Developmental Biology **207,** 215–228 (1999)
Article ID dbio.1998.9124, available online at http://www.idealibrary.com on IDEAL®

Patterning of *Caenorhabditis elegans* Posterior Structures by the *Abdominal-B* Homolog, *egl-5*

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The *Caenorhabditis elegans* body axis, like that of other animals, is patterned by the action of *Hox* genes. In order to examine the function of one *C. elegans Hox* gene in depth, we determined the postembryonic expression pattern of *egl-5*, the *C. elegans* member of the *Abdominal-B Hox* gene paralog group, by means of whole-mount staining with a polyclonal antibody. A major site of *egl-5* expression and function is in the epithelium joining the posterior digestive tract with the external epidermis. Patterning this region and its derived structures is a conserved function of *Abd-B* paralog group genes in other animals. Cells that initiate *egl-5* expression during embryogenesis are clustered around the presumptive anus. Expression is initiated postembryonically in four additional mesodermal and ectodermal cell lineages or tissues. Once initiated in a lineage, *egl-5* expression continues throughout development, suggesting that the action of *egl-5* can be regarded as defining a positional cell identity. A variety of cross-regulatory interactions between *egl-5* and the next more anterior *Hox* gene, *mab-5*, help define the expression domains of their respective gene products. In its expression in a localized body region, function as a marker of positional cell identity, and interactions with another *Hox* gene, *egl-5* resembles *Hox* genes of other animals. This suggests that *C. elegans*, in spite of its small cell number and reproducible cell lineages, may not differ greatly from other animals in the way it employs *Hox* genes for regional specification during development.

***Key Words:* *Hox* genes; nematode; C. elegans; development.**

INTRODUCTION

The many known similarities between species in the molecular mechanisms of development leave no doubt that many of these similarities are the result of common descent rather than convergent evolution. This conclusion implies that a long period of metazoan evolution occurred before the separation of the major animal phyla. What were the characteristics of the primitive ancestor in which these ubiquitous developmental mechanisms first evolved? Comparisons of modern representatives of different phyla can reveal conserved features that may reflect the characteristics of this unexpectedly complex creature.

Among the first discovered and still one of the most striking shared developmental mechanisms is patterning of the anteroposterior body axis and other body structures by homeodomain transcription factors encoded by the *Hox* genes. The ubiquity and importance of *Hox* gene patterning suggests that the mechanisms underlying it may have constituted some of the key innovations spurring metazoan evolution (Slack *et al.*, 1993). Comparisons of *Hox* gene structure, regulation, and action in a variety of species can help to define a set of common core properties that might shed light on the nature of these key innovations (Kenyon, 1994; Gellon and McGinnis, 1998).

Hox genes define body regions. In this role they are not associated with any particular differentiated cell type or body pattern. Other frequently shared properties include the clustering of *Hox* genes at a single chromosomal site; a colinearity of chromosomal map position and domain of action in the body; a common transcriptional orientation

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0012-1606/99 $30.00

with 5′ end toward the "posterior" end of the cluster; expression domains which overlap, beginning at an anterior boundary and extending toward the posterior; and a hierarchy of action in which more posteriorly expressed genes often take precedence over more anteriorly expressed genes (McGinnis and Krumlauf, 1992; Krumlauf, 1994; Lawrence and Morata, 1994). While none of these properties is so absolutely conserved among species as to indicate an essential role, their recurrence suggests some relationship to the regulation or mechanism of action of *Hox* genes or to their evolutionary origin.

Here we describe a study of the expression pattern of *egl-5*, the *Caenorhabditis elegans* member of the *Abdominal-B* paralog group and compare the expression and function of *egl-5* to the expression and function of *Abd-B* orthologs in other organisms. Studies of *Hox* genes in *C. elegans* could be especially valuable to understanding *Hox* gene function and evolution, because *C. elegans* is experimentally accessible and suitable for analysis at the single cell level and because nematodes may be an outgroup to both Arthropods and Chordates (Sidow and Thomas, 1994; Winnepenninckx *et al.*, 1995; but see Aguinaldo *et al.*, 1997). Conclusions from studies in nematodes may be applicable to other species provided *Hox* genes function in the nematode, with its small cell numbers and reproducible cell lineages, in ways that are similar to their functions in larger animals with apparently greater degrees of cellular flexibility. We find that for *egl-5* this appears to be the case.

C. elegans has four *Hox* genes mapping together in a 310-kb region of chromosome III (see Salser and Kenyon, 1994; Ruvkun, 1997, for reviews of *C. elegans Hox* genes). Sequence comparisons as well as comparison of predicted three-dimensional structures of the proteins indicate the four *C. elegans* genes are respectively members of the paralog groups defined by *Drosophila labial* (*ceh-13*), *Deformed* (*lin-39*), *Antennapedia* (*mab-5*), and *Abdominal-B* (*egl-5*) (Bürglin, 1995; Sharkey *et al.*, 1997). It is not known whether the four *C. elegans Hox* genes represent an early form of the *Hox* cluster or a degenerated form that has lost ancestral genes, possibly concomitant with evolution to small cell number and reproducible cell lineages.

Genetic analysis has shown that *egl-5* is like the *Abd-B* homologs of other animals in being involved primarily in patterning cell fates in the posterior body region and tail (Chisholm, 1991). Here we provide a description of the postembryonic expression pattern of EGL-5, including regulatory interactions with the *Antennapedia* homolog *mab-5*, determined by means of staining with an anti-EGL-5 polyclonal antibody. The data presented define the cellular focus of *egl-5* function and serve as a basis for further studies of *egl-5* regulation. We find that in a number of ways *egl-5* appears to be typical of *Hox* genes in other animals. Therefore, future studies of the genes that control EGL-5 expression and function may lead to insights regarding the underlying mechanism of action of *Hox* genes.

MATERIALS AND METHODS

Nematodes

The nematodes used in this study were all derivatives of strain Bristol N2. They were maintained at 20°C using standard methods (Brenner, 1974; Lewis and Fleming, 1995). All strains carried the *him-5(e1490)V* mutation, which elevates X chromosome nondisjunction, boosting the frequency of male self-progeny to some 30% (Hodgkin *et al.*, 1979). In the text, animals bearing only the *him-5(e1490)* mutation will be considered and referred to as wild type, and *him-5(e1490)* is not repeated in genotypes. The following additional mutations were used: *egl-5(u202)III* [a 7-bp insertion within the homeodomain, a null allele (Chisholm, 1991; Wang *et al.*, 1993)]; *egl-5(n486)III* [R52C, a hypomorphic allele (Wang *et al.*, 1993)]; *lin-32(e1926)X* [L24F, a hypomorphic allele (Zhao and Emmons, 1995)]; *mab-5(e1239)III* [intron 1 donor splice site mutation, a putative null allele (Salser and Kenyon, 1996)]. Worms homozygous for *lin-15(n765)X* (Huang *et al.*, 1994) were used for transformation.

Generation and Affinity Purification of Anti-EGL-5 Antibodies

Anti-EGL-5 antibodies were raised in rabbits by injection of a GST:EGL-5 fusion protein isolated from *Escherichia coli* (HRP, Inc., Denver, PA). For generation of the fusion protein, the coding sequence of a full-length *egl-5* cDNA (clone 160.111, gift of the Kenyon laboratory, see Wang *et al.*, 1993) was amplified by PCR, ligated into pGEX-KG (Guan and Dixon, 1991) in frame with and C-terminal to the GST coding sequence to generate plasmid EM#276, and introduced into *E. coli* strain BL21 (Novagen). The GST::EGL-5 fusion protein generated was purified by affinity chromatography on glutathione–Sepharose 4B (Pharmacia) (Smith and Johnson, 1988).

For affinity purification of antibodies, antiserum was incubated for 30 min at 56°C to inactivate complement factors and adsorbed 1 h at 37°C with *E. coli* acetone powder (25 mg/ml) [prepared from *E. coli* strain BL21 carrying plasmid pGEX-KG and induced to produce GST (Smith and Johnson, 1988)] (Miller and Shakes, 1995). Ten milliliters of pretreated antiserum was mixed with 3 ml of Sepharose 4B carrying immobilized GST::EGL-5. Anti-EGL-5 antibodies were eluted in 100 mM glycine, pH 2.5, and neutralized with 1/10 vol of 1 M Tris, pH 8.0. Under these conditions, GST::EGL-5 remained stably bound to the Sepharose 4B.

In Western blots, purified anti-EGL-5 antibodies stained a species of 25–26 kDa, corresponding to the predicted size of EGL-5 (data not shown). This species was present in extracts of wild-type worms but absent from extracts of *egl-5(u202).* No staining was observed in immunohistochemical staining of whole-mount *egl-5(u202)* nematodes.

