattern of *unc-86* expression piece by piece using regulatory pathways that are unique to particular sets of cell lineages. As described below, these sets of cell lineages are spatially clustered suggesting that spatially localized cues activate particular transcription factors that bind to corresponding *unc-86* regulatory regions to activate *unc-86* expression. Once expression of *unc-86* is initiated, *unc-86* autoregulation can confer continued UNC-86 expression. In this way, the

initial activators of asymmetric *unc-86* expression may be active only transiently to trigger *unc-86* autoregulation.

Asymmetric regulation of unc-86 is controlled by genes specific to particular cell lineages

The analysis of *unc-86* transcriptional regulatory sequences predicts that distinct transcription factors activate *unc-86* expression in particular cell lineages. In agreement with such a model, we found mutations in a set of transcription factor and cell lineage control genes that perturb the asymmetric activation of *unc-86* in a subset of the cell lineages that normally express the gene. For example, a *lin-11* mutant shows symmetric *unc-86* expression in the interneuron AVG as well as in its sister neuron RIR, which normally expresses *unc-86*. Therefore, *lin-11* may function in the neuroblast parent of RIR and AVG to generate asymmetric daughters that express distinct sets of genes, including *unc-86* in RIR only. In such a model, *lin-11* would function before *unc-86* in this particular cell lineage, but not necessarily as a direct regulator of *unc-86* expression. However, it is also possible that *lin-11* may function in AVG to repress *unc-86* expression. Like the *unc-86* downstream gene *mec-3*, whose gene product interacts with UNC-86 protein to regulate maintenance of *mec-3* gene expression (Xue et al. 1993), *lin-11* encodes a LIM homeo domain protein (Freyd et al. 1990). A model that LIN-11 normally antagonizes the UNC-86 autoregulatory activity in the AVG neuron is appealing because of the known association of POU and LIM proteins in maintenance of *mec-3* expression (Xue et al. 1993). However, our data do not favor a function for *lin-11* in the prevention of *unc-86* maintenance because we have not observed transient *unc-86* expression in AVG in wild-type or *unc-86* mutants (R. Baumeister and G. Ruvkin, unpubl.). We, therefore, favor the model that LIN-11 either functions in the neuroblast parent of AVG and RIR, or in AVG to inhibit the function of an unidentified transcription factor that can activate *unc-86* expression in both RIR and AVG. There is precedent for such combinatorial control of neurogenesis by LIM proteins (Tsuchida et al. 1994; Bach et al. 1995).

ham-1 controls asymmetric activation of *unc-86* in the HSN/PHB cell lineage. It is probable that *ham-1* represses *unc-86* in the PHB neuron, or acts earlier to specify an asymmetric division by the neuroblast parent of HSN and PHB. It is not known whether *ham-1* encodes a direct transcriptional regulator analogous to *lin-11* or whether it regulates the activity of such a regulator.

ham-1 and *lin-11* appear to act as repressors of *unc-86* expression in these two cell lineages. On the other hand, the rather large DNA segments used in the *unc-86* promoter analysis revealed only examples of positive regulation of *unc-86* activation (Fig. 3). Only high copy transgenes revealed other examples of possible *unc-86* repression in the particular cell lineages (Fig. 4G). It is possible that negative regulatory elements in the *unc-86* regulatory region, for example, that interact with LIN-11 or HAM-1, are closely associated with positive elements, as

has been observed in the case of *Drosophila eve* (Stanojevic et al. 1991).

Our analysis of other mutants revealed candidates to mediate the positive regulation of *unc-86* in particular cell lineages. For example, a mutant in the HOM-C gene *egl-5* generates HSN neurons that do not express *unc-86*. *unc-86* regulation in the other 55 neurons that express the gene is normal in this *egl-5* null mutant. Therefore *egl-5* acts upstream of *unc-86* only in the HSN. *egl-5* affects a broader range of HSN functions, consistent with it acting upstream of *unc-86* in HSN specification (Desai et al. 1988). The other HOM-C genes, *mab-5* and *lin-39*, do not affect *unc-86* expression. Therefore each HOM-C gene is unlikely to provide the spatial cues for *unc-86* up-regulation that we noted from the *unc-86* promoter analysis. However, given that the expression of the HOM-C genes overlaps in some cells and evidence for combinatorial control of cell fate by this gene cluster, it is possible that analysis of animals mutant for multiple members of the HOM cluster genes might show abnormal activation of *unc-86* in the spatial domains specified by these genes.

