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PAG-3, a Zn-finger transcription factor, determines neuroblast fate in *C. elegans*

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SUMMARY

During *Caenorhabditis elegans* development, the patterns of cell divisions, cell fates and programmed cell deaths are reproducible from animal to animal. In a search for mutants with abnormal patterns of programmed cell deaths in the ventral nerve cord, we identified mutations in the gene *pag-3*, which encodes a zinc-finger transcription factor similar to the mammalian Gfi-1 and *Drosophila* Senseless proteins. In *pag-3* mutants, specific neuroblasts express the pattern of divisions normally associated with their mother cells, producing with each reiteration an abnormal anterior daughter neuroblast and an extra posterior daughter cell that either terminally differentiates or undergoes programmed cell death, which accounts for the extra cell corpses seen in *pag-3* mutants. In addition, some neurons do not adopt their normal fates in *pag-3* mutants. The phenotype of *pag-3* mutants and the expression pattern of the PAG-3 protein suggest that in some lineages *pag-3* couples the determination of neuroblast cell fate to subsequent neuronal differentiation. We propose that *pag-3* counterparts in other organisms determine blast cell identity and for this reason may lead to cell lineage defects and cell proliferation when mutated.

Key words: Cell lineage, Neuroblast fate, Programmed cell death, *C. elegans*

INTRODUCTION

The known and essentially invariant cell lineage of the nematode *Caenorhabditis elegans* has provided a basis for analyzing at single-cell resolution the mechanisms that establish cell lineages and cell fates during metazoan development (Sternberg and Horvitz, 1984). Programmed cell death is a major aspect of animal development, and abnormalities in programmed cell death contribute to a variety of human diseases (reviewed by Lockshin et al., 1998). Genetic and molecular analyses of *C. elegans* have defined a pathway for programmed cell death (Metzstein et al., 1998). This pathway is extensively conserved between animals (Horvitz, 1999; Ranger et al., 2001). How the process of programmed cell death is controlled during development is less well understood. Two genes, *ces-1* and *ces-2*, regulate programmed cell death in *C. elegans* and have mammalian homologs with conserved functions (Ellis and Horvitz, 1991; Inaba et al., 1996; Inukai et al., 1999; Metzstein et al., 1996; Metzstein and Horvitz, 1999). Mutations that affect the mammalian homolog of *ces-2* can cause leukemia in children, perhaps in part by rendering malignant lymphoblasts resistant to programmed cell death (Inaba et al., 1996).

To define additional genes that control the programmed deaths of specific cells in *C. elegans* we have sought mutations that alter the pattern of cell deaths in the ventral nerve cord. A newly hatched *C. elegans* larva contains 22 motoneurons in its ventral cord and associated ganglia (Sulston and Horvitz, 1977; White et al., 1986). During postembryonic development, 12 ventrolateral P blast cells migrate into the ventral cord and undergo a series of divisions. The divisions of the P cells, together with those of a 13th blast cell, W, produce 65 neural cells, 10 of which undergo programmed cell death (Sulston and Horvitz, 1977) (Fig. 1). Seven of the 10 cell deaths are of cells at equivalent positions in the W and P cell lineages. Specifically, the six Pn.aap (Pn.aap, the posterior daughter of the anterior daughter of the anterior daughter of any P cell) cells derived from the P lineages in the anterior and posterior ventral cord (P1, P2 and P9-P12) and W.ap, a cell lineally equivalent to the Pn.aap cells, undergo programmed cell death; the six Pn.aap cells in the midbody (from P3-P8) survive and differentiate to become VC motoneurons.