Immunohistochemical Staining of Whole-Mount Worms

Synchronous worm cultures were established by isolating eggs by alkaline hypochlorite treatment of gravid worms as described by Lewis and Fleming (1995). Eggs were allowed to hatch overnight in M9 buffer (Sulston and Hodgkin, 1988). The arrested L1 larvae were then transferred to plates seeded with bacteria and allowed to grow to the desired stage of development. Since different batches of arrested L1 larvae recovered from starvation with variable lag times, the stage of development of synchronous cultures was

monitored by direct Nomarski observation of at least 10 males. The V5, V6, and B lineages were generally scored to define the developmental stage. Developmental times referred to in the text and figures are the times of posthatching development given by Sulston and Horvitz (1977) and Sulston *et al.* (1980) that correspond to the stages of development actually observed.

Immunostaining was by a variation of the fixation, permeabilization, and staining protocols of Finney and Ruvkun (1990) optimized for our antibody. For fixation and permeabilization, worms (100–200 μl) were washed in M9 buffer and resuspended in a volume of 250 μl, followed by the addition of 800 μl of 1.25× modified Ruvkun fixation buffer (25 mM NaCl, 100 mM KCl, 2.5 mM EGTA, 0.625 mM spermidine–HCl, 0.25 mM spermine, 0.625% β-mercaptoethanol, 18.75 mM Pipes pH 7.4, 1.25% paraformaldehyde) and 200 μl of a MeOH–EGTA solution (90% MeOH, 25 mM EGTA). The suspension was incubated 5 min at room temperature, immediately frozen in dry ice–EtOH, and thawed under a stream of tap water. The cycle of incubation, freezing, and thawing was repeated twice to total of three freeze–thaw cycles and an overall fixation time of 15 min. The fixing solution was removed and the worms were washed twice in TTB (100 mM Tris–HCl, pH 7.4, 1% Triton X-100, 1 mM EDTA), incubated for 15 min at 37°C in 1% β-mercaptoethanol in TTB, washed twice in borate buffer (50 mM H_3BO_3, pH 9.5), and incubated for 1 h at 37°C in 10 mM DTT in borate buffer. The worms were then washed once in borate buffer and incubated for 1 h at room temperature in 1% H_2O_2 in borate buffer. Finally, the worms were washed once in borate buffer and twice in AbB (1× PBS, 0.1% BSA, 0.5% Triton X-100, 0.05% NaN_3, and 1 mM EDTA) and kept in AbA (AbB with 1% BSA) at 4°C if not immediately immunostained (PBS is 137 mM NaCl, 2.7 mM KCl, 10.14 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.2–7.4). Fixation and permeabilization were monitored for every batch of worms by staining an aliquot with anti-histone monoclonal antibody (MAB052, Chemicon, diluted 1:1000 in AbA) (Salser *et al.,* 1993). Successful fixation and permeabilization were indicated by uniform staining of all nuclei.

In order to reduce background, prior to use in immunohistochemistry assays, affinity-purified anti-EGL-5 antibodies were preadsorbed with extract of *egl-5(u202)* nematodes. Antibodies were diluted 1:25 in worm extract and incubated for 2–4 h at 37°C. Worm extract was prepared by sonicating *egl-5(u202)* nematodes (400–500 μl) in 500 μl PBS (defined above). Debris was removed by a 5-min microcentrifuge spin and NaN_3 was added to the supernatant to a final concentration of 0.01%.

For immunohistochemical staining, 50–100 μl of fixed, permeabilized worms was incubated for 1 h at 37°C with preabsorbed anti-EGL-5 Ab (diluted 1:100 in AbA). After reaction with primary antibody, worms were washed three times in AbB, incubated for 1 h at 37°C with Cy3-conjugated AffiniPure goat anti-rabbit IgG (H + L) secondary antibody (Jackson ImmunoResearch, diluted 1:100 in AbA), washed twice in AbB, and left in AbA. In most experiments, MH27 monoclonal antibody was added to the primary antibodies (Francis and Waterston, 1991, diluted 1:50 in AbA) in order to help in the identification of hypodermal cell boundaries. FITC-labeled goat anti-mouse IgG + IgM (H + L) secondary antibody (Jackson ImmunoResearch, diluted 1:100 in AbA) was used to visualize reactions with the monoclonal antibodies.

After immunostaining, DAPI was added to the nematode suspension to a final concentration of 1 μg/ml. The larvae were mounted in SlowFade antifade (Molecular Probes) over a 2% agarose pad in PBS and observed with a Zeiss Axioplan immunofluorescence microscope.

Construction of the egl-5::gfp Reporter

The *egl-5::gfp* reporter used (EM#278) contains 13.2 kb of 5′ flanking genomic DNA and *egl-5* coding sequence up to the middle of exon 3, joined in frame to the *gfp* cassette from plasmid pPD96.67 (Fire laboratory Vector Kit). EM#278 was introduced into *lin-15(n765)* hermaphrodites together with plasmid pL15EK (Clark *et al.,* 1994) carrying the wild-type *lin-15* gene as coinjection marker (Mello and Fire, 1995). Extrachromosomal arrays were integrated by gamma irradiation (Mello and Fire, 1995), yielding integrated transgene *bxIs7* in nematode strain EM514. Worms were observed either with a Zeiss Axioplan microscope equipped for epifluorescence or with a Bio-Rad MRC 600 scanning laser confocal microscope.

RESULTS

Initiation of egl-5 Expression during Embryogenesis and Postembryonic Development

In the young larva (10 h posthatching), staining with anti-EGL-5 antibodies was observed in the nuclei of five rectal epithelial cells, three left–right pairs of neurons, and four to six bilateral pairs of body-wall muscle cells. With the exception of the pair of HSN neurons, which are in the mid-body adjacent to the gonad, all these cells are in the posterior body region (Fig. 1A). Previously, expression of an *egl-5* reporter gene was observed in the same set of cells during late embryogenesis or at hatching (Wang *et al.,* 1993; Ahringer, 1996). In the late embryo, all these cells, including the HSN neurons, lie adjacent to one another in a small region around the presumptive anus (Fig. 1B). The more anterior *Hox* genes *mab-5* and *lin-39* are expressed in adjacent, successively more anterior clusters of cells (Cowing and Kenyon, 1992; Wang *et al.,* 1993; Clark *et al.,* 1993). The fourth member of the *C. elegans Hox* gene cluster, *ceh-13,* is expressed in a variety of embryonic lineages mostly in the anterior region (Wittmann *et al.,* 1997). Therefore, as in other organisms, *C. elegans Hox* genes are expressed in ordered domains along the anteroposterior axis of the embryo.

Postembryonically, expression of *egl-5* is initiated in four additional lineages or tissues (Fig. 1A). Most of these cells are also in the posterior region. The exceptions are cells of the male gonad, which initiate expression of EGL-5 when they are still in the mid-body. These cells migrate toward the posterior body, so even in this case *egl-5* expression remains associated with differentiation of posterior structures.

In all cells where *egl-5* expression is initiated, the gene appears to remain on throughout postembryonic development and adulthood. All antibody staining observed was nuclear. Although we did not trace out every cell of several complex cell lineages expressing EGL-5, judging by the number of staining cells observed, expression appears to remain on in all branches. We observed no instance where *egl-5* expression was initiated and later turned off. Therefore, in addition to being found in a restricted body region, *egl-5* is like *Hox* genes of other animals in possibly acting as