Mutations in *lin-32*, a member of the *achaete/scute* (*ac/sc*) class of neurogenic transcription factor, and *vab-3*, a Pax-6 homolog, affect several neural lineages which produce *unc-86*-expressing sensory neurons but do not affect other *unc-86*-dependent cell lineages (Fig. 6). All of the lineages affected by these genes produce sensory neurons, consistent with the function of their homologs in other animals (Jarman et al. 1993; Glaser et al. 1994; Halder et al. 1995). However, because only non-null alleles of these genes have been isolated, we cannot yet rule out that the unaffected cell lineages have a lower threshold for *lin-32* or *vab-3* gene activity. Although the effects of these mutants on *unc-86* expression may reflect an earlier function in neuroblast specification, it is also possible that each of these transcription factors directly regulates *unc-86*.

These results suggest that cell lineage-specific regulators bind to distinct enhancers distributed across the *unc-86* regulatory region. The enhancer elements that respond to HAM-1 and EGL-5 are likely to be located in the -5.1 - to -2.8 -kb and $+2.8$ - to $+5.3$ -kb segments that establish *unc-86* expression in the HSN cell lineage. The *unc-86* regulatory region that responds to LIN-11 (or a gene regulated by LIN-11) to silence *unc-86* expression in AVG may correspond to the -2.6 - to -0.8 -kb and $+2.8$ - to $+5.3$ -kb positive regulatory *unc-86* segments that establish expression in RIR, the sister of AVG (Fig. 3A).

unc-86 integrates spatial and cell lineage control signals

Asymmetric activation of *unc-86* is transcriptional and occurs within 10 min of the birth of an *unc-86*-expressing neuroblast or neuron (Finney and Ruvkun 1990). We suspect therefore that particular transcription factors inherited from the mother of that neuroblast or neuron are either asymmetrically segregated to, or activated in, one

daughter cell to activate *unc-86* expression. Therefore, any mechanism that establishes this asymmetry acts at the division of the mother cell to regulate the dowry of transcription factors inherited by *unc-86*-expressing daughter cells. For that reason, we inspected the location of the mothers of *unc-86*-expressing cells (Fig. 7) generated in embryogenesis.

We found that the mothers of the *unc-86*-expressing neurons or neuroblasts are clustered at various positions in the embryo. This is most obvious in the anterior half of the embryo, where these mother cells form stripes perpendicular to the antero-posterior axis (Fig. 7). The spatial clusters of neuroblasts correspond quite accurately with the sets of neurons whose *unc-86* expression is regulated by particular segments in the *unc-86* promoter. For example, gene segments that activate *unc-86* expression in the neurons of the anterior ganglion are always accompanied by expression of the AIM neurons in the ventral ganglion, which is located further posterior in an L1 animal (Figs. 6 and 7). In the embryo, the mother cells of AIM and those of RIP, URYV, IL2V, and URAV are all located in the same antero-posterior position in the embryo.

We suggest that all the *unc-86*-expressing cells derived from this spatial cluster use a common regulatory mechanism mediated by particular *cis* elements in the *unc-86* regulatory region. Consistent with this assumption, mutations in the *unc-86* regulator *vab-3* only affect neurons derived from the two anterior-most clusters of cells. In an analogous manner, *lin-11* mutants only affect expression of RIH and RIR and their sisters, which are derived from two mother cells that are neighbors in a central position at the ventral side of the embryo.