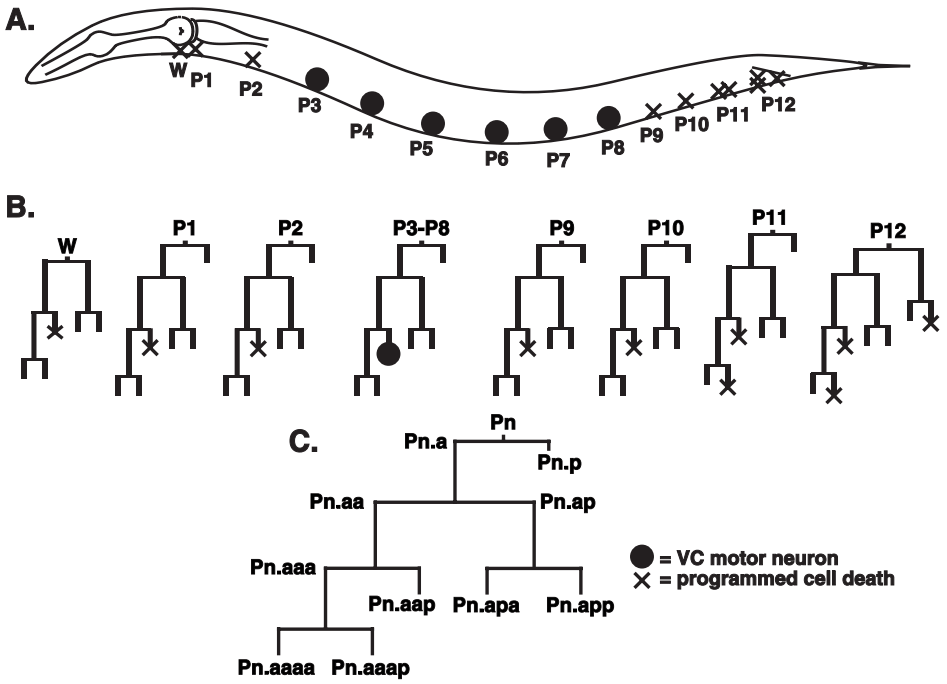

Fig. 1. Postembryonic development of the ventral nerve cord in *C. elegans*. (A) X: the positions of the ten cells that undergo programmed cell death. Filled circles indicate the six cells in the midbody that survive and differentiate to become VC motoneurons. Anterior is leftwards and ventral is downwards. (B) The postembryonic cell lineages are shown schematically, with vertical lines representing time and horizontal lines representing a cell division along the anteroposterior body axis. Each P cell divides to produce an anterior daughter, Pn.a, which is a neuroblast, and a posterior daughter, Pn.p, which is hypodermal. W, a neuroblast in the retrovesicular ganglion, follows a division pattern identical to that of Pn.a cells. (C) The nomenclature used to identify cells in the P cell lineages. Cell lineages are from Sulston and Horvitz (Sulston and Horvitz, 1977).

In our search for mutants abnormal in ventral cord cell deaths, we have identified alleles of the gene *pag-3*. We describe the effects of mutations in *pag-3* on programmed cell deaths in the ventral nerve cord and the role of *pag-3* in the determination of neuroblast cell fate and in neuronal differentiation.

MATERIALS AND METHODS

Isolation of *pag-3(n3098)*

ced-1 hermaphrodites were mutagenized with ethylmethane sulfonate (Brenner, 1974). A synchronized population of F2 progeny was examined as late L2/early L3 larvae using Nomarski microscopy (Sulston and Horvitz, 1977). Animals with abnormal numbers or patterns of cell corpses in the ventral cord were recovered from the slide for analysis. A single allele of *pag-3, n3098*, was isolated after screening approximately 9000 animals, or 4500 mutagenized haploid genomes.

Strains, alleles and cell lineage analysis

All strains were maintained at 20°C as described by Brenner (Brenner, 1974). Cell nomenclature and cell lineage analysis were as described by Sulston and Horvitz (Sulston and Horvitz, 1977), except that 'P0.a' is now known as W (Sulston et al., 1983). Unless otherwise indicated, mutations used are cited by Riddle et al. (Riddle et al., 1997). Mutations used were:
LGI, *ced-1(e1735)*;
LGII, *wdIs4* (the integrated $P_{unc\text{-}4}$*gfp* plasmid pNE-1, generously provided by David Miller);
LGIII, *wdIs1* (the integrated $P_{unc\text{-}4}$*lacZ* plasmid pNC4-22Lz) (Miller et al., 1992), *lin-39(n1760), mab-5(e1239)* and *egl-5(n945)*;
LGIV, *ced-3(n717)*;
LGV, *oyIs14* (Hao et al., 2001) kindly provided by T. Sarafi-Reinach and P. Sengupta;
LGX, *nIs106* (Reddien et al., 2001), *lon-2(e678); pag-3(ls64, ls20)* (Jia et al., 1996), *lin-15(n765)* and *mnDp1 X;V/+;mnDf19* (Meneely and Herman, 1979).