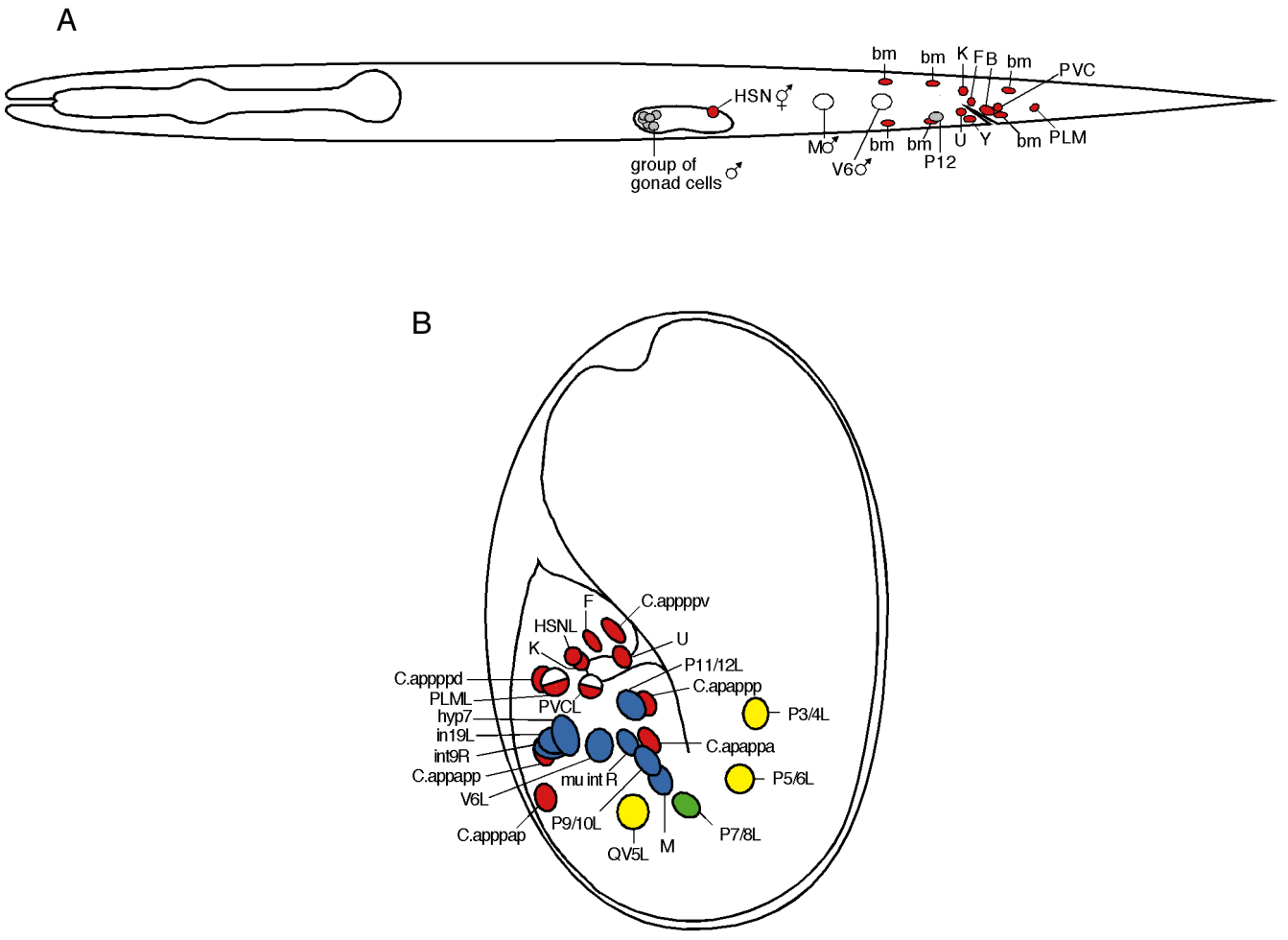

FIG. 1. (A) Schematic summary of postembryonic expression of *egl-5* (left and central nuclei of an early L1 larva). Nuclei of cells that express *egl-5* at hatching are represented in red, of cells that start expressing *egl-5* later in L1 in gray, and of cells whose descendants start expressing *egl-5* during L2 as open ellipses. Cells or cell lineages that show sex-specific expression are indicated. HSN, hermaphrodite specific neuron; PLM, posterior lateral microtubule neuron; PVC, an interneuron; bm, body-wall muscle cells. All the other cells shown are postembryonic blast cells. (B) Positions during late embryogenesis of cells that express *Hox* genes (left side and central nuclei only). The diagram of nuclear positions at the beginning of embryonic morphogenesis (430 min after first cleavage) is taken from Sulston *et al.* (1983). The time during embryogenesis when expression in these cells begins has not been fully established. Red, *egl-5*; blue, *mab-5*; yellow, *lin-39*; green, *mab-5* and *lin-39*. The *mab-5* expression pattern is from the reporter gene studies of Cowing and Kenyon (1992); the *lin-39* expression pattern is from the reporter gene study of Wang *et al.* (1993). Not all *lin-39*-expressing cells are shown. The mothers of PLM and PVC, which also generate daughters that do not express *egl-5*, are represented as half-colored circles. The B and Y blast cells are in the same perianal region on the right-hand side. The descendants of the C blastomere shown correspond to the body-wall muscle cells in A.

a relatively fixed marker of positional cell identity (Lawrence and Morata, 1994). The data supporting these conclusions are presented in the following sections.

Expression of egl-5 in Postembryonic Cell Lineages and Tissues

Rectal epithelial cells. A major site of EGL-5 expression is in the rectal epithelium. At hatching, the rectal epithelium comprises five cells, K, F, B, U, and Y, which join the intestinal–rectal valve to the external epidermis (Fig. 1A) (White, 1988). In hermaphrodites, these cells remain as rectal epidermis and do not divide with the exceptions of Y, which differentiates into the PDA motorneuron, and K, which divides once, giving rise to a cell that remains part of the dorsal rectal epithelium and a cell that becomes the DVB motorneuron. In males, in addition to K, the remaining cells divide extensively to produce genital structures and male-specific neurons (Sulston *et al.*, 1980).

There is strong expression of EGL-5 in all these rectal epithelial cells in both sexes (Figs. 2A and 2F). In the hermaphrodite, although three of the cells (B, U, and F) do

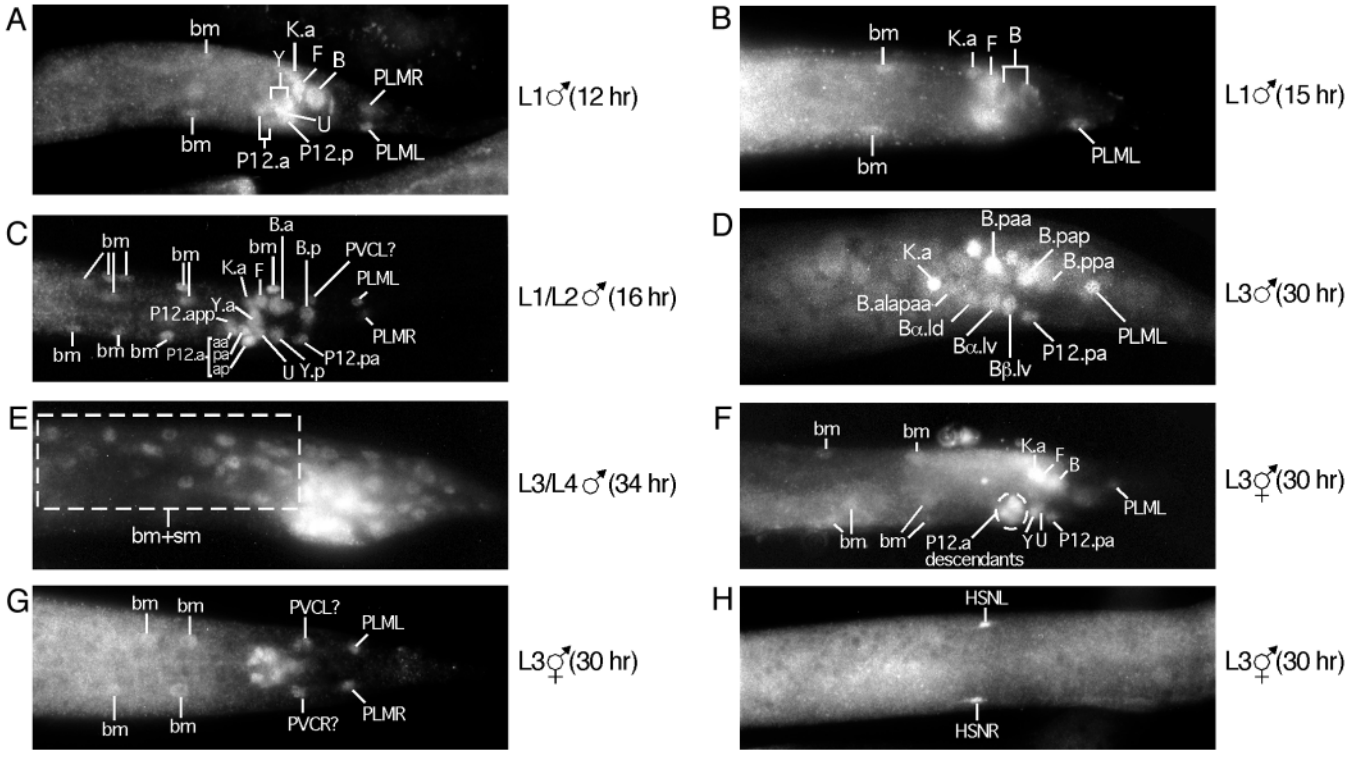

FIG. 2. Expression of EGL-5 in the rectal epithelium, tail ganglia, and muscle cells of males and hermaphrodites. Representative photomicrographs are shown of larval worms stained with anti-EGL-5 antibody. Larval stage, sex, and hours of postembryonic development are given to the right. For the identities and lineal relationships of the cells shown, see Sulston and Horvitz (1977) and Sulston *et al.* (1983). bm, body-wall muscle nuclei; sm, sex muscle nuclei. All photomicrographs show a lateral view of the posterior body region, except G, which is a ventral view, and H, which is a ventral view at mid-body. In B, the B cell is dividing; immunostaining surrounds the central region of condensed chromatin of the newborn nuclei.

not divide or change their differentiated state, and there is no apparent defect traceable to them in *egl-5* mutants, expression continues throughout postembryonic development and into adulthood. In the male, we observed antibody staining in B.a and B.p, as well as Y.a and Y.p in late L1 and early L2 (Figs. 2B and 2C). At later times, we did not attempt to identify all the cells in these lineages. However, considering the number of stained nuclei in the perianal region and adjacent ganglia, it seems likely that most, if not all, B, Y, U, F, and K descendants express *egl-5* (Figs. 2D and 2E).