At least within the two anterior clusters, the polarity of the asymmetry to which the *unc-86* promoter responds seem to be already programmed in the mother cells, because the *unc-86*-expressing daughter cells always arise from the faces of the mother cells which are oriented towards one another. This polarization of mother neuroblasts could be due to an external oriented signal along the line demarked by the oriented neuroblasts or could correspond to an earlier boundary - for example a segment boundary which has been implicated as signaling centers in *Drosophila* disc development (Tabata and Kornberg 1994). It is striking that *lin-11* is necessary for such a cell lineage polarization, triggered by anchor cell signaling in the developing vulvae (Freyd et al. 1990). *lin-11* could propagate an analogous polarization cue in the cell lineage that generates the AVG/RIR sister neurons.

A common pathway for neurogenesis

Homologs of *unc-86* that are expressed and function in the developing nervous system have been identified in *Drosophila* (Treacy et al. 1992; Yang et al. 1993), mouse (Xiang et al. 1993), and human (Gerrero et al. 1993). In *Drosophila*, POU proteins have been shown to function like *unc-86* in asymmetric neuroblast specification (Yeo et al. 1995; Bhat et al. 1995). The emerging view (Jan and

Figure 7. Location of mother cells that activate *unc-86* expression asymmetrically in one of two daughters. Neuroblasts only on dorsal and ventral views of a 260-min embryo are shown. Cells whose daughters will express *unc-86* are shaded, with the shaded half indicating the position of the daughter after cell division. Only one of two bilaterally symmetric cells is labeled. The clusters of neuroblasts that express fusion genes bearing particular *unc-86* gene segments are boxed, with the name of the segments indicated. Genes that affect *unc-86* expression in these cells are also indicated. Not shown are the neuroblasts that generate the URX, IL, and NSM neurons and the neuroblasts that divide postembryonically. (Solid arrows) Position of ADL(L/R) neurons, where *unc-86* expression is regulated negatively (see text). (Footnoted cells) ¹Shaded cells already express *unc-86* at this stage. They are neurons (PVR) or neuroblasts that will divide again to generate BDU/ALM and FLP/AIZ/RMG. ²The granddaughters of these neuroblasts will express *unc-86*. The cell positions and names taken from Sulston et al. (1983).

Jan 1994) is that both cellular interactions mediated by a Notch-signaling system coupled to *ac/sc* transcription factors, and segregated factors, such as Numb and Prospero (Hirata et al. 1995; Knoblich et al. 1995; Spana and Doe 1995) have important roles in the generation of this neural cell lineage asymmetry in *Drosophila* (Posakony 1994). Homologs of these genes (Bürglin 1994) may mediate aspects of asymmetric *unc-86* expression in *C. elegans*, and of analogous POU proteins across phylogeny.

Homologs of the genes that regulate *unc-86* expression in particular subdomains of its expression pattern, the *ac/sc* gene *lin-32*, the LIM gene *lin-11*, the Pax gene *vab-3*, and the HOM-C gene *egl-5* also regulate neurogenesis in vertebrates and other invertebrates (Guillemot et al. 1993; Ferreiro et al. 1994; Glaser et al. 1994). Consistent with a model that in other species, homologs of these genes regulate the expression of *unc-86* homologs, expression of murine Pax-6 in the developing eye (Walther and Gruss 1991) precedes expression of the *unc-86* homolog, *brn-3* (Xiang et al. 1993). In addition, genes that are regulated by *unc-86*, for example, the *mec-1*, *mec-4*, and *mec-7* genes in mechanosensory neurons, are also conserved across phylogeny (Hamelin et al. 1992; Canessa et al. 1993; Huang et al. 1995). Mutations in the human POU gene Brn-4 cause defects in auditory neurogenesis (de Kok et al. 1995) that may be mechanistically related to the defects in mechanosensory neuron development in *unc-86* mutants (Chalfie and Au 1989). Therefore *unc-86* and its homologs may have a universal role in coupling neuroblast cell lineage control signals to the expression of neuron-type specific genes during neurogenesis.