Construction and analysis of *pag-3/Df* animals

ced-1(e1735) males were mated with *mnDp1/+; mnDf19* hermaphrodites, and non-Unc (*mnDp1*-carrying) cross progeny males were mated with *ced-1(e1735); dpy-17(e164) unc-32(e189); pag-3* hermaphrodites. Non-Dpy non-Unc-32 Pag-3 hermaphrodites were picked as late L2 Unc larvae, and the patterns of cell corpses in the ventral cord of Ced animals examined using Nomarski optics. Similar results were obtained with a second deficiency, *mnDf43*, which also deletes the *pag-3* locus (Meneely and Herman, 1979). To construct *pag-3/pag-3/+* animals, crosses were performed as above, except non-Dpy non-Unc cross progeny males were mated with *ced-1; dpy-17 unc-32; pag-3* hermaphrodites, and non-Dpy non-Unc Ced animals were scored.

Antibody preparation and staining of fixed animals

A DNA fragment encoding the Zn-finger domains of PAG-3 (amino acids 124-333, PAG-3ZC) was amplified from the *pag-3* cDNA (Jia et al., 1997) and cloned into pET21b(+) (Novagen) to construct a HIS-tagged fusion protein. The expressed protein was partially purified using the method described by the manufacturer.

PAG-3 antibodies were purified from rabbit serum as described (Koelle and Horvitz, 1996). The specificity of the antiserum was demonstrated by recognition on western blots of an approximately 33 kDa band in *E. coli* expressing PAG-3ZC and a 47 kDa band in extracts of wild-type *C. elegans*. Fixed *pag-3(n3098)* animals did not stain with the antiserum, but *dpy-17(e164)* mutants fixed in the same tube did stain. For whole-mount staining, animals were fixed with Bouin's fixative as described (Nonet et al., 1997). Primary antiserum was diluted 1:50 for staining and detected with FITC- or Texas Red-conjugated goat anti-rabbit secondary antibody. For experiments with PAG-3 antiserum and the $P_{unc\text{-}4}$*lacZ* construct *wdIs1* co-staining, β-galactosidase was detected in fixed animals using a 1:500 dilution of a α-*lacZ* monoclonal antibody (Promega). FMRFamide staining was done as described (Li and Chalfie, 1990), using collagenase for permeabilization. Antiserum against FMRFamide was kindly provided by Chris Li.

RESULTS

Mutations in *pag-3* affect the pattern of cell deaths in the ventral cord

To visualize the pattern of programmed cell deaths in the *C. elegans* ventral cord, we used a mutation in the gene *ced-1*, which encodes a receptor that allows engulfing cells to recognize dying cells (Zhou et al., 2001). In *ced-1* mutants cell corpses persist rather than being engulfed and degraded and are easily detected using Nomarski differential interference contrast microscopy (Hedgecock et al., 1983). We mutagenized *ced-1* hermaphrodites with ethylmethane sulfonate (Brenner, 1974) and examined the patterns of cell corpses in the ventral nerve cords of F2 progeny.

In our screen, we identified a mutation, *n3098*, that recessively conferred an increased number of cell corpses in the anterior ventral cords of many animals (Fig. 2). In the posterior ventral cord, some individual *n3098* mutants had an increased number of cell corpses, while others had fewer than the normal number of corpses. Not all postembryonic cell deaths were affected by *n3098*, as the number of corpses generated by the Q neuroblasts (Sulston and Horvitz, 1977) was normal (1.6±0.5 versus 1.7±0.5 and 1.3±0.1 versus 1.4±0.1 for *ced-1* versus *ced-1; n3098* mutants for QL and QR, respectively).