B, Y, U, F, and K and their descendants are defective in various ways in *egl-5* mutants (Chisholm, 1991). In *egl-5* hermaphrodites the fates of Y and K are abnormal: Y remains an epithelial cell, does not differentiate into PDA, and may divide in some animals, while K does not divide and DVB is not made. In *egl-5* males, the structures that arise from B, Y, U, and F, namely, the spicules, gubernaculum, and proctodeal chamber, are absent or grossly abnormal, and K exhibits the same defect as in hermaphrodites. In addition, the cellular retraction process that remodels the posterior region at the end of the L4 larval stage is abnormal, resulting in males with rounded tails and little or no fan. Defective retraction could be the result of abnormalities in descendants of B.p, as these cells constitute a significant proportion of the volume of the tail, particularly on the ventral side where the greatest retraction occurs (Sulston *et al.*, 1980). In *egl-5* males, B, Y, U, and F still divide, but the lineages do not resemble wild-type lineages, suggesting that there is abnormal assignment of cell fates from the earliest divisions. Finally, both males and hermaphrodites are defective in defecation, indicating there are defects in the analia that have not been fully defined.

Ventral neuroectoblast P12. *egl-5* mutants are defective in the posterior ventral neuroectoderm (Chisholm, 1991). The two posterior ventral neuroectoblast cells P11 and P12 are initially equivalent left/right homologs. On migrating to the ventral midline, their respective fates are assigned by cell interaction (Sulston and White, 1980). At first, both cells express the *Hox* gene *mab-5* (Salser *et al.*, 1993). EGL-5 staining first appeared in P12.a and P12.p in 12-h worms (Fig. 2A). Staining continued in P12 descendants in 15-h and older worms (Figs. 2C, 2D, and 2F) and apparently persists into adulthood. Around the time EGL-5 staining first appears, MAB-5 antibody staining disappears in P12 descendants but persists in P11 descendants (Salser

et al., 1993). Turn off of *mab-5* expression does not occur in an *egl-5* mutant and hence requires *egl-5* gene function (Salser *et al.*, 1993). In *egl-5* mutants, P12 takes the normal fate of P11, expressing a P11-like lineage (Chisholm, 1991).

Mechanosensory neurons. *egl-5* worms are insensitive to both light touch (Mec phenotype) and heavy touch (Tab phenotype) in the tail (Chisholm, 1991). These phenotypes are due to one or more presumptive defects in a neuronal circuit involving two PLM mechanosensory neurons in the tail and two PVC interneurons in the lumbar ganglia (Chalfie *et al.*, 1985). Expression of EGL-5 was detected in the PLM neurons throughout larval development in both sexes (Figs. 2A–2D, 2F, and 2G). In addition, two cells expressing EGL-5, one in the anterior region of each lumbar ganglion, are presumptively the PVC interneurons (Figs. 2C and 2G). The Mec and Tab defects could therefore be the result of defects in both the PLM and the PVC components of the touch circuitry.

Muscle cells. In males and hermaphrodites, a single mesoblast cell called M generates both body-wall muscles and sex-specific muscles during postembryonic development (Sulston and Horvitz, 1977). M divides during L1, generating body-wall muscle cells and sex-specific mesoblasts. The sex-specific mesoblasts divide during L3, giving rise to vulval and uterine muscles in the hermaphrodite and to a variety of sex-specific muscles of the tail in the male (Sulston and Horvitz, 1977; Sulston *et al.*, 1980).

Expression of EGL-5 was detected in 4 to 6 left/right pairs of posterior body-wall muscle cells in L1 larvae at the earliest times examined (10–12 h) (Fig. 2A). By late L1 or early L2 (1516 h posthatching) it is likely that both embryonic and postembryonic muscle cells or mesoblasts are expressing EGL-5, since staining was detected in at least 12 left/right pairs of nuclei, of which only 5 dorsal and 3 ventral can be embryonic (Fig. 2C). Staining is strongest in the most posterior nuclei and tapers off toward the anterior. Staining in posterior body-wall muscle cells remains throughout larval development and into adulthood in both sexes. The expression of EGL-5 in posterior body-wall muscles as well as in the posterior ventral nervous system (preanal ganglion) suggests a possible role for *egl-5* in defining neuromuscular junction specificity. A defect in this function could be associated with the uncoordinated phenotype observed in *egl-5* mutants (Chisholm, 1991).

In males, by the time the sex mesoblasts are dividing in L3 (28–34 h posthatching), there is strong EGL-5 expression in the sex-specific muscle lineages and sex-specific muscles (Fig. 2E). These muscles include the diagonal muscles as well as muscles of the spicules, gubernaculum, and other sex-specific muscles, which were not individually identified; possibly all the sex muscles express EGL-5. Staining in the sex-specific muscles persists through adulthood. Although in *egl-5* males the lineages of the sex muscle precursors are apparently normal at least until the penultimate divisions, the arrangement of the sex muscles is abnormal, with muscles misattached or absent (Chisholm, 1991). No staining was observed in sex-specific muscles or sex-specific muscle lineages in the hermaphrodite, and these are apparently normal in *egl-5* mutants.

HSN neurons. Developmental defects in the two HSN neurons are responsible for the egg-laying-defective (Egl) phenotype of *egl-5* hermaphrodites (Desai *et al.*, 1988). In *egl-5* mutants, HSNs lack serotonin and do not migrate during embryogenesis the full distance between their birthplace in the tail to the mid-body (Desai *et al.*, 1988; Chisholm, 1991). In wild-type hermaphrodites, EGL-5 was present in both HSN neurons from L1 onward and throughout adulthood (Fig. 2H). This continuous expression indicates that the requirement for *egl-5* in defining multiple independent properties of HSN neurons could be because of an early function in the pathway of HSN specification, a late function in transcription of one or more differentiation genes, or in both of these processes.

Male gonad. The male somatic gonad is grossly abnormal in *egl-5* mutants (Chisholm, 1991). In normal development, the somatic gonad is formed by descendants of two cells, Z1 and Z4, which in both sexes lie in the gonad primordium in the central ventral body. In the male, Z1 and Z4 give rise to 56 cells during a gonadogenesis process that comprises three stages: an early mitotic stage from mid-L1 to early L2, during which Z1 and Z4 divide three times, an amitotic period from early to late L2, and a late mitotic stage during L3 (Kimble and Hirsh, 1979). Defects in the male gonad in *egl-5* mutants stem from lineage delays and defects in the late mitotic stage (Chisholm, 1991).

EGL-5 is first detected in the male gonad in late L1 (12–15 h posthatching) in a group of 6 cells at the anterior end (Fig. 3A). At the time staining first appears, Z1 and Z4 have generated six descendants, but these are not clustered in the same arrangement as is the group of staining cells. It appears that EGL-5 must be expressed in a group of cells in one region that consists of both somatic cells and germ cells. During the amitotic period, as the gonad grows to the anterior, this group of six cells remains clustered and expresses EGL-5 (Fig. 3B). Later, at the beginning of the late mitotic period, as the gonad reflexes toward the posterior, the stained nuclei lose their clustered arrangement, becoming scattered (Fig. 3C). By 34 h, EGL-5 expression can be detected in several dividing cells that are forming the primordium of the seminal vesicle as well as in two large nuclei in the valve region (Fig. 3D). In the nuclei of the dividing cells, the anti-EGL-5 staining surrounds a condensed chromatin. This staining pattern remains up to the end of the late mitotic period (35–37 h posthatching), when antibody staining can be detected not only in the cells of the seminal vesicle and in valve cells, but also in sperm cells (Fig. 3E). With the exception of the valve cells, no staining in cells of the vas deferens was observed.