Materials and methods

Construction of *unc-86*-GFP and *lacZ* fusion genes

ABC₁₂₃₄WXY was constructed by cloning a *SpeI* fragment containing the *unc-86* promoter and part of the transcribed region into vector pPD22.04 (Fire et al. 1990). Construct C₃₄WXYZ deletes sequences upstream of the *EcoRI* site at -422 and adds a *SpeI*-*EcoRI* fragment from the *unc-86* 3' region to the reporter gene downstream of its polyadenylation site. GFP reporter plasmids were constructed by replacing the *KpnI*-*SphI* fragment containing the *lacZ* gene with the respective GFP reading frame from plasmid TU61 (Chalfie et al. 1994). ABC₁₂₃₄ is a *Bam*HI-*Xma*I deletion of ABC₁₂₃₄WXY, with fragments C₁₂₃₄ reinserted as a PCR fragment from primers RB15 (5'-CAGGAAGTCAAGGTTGAGGATCC) and RB14 (5'-GGTCATTTT-TGAAGCGGTTGTCG). ABC₁₂₃₄1 and ABC₁₂₃₄2 are ABC₁₂₃₄ with the *DsaI*-*Xma*I fragment replaced by a synthetic fragment CACGGACATGACCCC and CACGGAAAAATGACCCC, respectively. AC₁₂₃₄ is a *Bam*HI-*Bgl*II deletion construct, and BC₁₂₃₄ is a *Sall*-*Bam*HI deletion derivative of ABC₁₂₃₄. C₁₂₃₄ is a *Hind*III deletion construct derived from ABC₁₂₃₄.

The *mec-7*/GFP and *pes-10*/*lacZ* fusion genes were constructed by ligation of a 2.3-kb *SpeI*-*Hind*III fragment (A': coordinates -5.1- to -2.8-kb) or a 1.9-kb *Hind*III fragment (B': coordinates -2.7 to -0.8 kb) from the *unc-86* upstream region, or a 2.5-kb *SpeI* *Eco*RI fragment (Z: coordinates +2.6 to +5.2 kb) from the *unc-86* 3' end into the polylinker of pPD95.25 or a GFP derivative of pPD52.102 (J. Kaplan, pers. comm.). These reporter genes bear proximal elements of the *pes-10* and *mec-7* genes, respectively, and the *unc-86* segments were inserted in either orientation upstream of these proximal elements.

Determination of the *unc-86* transcriptional start

The correct *unc-86* mRNA start site was determined by PCR

amplification of first-strand reverse-transcribed total RNA and RNA-linker ligation (Innis et al. 1990). The longest ligation product starts with the (G)GGACATGC at nucleotide 5095 (numbers according to Fig. 3). Consistent with this result, only primers mapping 3' to the identified start site allowed amplification of spliced products.

Expression analysis of reporter genes

Fusion gene DNA was co-injected with visible genetic marker plasmids as described (Mello et al. 1991). Most transformations used 10 ng/μl reporter gene (low-copy number). In transformations that surveyed negative regulatory elements, reporter genes were injected at 200 ng/μl (high-copy number). In general fusion gene expression in a particular neuron or neuroblast was viewed in numerous animals and multiple lines. To ensure a better reproduction of the weak expression levels, some photographs were taken from transgenic animals injected at 200 ng/μl.

lacZ and *unc-86* expression was detected as indirect immunofluorescence using anti-β-galactosidase (Promega) and anti-UNC-86 specific antibodies as described (Finney and Ruvkun 1990). Cells stained with anti-UNC-86 or anti-β-galactosidase antibodies were identified based on the position of their nuclei with respect to DAPI staining. GFP expressing cells were identified based on their position and the morphology of cell body and axon. Because of their rapid migration in L1 and the lack of a fixed final position, the four nuclei of the T lineage, PHC(L/R) and PLN(L/R), could not be identified unambiguously in fixed animals. However, the correct number of tail cells expressing the *lacZ* gene and the occasional detection of staining in the dying T.pppp cell indicated expression in the T lineage. One discrepancy from the reported *unc-86* expression pattern (Finney and Ruvkun 1990) was detected. The two pharyngeal neurons I1(L/R), but not I2(L/R), as reported, stain with *unc-86* antibodies (confirmed by S. Shaham, pers. comm.). Figure 1 contains the corrected expression pattern.

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Lineage-specific regulators couple cell lineage asymmetry to the transcription of the *Caenorhabditis elegans* POU gene *unc-86* during neurogenesis.

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