The additional corpses seen in the ventral cords of *n3098* mutants resulted from programmed cell deaths, as mutations in the *ced-3* gene, which encodes a caspase required for programmed cell death in *C. elegans* (Yuan et al., 1993), prevented their appearance (data not shown). *n3098* mutants had sluggish and uncoordinated movement. *ced-3; n3098* mutants (but not *ced-3* mutants) were also sluggish and uncoordinated, indicating that the movement defect was not caused by the deaths of ventral cord motoneurons required for normal movement. The *n3098* mutation mapped to the right arm of LG X. The movement defect of *n3098* mutants and the map location of *n3098* suggested it might be an allele of the gene *pag-3*, originally identified in mutants that overexpressed a $P_{mec\text{-}7}$*lacZ* transgene in the BDU neurons (Jia et al., 1996). *pag-3* is predicted to encode a 336 amino acid C2H2 zinc-finger protein with greater than 80% amino acid identity in its five Zn-finger domains to the mammalian Gfi-1 and Gfi-1B proteins (Jia et al., 1997; Tong et al., 1998). Genetic mosaic analysis suggests that *pag-3* is required in the nervous system for normal movement (Jia et al., 1996; Jia et al., 1997).

We found that *n3098* mapped to the region of *pag-3* and failed to complement the movement defect of *pag-3(ls20)* mutants. In addition, in a *ced-1* background, *pag-3(ls20)* mutants, like *n3098* mutants, had an abnormal pattern of cell corpses in the ventral cord (Fig. 2). *ced-1; pag-3(ls20)* mutants had more corpses in the ventral cord than did *ced-1; pag-3(n3098)* mutants, suggesting that at least one of these alleles is not null. Analysis of the phenotype of mutants heterozygous for the *pag-3(n3098*) and *pag-3(ls20*) alleles in *trans* to chromosomal deficiencies that delete the *pag-3* locus suggested that *n3098* is a strong loss-of-function or null allele (Fig. 2). We determined the sequence of *pag-3* genomic DNA from *pag-3(n3098)* mutants and identified a G-to-A mutation changing the codon for tryptophan at amino acid 113 to an opal stop codon, which is located before the sequences encoding the Zn-finger domains of PAG-3. *pag-3(ls20)* homozygotes had