No expression of EGL-5 was detected in the developing hermaphrodite gonad, and there is no obvious gonadal defect in the hermaphrodite in *egl-5* mutants.

Lateral hypodermis. In *egl-5* mutants there are two cell-autonomous defects in the male seam (a lateral row of hypodermal cells that also serves as a neuroectoderm): one

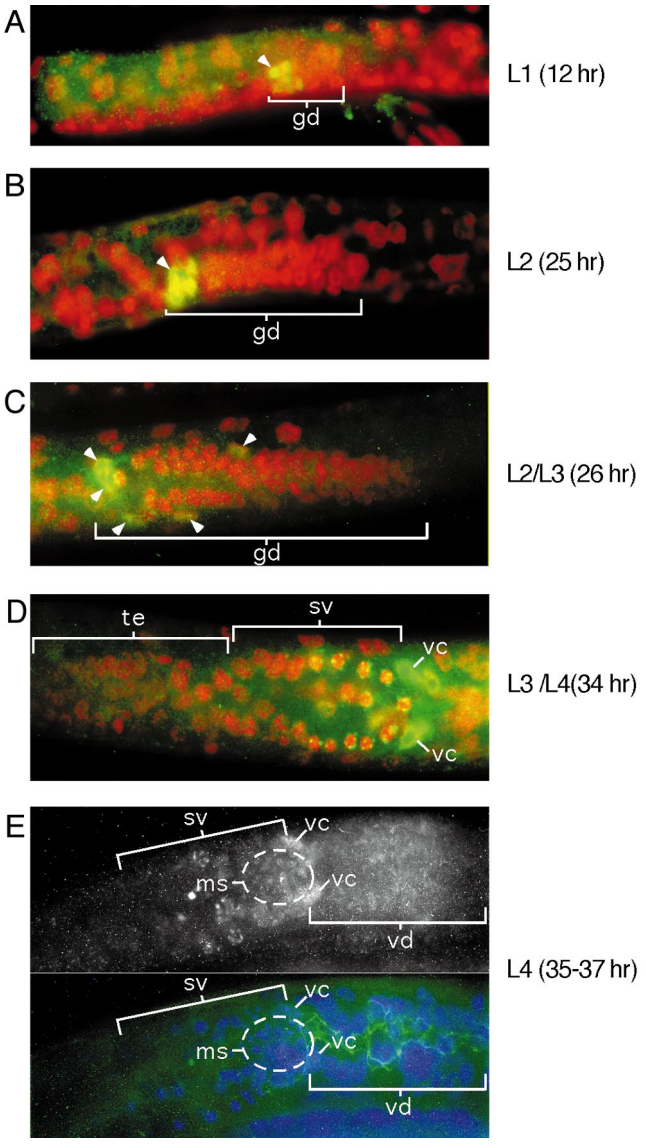

FIG. 3. Expression of EGL-5 in the male gonad. False color photomicrographs: green, fluorescence of secondary antibody; red, DAPI nuclear staining; yellow or yellowish-green, nuclei staining positively for EGL-5. Arrowheads indicate clustered, positively staining nuclei (in A and B) which later become dispersed (in C). In E, top shows anti-EGL-5 staining; bottom shows DAPI nuclear staining (blue) and adherens junctions at cell boundaries stained with monoclonal antibody MH27 (green). gd, gonad; te, testes; sv, seminal vesicle; vc, valve cell; ms, mature sperm; vd, vas deferens. A and B, lateral view; C–E, ventral view.

of nine sensory rays is absent (ray 6) due to failure of expression of the cell sublineage giving rise to this ray, and the morphological identities of rays 2–5 are abnormal (Chisholm, 1991; Chow and Emmons, 1994). In addition, ray 1 remains lateral and does not migrate posteriorly to its normal position in the fan; the autonomy or nonautonomy of this defect was not determined (Salser and Kenyon, 1996).

EGL-5 is expressed in the male seam from mid-L2. EGL-5 first appears in V6.ppp at 20–22 h of postembryonic development (Fig. 4B). Staining persists in V6.pppa and V6.pppp, although at a lower level (Fig. 4C). Intensity of staining increased in R5 and R6 and to a lesser extent in R4 (Fig. 4D). At 31 h, staining also appeared in R3 (Fig. 4E). Later identification of staining cells in ray sublineages was not possible due to the intense fluorescence of B-lineage cells lying in the same region. However, it was possible to observe expression of a reporter gene using the confocal microscope in R4, R5, and R6 and also in cells of the R5 and R6 sublineages (Figs. 4F and 4G). No staining was observed in the V5 lineage, including in cells of ray 1. Either expression of EGL-5 is below the limit of detection or there is no expression in this lineage and the ray 1 migration defect in *egl-5(−)* is a cell-nonautonomous effect. The hermaphrodite seam is normal in *egl-5* mutants (Chisholm, 1991), and no staining was observed in hermaphrodite seam cells ($n > 100$).

The male seam cell expression pattern can be understood in the context of the role *egl-5* plays, together with *mab-5*, in defining the identities of the V-rays [the rays descended from seam cells V5 and V6, namely, rays 1–6 (Fig. 5)]. Rays are generated by the repetition of a cell sublineage and contain the same three cell types (Sulston *et al.*, 1980). Yet they differ by their position, morphology, neurotransmitter expression, and undoubtedly other characteristics. Genetic studies have shown that *egl-5* and *mab-5* play a role in defining these characteristic differences among the V-rays (Chow and Emmons, 1994; Salser and Kenyon, 1996).

The expression patterns of EGL-5 and MAB-5 in the seam lineages are consistent with the conclusions drawn from genetic studies. EGL-5 and MAB-5 are present in different subsets of V-rays, with apparent overlap in ray 4 and no expression of either gene in ray 1 (Fig. 5). Salser and Kenyon (1996) showed that the alternating OFF–ON pattern of MAB-5 in rays 1–4 was essential to specification of the morphological identities of these rays. If MAB-5 was ectopically expressed from a transgene, the morphological identity of ray 1 was transformed to that of ray 2, and the morphological identity of ray 3 was transformed to that of ray 4. Chow and Emmons (1994) showed, conversely, that decreased *mab-5* gene function caused transformation of ray 4 to ray 3.

EGL-5 appears to have a similar essential role in a different subset of the V-rays. Decreased numbers of *egl-5(+)* gene copies resulted in transformation of the identity of ray 6 to that of ray 4. The frequency of this transformation was raised by increased numbers of *mab-5(+)* gene copies (Chow and Emmons, 1994). This result is consistent with the observation that ray 6 expresses EGL-5 at a high level, whereas ray 4 expresses EGL-5 at a lower level and also expresses MAB-5. The frequency of transformation of ray 4 to ray 3 caused by decreased number of *mab-5(+)* gene copies was raised by increased numbers of *egl-5(+)* gene copies (Chow and Emmons, 1994). This is consistent with the observation that ray 3 expresses EGL-5.