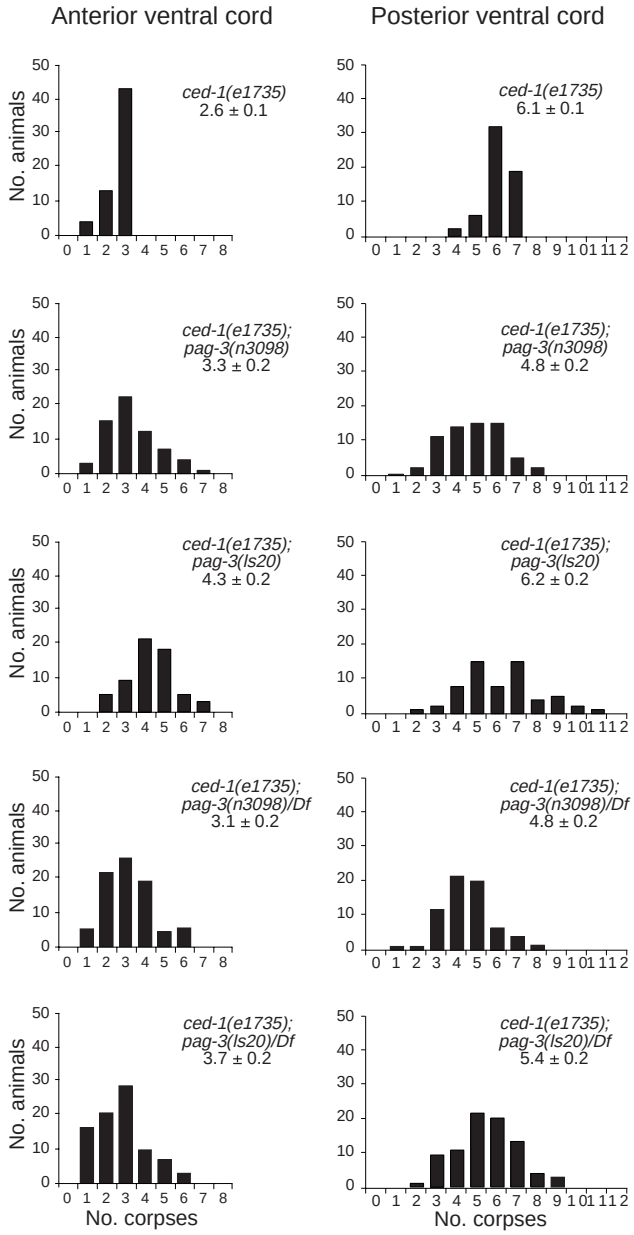

Fig. 2. Numbers of cell corpses in *pag-3* mutants. The numbers of cell deaths in *pag-3* mutants was assayed by counting cell corpses in the ventral cords of 60-65 early L3 *ced-1; pag-3* hermaphrodites using Nomarski microscopy. The difference between the observed number of corpses in *ced-1* mutants and the number of deaths that occur in the corresponding lineages of developing animals can be accounted for by an incomplete block in engulfment of cell corpses in the *ced-1* mutants and by a role for engulfment in cell killing (Ellis et al., 1991; Reddien et al., 2001). In *pag-3* mutants, no cell corpses were observed in regions of the ventral cords that lack cell deaths in wild-type animals. For the analysis of *pag-3* alleles, we constructed strains heterozygous for the indicated *pag-3* alleles and for a small chromosomal deficiency (*Df*) which deletes the *pag-3* locus (see Materials and Methods). Using an unpaired Student's *t*-test, we found the differences in mean corpse number±s.e.m. between *pag-3(ls20)* and *pag-3(ls20)/Df* to be significant ($P<0.02$ for both anterior and posterior ventral cord data), while those between *pag-3(n3098)* and *pag-3(n3098)/Df* were not ($P<0.40$ and $P<0.77$, anterior and posterior, respectively). Anterior ventral cord, cell corpses generated by W, P1 and P2; posterior ventral cord, corpses generated by P9-P12.

more corpses than did *pag-3(ls20)*/*Df* mutants, suggesting that *pag-3(ls20)*, while generally acting as a strong loss-of-function allele, may cause abnormalities in addition to those resulting from a complete loss of *pag-3* function (e.g. by a chromosomal deficiency that spans the *pag-3* locus). *pag-3(ls20)/pag-3(+)* and *pag-3(ls20)/pag-3(ls20)/pag-3(+)* mutants (the latter wild-type allele was provided on an attached chromosomal duplication – see Materials and Methods) had a wild-type pattern of cell corpses. The *ls20* allele is an H228Y mis-sense mutation affecting the fourth Zn finger (Jia et al., 1997). The corresponding Zn finger in Gfi-1 is essential for DNA binding (Zweidler-Mckay et al., 1996), consistent with our hypothesis that the PAG-3(ls20) protein interacts abnormally with DNA or other proteins and interferes with the function of a multiprotein complex in a way that is recessive to a single wild-type allele of *pag-3*. Taken together, our data indicate that mutations in *pag-3* can affect the pattern of cell corpses in the ventral nerve cord of *C. elegans* and that *pag-3(n3098)* is a strong loss-of-function or null allele.

Differentiation of the BDU interneurons in *pag-3(n3098)* mutants is probably abnormal

Previously described mutations in *pag-3* lead to misexpression of touch neuron genes in the BDU interneurons (Jia et al., 1996). We observed that *pag-3(n3098)* mutants similarly expressed an integrated $P_{mec\text{-}7}$*lacZ* reporter in the BDU interneurons, which do not normally express *mec-7* (Jia et al., 1996), and we quantified this effect. In 50 wild-type animals examined carrying the $P_{mec\text{-}7}$*lacZ* reporter, 0/100 BDU interneurons expressed β-galactosidase, whereas 92/100 ALM mechanosensory neurons did so. By contrast, in 50 *pag-3(n3098)* mutants 39/100 BDU and 83/100 ALM neurons expressed β-galactosidase, similar to the previously reported phenotypes of mutants with other mutations in *pag-3* (Jia et al., 1996) (data not shown). The ALM and BDU neurons of wild-type animals and *pag-3(n3098)* mutants were easily identified by their axonal morphologies and nuclear positions, and no animal had more than two ALM or BDU neurons. These data suggest that the misexpression of mechanosensory genes in the BDU interneurons results from a defect in neuronal differentiation, as previously proposed (Jia et al., 1996).