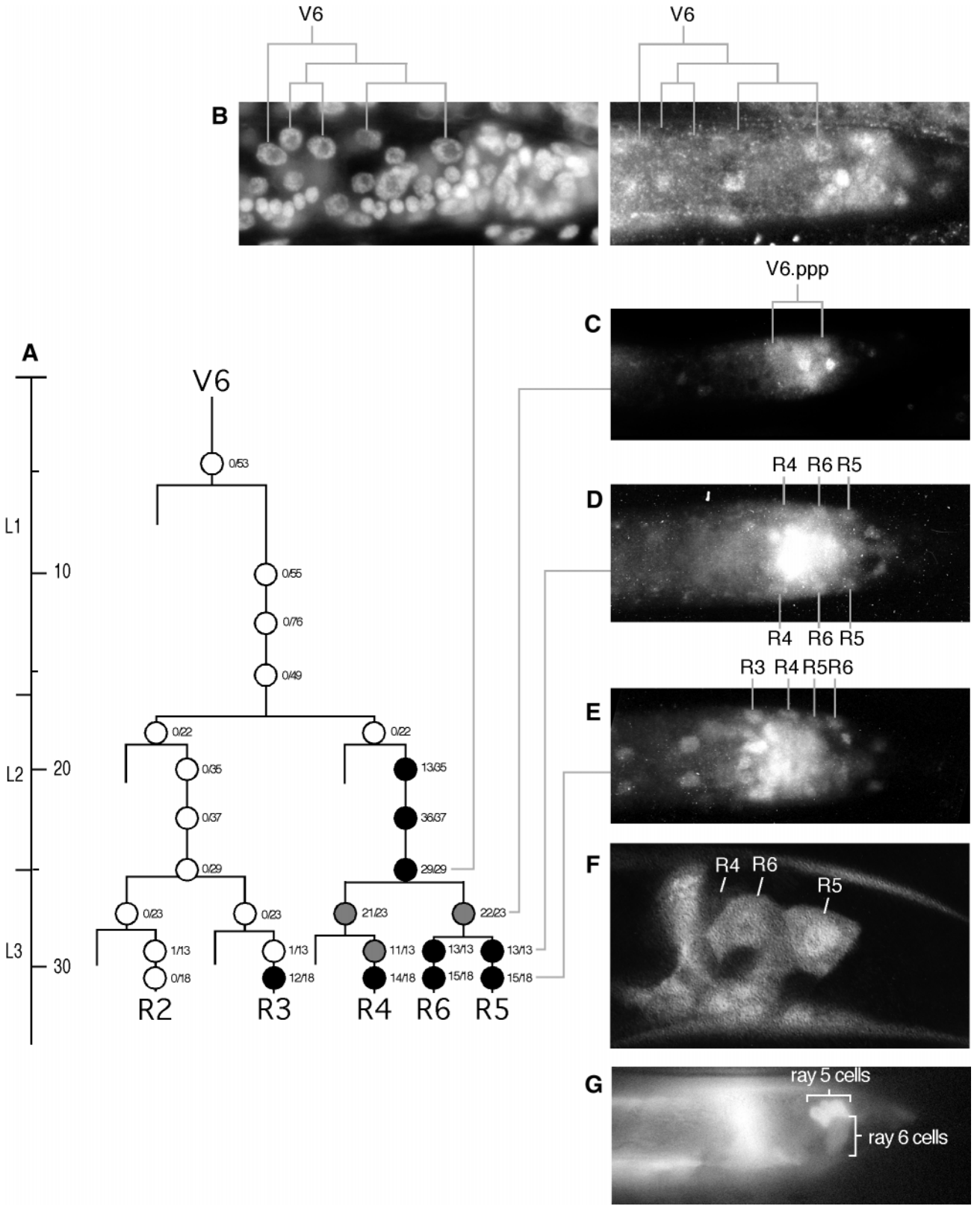

FIG. 4. Expression of EGL-5 in the male V6 lineage. (A) EGL-5 antibody staining at various points in the V6 cell lineage. Each circle represents one analyzed time point. Black circles indicate an apparently higher expression level than that indicated by gray circles. The fraction beside each circle represents the number of stained cells over the total number of cells examined. Unlabeled anterior lineage branches are cells that fuse with the hypodermal syncytium and showed no staining. (B–E) Representative photomicrographs at the times indicated on the lineage diagram. (B) Lateral view: left, DAPI nuclear staining; right, immunostaining. (C) Dorsolateral view. (D) Dorsal view. (E) Ventrolateral view. (F and G) *egl-5::gfp* reporter expression in the ray sublineages. F, 30 h posthatch; G, 35 h posthatch.

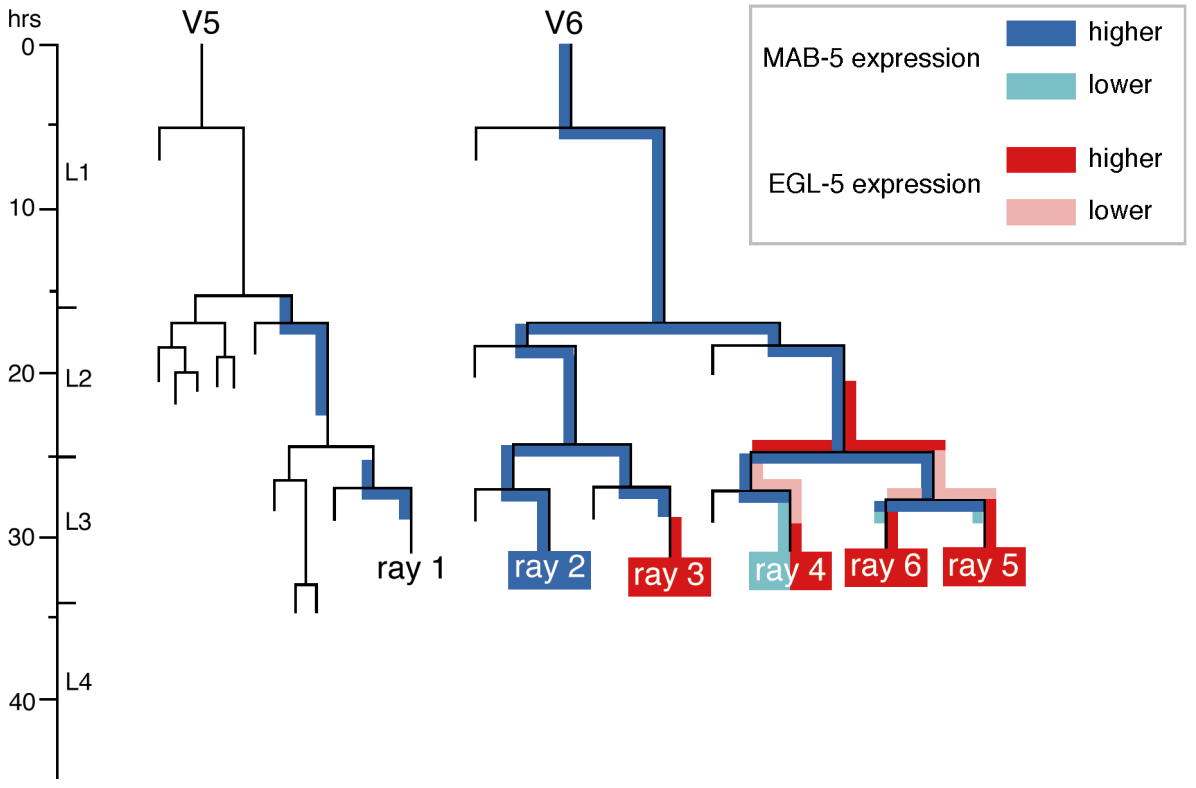

FIG. 5. MAB-5 and EGL-5 expression in the male V5 and V6 lineages. The MAB-5 expression pattern is from Salser and Kenyon (1996). Expression in ray sublineages, not shown in the lineage diagrams, is indicated as colored boxes.

In an *egl-5* null mutant, rays 2–5 cluster together at the position of ray 2, suggesting that all these rays have adopted the identity of ray 2 (Chisholm, 1991; Chow and Emmons, 1994). This phenotype is consistent with the observation that in wild type, ray 2 is the only ray in the V6 lineage that does not express EGL-5. *egl-5* might therefore be necessary for V6-rays to take an identity different from ray 2 identity. It is also possible that *egl-5* functions earlier in the lineage, in V6.ppp, to cause this cell to take a pathway different from V6.pap, resulting in generation of rays 4–6 instead of rays 2 and 3.

Role of egl-5 in Expression of the Ray Sublineage

In addition to roles in generation of ray 6 and in determination of ray morphological identities, a broader role for *egl-5* in expression of the ray sublineage was suggested by an earlier observation. In *mab-5* mutants, all V-rays are absent because the ray sublineage is not expressed (Kenyon, 1986). This loss of the ray sublineage can be rescued by expression of a *mab-5* transgene during L1 (Salser and Kenyon, 1996). However, transgene rescue only occurred in an *egl-5(+)* background. In a *mab-5 egl-5* double mutant, L1 expression of MAB-5 from a transgene was incapable of rescuing the ray defect. Thus early action of *mab-5* appears to potentiate a late action of *egl-5*, which results in expression of the ray sublineage.

There was a possibility that this inferred role of *egl-5* in expression of the ray sublineage after heat-shock induction of a *mab-5* transgene did not accurately reflect the role of *egl-5* in wild type. However, we obtained further evidence in support of a role of *egl-5* in expression of the ray sublineage by examination of *egl-5*;*lin-32* double mutants. *lin-32* encodes a bHLH transcription factor necessary for expression of the ray sublineage (Zhao and Emmons, 1995). In weak alleles of *lin-32*, such as *lin-32(e1926)*, approximately 80% of the V-rays are lost. However, in *egl-5*;*lin-32(e1926)* double mutants, there are no V-rays [genotype, *egl-5(n486);lin-32(e1926)*, n = 100 sides; in an *egl-5* background, rays are scored as papillae during late L4; T-ray frequency (20%) was unaffected]. Therefore the V-rays expressed in a *lin-32(e1926)* background are dependent on *egl-5(+)* function. This can explain why the rays that do form in a *lin-32* hypomorph are from the branches that express EGL-5 (Fig. 5) (Zhao and Emmons, 1995).

If *egl-5* causes expression of the ray sublineage, why are all V-rays present, with the exception of ray 6, in an *egl-5* mutant? Possibly expression of MAB-5 late in the V6 lineage is sufficient to induce the ray sublineage. Alterna-

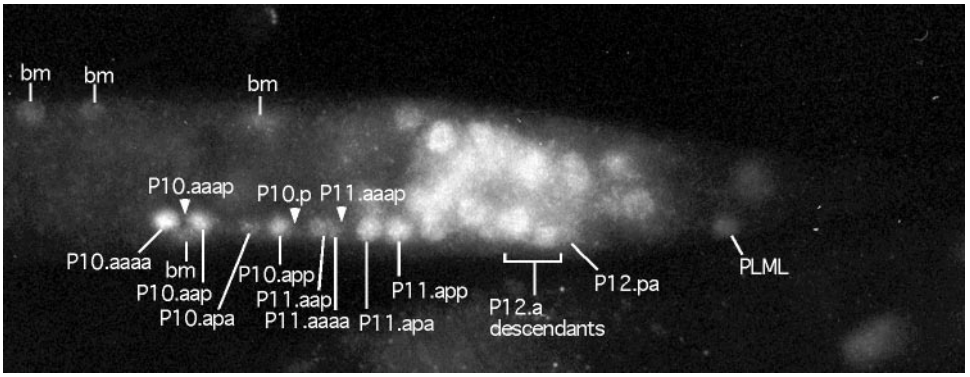

FIG. 6. Expression of EGL-5 in P10 and P11 lineages of *mab-5* mutants. Shown is anti-EGL-5 staining of a 25 h posthatch *mab-5(e1239)* male. Compare the immunostaining of P10 and P11 descendants in the ventral chord and preanal ganglion to Fig. 2C. Three cells that showed no staining are indicated by arrowheads.

tively, in an *egl-5* mutant, expression of *mab-5* may not be turned off in the ray 3 and ray 5 lineage branches, as it is in wild type (see Fig. 5 and Discussion).

Regulatory Interactions with the Antennapedia Homolog mab-5

The loss of all V-rays in a *mab-5* mutant in spite of the apparent role of *egl-5* in generating some V-rays discussed above would be explained if one function of *mab-5* is to turn on *egl-5*. Such a regulatory relationship is also suggested by the *egl-5(+)* requirement for rescue of *mab-5(−)* by a *mab-5* transgene (Salser and Kenyon, 1996). Indeed, we found after immunostaining of *mab-5(e1239)* males, 0/42 L2 (V6.ppp stage) and 0/18 L3 (Rn stage) animals showed EGL-5 staining in seam cells. Thus *mab-5* is required for expression of *egl-5* in the seam. EGL-5 staining in *mab-5* appeared normal in all other tissues with one exception, the ventral hypodermis. Therefore, *mab-5* is not necessary for establishment or regulation of *egl-5* expression during embryogenesis or postembryonically in the gonadal and muscle cell lineages.

In the ventral hypodermis a regulatory relationship between *mab-5* and *egl-5* was found that was the opposite of that in the seam: *mab-5* repressed *egl-5*. In wild type, MAB-5 is present in descendants of P7–P11 (Salser *et al.*, 1993), whereas EGL-5 is present in descendants of P12 (this work). In a *mab-5* mutant, not only was EGL-5 expressed in the descendants of P12, as in wild type, but also in descendants of P10 and P11, in both males ($n = 73$) and hermaphrodites ($n = 33$) (Fig. 6). Therefore, *mab-5* is necessary for preventing expression of *egl-5* in these cells.

DISCUSSION

The Ancestral Function of the Abd-B Paralog Group

From the overall characteristics of *egl-5* expression and function described here and in earlier studies it appears that *egl-5* has many properties in common with *Hox* genes in other animals. e*gl-5* is initially turned on in adjacent, lineally unrelated cells in a restricted region of the posterior embryo. Once turned on, it remains on throughout development. It is expressed in and necessary for differentiation of a variety of mesodermal, epidermal, and neuronal cells. Origin in one body region, continuous expression, and lack of association with any particular cell fate suggest that *egl-5* function can be thought of as specifying a positional cell identity, a primary characteristic of *Hox* genes. As for *Hox* genes in other animals, a variety of cross-regulatory interactions between *egl-5* and the next most anterior *Hox* gene, *mab-5*, help to establish the expression domains of these two proteins. From these results it does not appear, at least with respect to *egl-5*, that *C. elegans* differs greatly from other metazoans in the way it utilizes *Hox* genes to pattern body regions.

The most striking characteristic that *egl-5* shares with *Abd-B* orthologs in other animals is its role in differentiation of the proctodeal region. This similarity, together with the more broadly conserved characteristics discussed above, supports the conclusion that *egl-5* is a true ortholog of *Abd-B* and the members of the vertebrate *Hox* gene posterior paralog groups. Assignment of *egl-5* to the *Abd-B* family is not obvious by sequence similarity alone. In spite of sequence similarity to *Drosophila Abd-B*, *egl-5* shares no characteristic residues with the vertebrate HOX10 to HOX13 consensus sequence (Sharkey *et al.*, 1997). In the phylogenetic analysis of homeodomains of Zhang and Nei (1996), *egl-5* falls as an outgroup to all the other *Hox* genes. The presence of 39 unrelated genes separating the four *C. elegans Hox* genes, and the inverted chromosomal order of two of the genes, might lead one to question whether it is reasonable to even speak of a *Hox* gene cluster in *C. elegans* at all. However, both the interposed genes and the inverted gene order can be explained by a single inversion. We believe the present data support the assumption that the *C. elegans Hox* genes are cognates of *Hox* cluster genes of other animals.

Like *Abd-B* orthologs of arthropods and vertebrates, *egl-5* is strongly expressed in the region around the anus, including the epithelium forming the junction between the hindgut and external epidermis, and in the genital primordia (Celniker, *et al.*, 1990; Delorenzi and Bienz, 1990; Kelsh *et al.*, 1993; Dollé *et al.*, 1991a,b; Roberts *et al.*, 1995; Sordino *et al.*, 1996; van der Hoeven *et al.*, 1996; Warot *et al.*, 1997; Kondo *et al.*, 1997; Mortlock and Innis, 1997). *Abd-B* function in this region is necessary for differentiation of the analia and genitalia (Chisholm, 1991; Celniker, *et al.*, 1990; Dollé *et al.*, 1991a; Warot *et al.*, 1997; Kondo *et al.*, 1997; Mortlock and Innis, 1997). Such phylogenetic conservation suggests that directing differentiation of the proctodeal region may have been the ancestral function of the founder of the *Abd-B* paralog group (van der Hoeven *et al.*, 1996).

Conserved expression and function of the *Abd-B* paralog group genes in the proctodeal region may have implications for the evolution of the *Hox* cluster (van der Hoeven *et al.*, 1996). Phylogenetic reconstruction of the relationships among *Hox* gene homeodomain amino acid sequences have indicated that the terminal genes of the cluster, the *labial* and *Abd-B* paralogs, are the most distantly related and that genes internal to the cluster likely arose at successively later times by gene duplication (Zhang and Nei, 1996; Martínez *et al.*, 1998). Thus the earliest form of the *Hox* cluster might have consisted of the *labial* and *Abd-B* paralogs. A primitive animal with a mouth and anus necessarily has two axial pattern elements: an anterior junction between ectodermal and endodermal tissues and a posterior junction (van der Hoeven *et al.*, 1996). The respective functions of the two *Hox* genes in a primitive *Hox* gene cluster might have been to transcribe genes for constructing the heterotypic cell junctions at the two ends of the digestive tract. Such a model would account for the essential characteristic of *Hox* genes, that they are expressed across tissue types in a single body region. Later, additional *Hox* genes that became available by gene duplication could be recruited to add internal pattern elements.

Other sites of EGL-5 expression and function are also consistent with a role in expression of cell recognition or adhesion molecules specific to the posterior region. In the gonad, EGL-5 is expressed in cells that migrate toward the posterior. In *egl-5* mutants, this migration is incomplete and the gonad fails to form a connection at the presumptive cloaca (Chisholm, 1991). The function of *egl-5* in this tissue could therefore be in either or both cell migration and attachment. However, *egl-5* also plays a role in the gonadal cell lineage, and the migration and morphogenesis defects could be secondary. Likewise, sex muscles that express EGL-5 are misattached in *egl-5* mutants, and many neurons in the posterior ganglia are miswired (Chisholm, 1991).

Role of egl-5 in Patterning the Rays

In addition to its role in the proctodeal region, gonad, and sex muscles, *egl-5* is necessary for patterning of the posterior lateral and ventral neuroepithelia. The axially repeated patterns of postembryonic cell lineages and differentiated cell types generated by these two tissues have been likened to the segments of more overtly segmented animals (Sulston *et al.*, 1983; Hedgcock and Hall, 1990). As in other animals, these *C. elegans* "segments" are patterned by *Hox* genes (Kenyon, 1986; Wang *et al.*, 1993; Salser *et al.*, 1993; Chow and Emmons, 1995). EGL-5 is expressed in and required for the differentiation of descendants of the penultimate lateral blast cell, V6, and the most posterior of the ventral epidermal blast cells, P12.

The function of *egl-5* in the lateral epidermis is to pattern the male sensory rays. Rays may be discriminated by their positions, morphologies, and the neurotransmitters expressed by their constituent neurons. The particular set of properties characteristic of a given ray may be viewed as defining a ray identity. Mutations in or misexpression of *mab-5* or *egl-5* result in posterior-to-anterior or anterior-to-posterior transformations in the morphological properties of subsets of the rays (Chow and Emmons, 1995; Salser and Kenyon, 1996). One result of such ray identity transformations is fusion of adjacent rays. Ray fusions are evidence that *egl-5* and *mab-5* directly or indirectly govern the expression of cell recognition or cell adhesion molecules in the rays. Mutation in *egl-5* also results in loss of the neurotransmitter dopamine from ray 5 (R. Lints and S. W. Emmons, unpublished). Thus *egl-5* governs expression of multiple ray properties, including the properties of ray neurons.

MAB-5 and EGL-5 expression overlaps in ray 4. Otherwise, the expression pattern of these genes in the rays is complementary (Fig. 5). From this pattern, it is possible to suggest a *Hox* code for ray identity: ray 1, *mab-5* OFF, *egl-5* OFF; ray 2, *mab-5* ON, *egl-5* OFF; ray 3, *mab-5* OFF, *egl-5* ON; ray 4, *mab-5* ON, *egl-5* ON; ray 5 and ray 6, *mab-5* OFF, *egl-5* ON. Transformations in ray identities observed previously in genetic studies are consistent with such a code (Chow and Emmons, 1995; Salser and Kenyon, 1996; Emmons, 1998). Additional functions are necessary to make the code complete, so as to specify up to nine unique ray identities. In other systems, *Hox* genes are known to pattern elements of the nervous system, including the properties of individual neurons, along the anteroposterior body axis (Lumsden and Krumlauf, 1996; Tanabe and Jessell, 1996). Possibly further studies of the specification of ray identities by *mab-5* and *egl-5* will shed light on the mechanisms underlying the role of *Hox* genes in the nervous system.

Establishment of egl-5 Expression and Interactions with the Hox Gene mab-5

Establishment of the *Hox* gene expression pattern during development is a primary step in the process that defines the morphology of an animal. For most of the cells and cell lineages where EGL-5 is expressed, we do not know what transcription factors or combinations of transcription factors are responsible for initiation of transcription at the *egl-5* promoter. In the seam, MAB-5 is necessary and could act by direct binding at the *egl-5* promoter. However,

elsewhere, establishment of *egl-5* expression did not require a functional *mab-5* gene. Hence it appears the *C. elegans Hox* gene expression pattern is not generally established by a cascade of positive interactions between *Hox* genes (Faiella *et al.*, 1994; Gould *et al.*, 1997). In most lineages, transcription factors of other classes must activate the *egl-5* promoter. In the posterior, ventral neuroectoblast P12, *egl-5* expression is activated as a consequence of the combined actions of an epidermal growth factor signaling pathway and a Wnt signaling pathway (Jiang and Sternberg, 1998). In the embryo, expression of an *egl-5::lacZ* reporter was eliminated or reduced by a mutation in *vab-7,* which encodes a *C. elegans* homolog of *Drosophila even-skipped* (*eve*) (Ahringer, 1996). Therefore, VAB-7 may help to activate the *egl-5* promoter in embryonic muscle lineages.

We found that once *egl-5* is turned on in a tissue, it remained on in that tissue throughout development. Thus *egl-5* is not subject to extensive postestablishment regulation, similar to that affecting *mab-5* and some other *Hox* genes (Salser and Kenyon, 1996; Mann, 1994; Castelli-Gair *et al.*, 1994; Castelli-Gair and Akam, 1995). Possibly *egl-5* transcription is maintained by an autoactivating feedback loop, as it is for some other *Hox* proteins (Pöpperl *et al.*, 1995).

Because the *Hox* genes act to define alternative domains of the body, it is expected that their domains of expression or action will be closely coordinated. Cross-regulatory interactions or prevalence of action of one *Hox* protein over another have been extensively documented in other animals (McGinnis and Krumlauf, 1992; Krumlauf, 1994). Negative interactions between *egl-5* and the next most anterior *C. elegans Hox* gene, *mab-5,* help to define the expression patterns of these two genes in the ventral ectoderm. In the ventral neuroectoblasts P10 and P11, *mab-5* represses *egl-5* (this work). In the ventral neuroectoblast P12, *egl-5* represses *mab-5* (Salser *et al.*, 1993). Thus mutual, negative, cross-regulatory interactions between these two genes serve to establish alternative expression domains in the ventral neuroectoderm. We suggest that *egl-5* may similarly negatively regulate *mab-5* in the seam, thus accounting for the somewhat complementary pattern of EGL-5 and MAB-5 in the V-ray lineages. It is not known whether these negative interactions are due to direct action of one of these *Hox* proteins at the promoter of the other, but this remains a likely possibility.

Future Problems: Regulation and Targets

For *Hox* genes, as for other regulatory transcription factor genes, understanding their action and function during development involves understanding their transcriptional regulation and determining their effects on gene targets. These two problems are interrelated, because one regulatory transcription factor gene is the target of another and because many transcription factors regulate their own promoters. Indeed, understanding the succession of cell states that eukaryotic cells pass through during tissue and cell fate specification involves understanding the cascade of transcription factor activation steps that leads to the establishment of a succession of distinct transcriptional cell states. We have shown that generation of cells characterized by different expression patterns of the two *Hox* proteins EGL-5 and MAB-5 is essential for establishment of differing ray identities in the postembryonic seam lineages (Emmons, 1998). Likewise, many of the transcription factors that define the alternative states of early *C. elegans* blastomeres have been identified (Schnabel and Priess, 1997). How transcriptional cascades work is a central problem in understanding development.

A key element in understanding transcription factor cascades is understanding how specific promoters are activated by combinations of transcription factors. The modification of transcription factor action by cell-specific factors is illustrated at many points in the function and expression of EGL-5. For example, *egl-5* is activated by *mab-5* in one cell and repressed by *mab-5* in another. If both these effects are direct, it means the sign of the action of MAB-5 at the *egl-5* promoter can be reversed by cell-specific cofactors (Biggin and McGinnis, 1997). In the B cell, while EGL-5 is expressed in both sexes, it has a profound developmental role only in the male. Apparently, the action of EGL-5 at target promoters in this cell is affected directly or indirectly by the state of activation of the terminal sex determination transcription factor TRA-1. Activation of *egl-5* by *mab-5* in the seam lineage is regulated by sexual, temporal, and spatial factors. MAB-5 is present in the V6 lineage continuously until mid-L3, yet it turns on *egl-5* expression at a precise time during L2. This activation event occurs in only one branch of the cell lineage. Furthermore, it occurs only in the male. This action of *mab-5* is therefore modulated by temporal factors, such as the products of the heterochronic genes (Ambros, 1997; Antebi *et al.*, 1998), and spatial factors, such as those regulated by the action of asymmetrical cell signals (e.g., Jiang and Sternberg, 1998; Lin *et al.*, 1998), as well as by the sex determination pathway. Understanding how inputs from this variety of developmental coordinates are integrated at target promoters is fundamental to understanding the specification of cell fates during development.

ACKNOWLEDGMENT

We thank members of the Emmons laboratory for their comments on the manuscript. This work was supported by a grant from the NIH (GM39353) to S.W.E. H.B.F. was a recipient of a Brazilian Research Council (CNPq) Postdoctoral Fellowship. Some nematode strains used in this work were received from the *Caenorhabditis* Genetics Center, which is funded by a grant from the NIH National Center for Research Resources. S.W.E. is the Siegfried Ullmann Professor of Molecular Genetics.

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Received September 16, 1998
Accepted October 14, 1998