pag-3 is required for cell-fate determination by the Pn.aa neuroblasts

To determine the cause of the abnormalities in the patterns of cell deaths in *pag-3* mutants, we directly observed the postembryonic cell lineages in the ventral cords of developing *pag-3(n3098)* and *pag-3(ls20)* larvae. As shown in Fig. 3, P-cell lineages in *pag-3* mutants had a reiterative cell lineage defect. Specifically, in the P-cell lineages of wild-type animals, the neuroblast Pn.aa divides to produce an anterior daughter Pn.aaa that is itself a neuroblast and a posterior daughter Pn.aap that terminally differentiates or undergoes programmed cell death. Each Pn.aaa neuroblast then divides to produce two terminally differentiated neurons. In *pag-3* mutants the Pn.aaa neuroblasts, rather than producing two terminally differentiated neurons, instead often produced an anterior daughter that was a neuroblast and a posterior daughter that underwent programmed cell death or terminally differentiated with a morphology typical for a neuron.

For example, seven of the nine anterior-most daughter cells (the W.aaa and Pn.aaaa cells) we observed in the W, P1 and P2 lineages of three *pag-3(n3098)* mutants underwent one or two extra rounds of division (Fig. 3B). The anterior-most daughter cells ultimately exited the cell cycle and adopted a nuclear morphology typical of a neuron. The Pn.aaap cells, the posterior daughters resulting from the Pn.aaa divisions, in the W, P1 and P2 lineages of wild-type animals survive to become motoneurons, but in *pag-3(n3098)* mutants instead often underwent programmed cell death, as do the Pn.aap cells in the wild type (Fig. 3B).

We also observed in the midbody and posterior ventral cord of *pag-3(n3098)* mutants extra cell divisions by Pn.aaaa cells and the programmed cell deaths of the P9- and P10.aaap cells, which survive in wild-type animals (Fig. 3B). Compared with the anterior ventral cord cell lineages, extra divisions by Pn.aaaa cells were rare in the more posterior lineages of *pag-3(n3098)* mutants. We observed only a single extra division in the P9-12 lineages of six *pag-3(n3098)* mutants (Fig. 3B). However, in three *pag-3(ls20)* mutants, five of 12 P9-12.aaaa cells underwent one extra round of division (Fig. 3C). The more frequent extra divisions by Pn.aaaa neuroblasts in *pag-3(ls20)* mutants and the increased number of posterior daughter cells generated by these divisions (some of which undergo programmed cell death) can account at least in part for the greater number of cell corpses in the ventral cords of *pag-3(ls20)* compared with *pag-3(n3098)* mutants. The greater likelihood of extra divisions by the Pn.aaaa neuroblasts in the anterior compared with the posterior ventral cord suggests that positional information influences the *pag-3* mutant phenotype.

The patterns of cell divisions and programmed cell deaths we observed are most easily explained by proposing that in *pag-3* mutants, the Pn.aaa neuroblasts adopt the fate of their mothers, the Pn.aa neuroblasts (Fig. 3D). The predicted consequences of this reiterative lineage defect are that the neurons normally generated by the Pn.aaa neuroblasts would be abnormal or absent and that extra Pn.aap-like cells would be produced.

The VA and VB motoneurons are abnormal or absent in *pag-3* mutants

The normal descendants (Pn.aaaa and Pn.aaap) of the Pn.aaa neuroblasts are the VA and VB motoneurons (Sulston and Horvitz, 1977). *pag-3* mutants are defective in both forwards and backwards movement, suggesting that the VA and VB motoneurons, which control backwards and forwards movement, respectively (Chalfie et al., 1985), are defective. We used reporter constructs expressed in the VA and VB motoneurons to test whether these neurons were present in *pag-3* mutants. At hatching and in young L1 larvae, the ventral nerve cord and associated ganglia contain nine DA, seven DB and six DD juvenile motoneurons (White et al., 1986). The postembryonic P cell lineages, which commence during the late L1 stage, add 12 VA, 11 VB and 13 VD motoneurons to the juvenile neurons, as well as additional cells (Sulston and Horvitz, 1977). We used an integrated $P_{unc\text{-}4}$*gfp* transgene (generously provided by David Miller), which is expressed in the DA and VA motoneurons (Pflugrad et al., 1997), to determine the number of VA motoneurons in *pag-3(n3098)* mutants. We counted the number of fluorescent nuclei in L3 larvae carrying the $P_{unc\text{-}4}$*gfp* transgene. From this number,

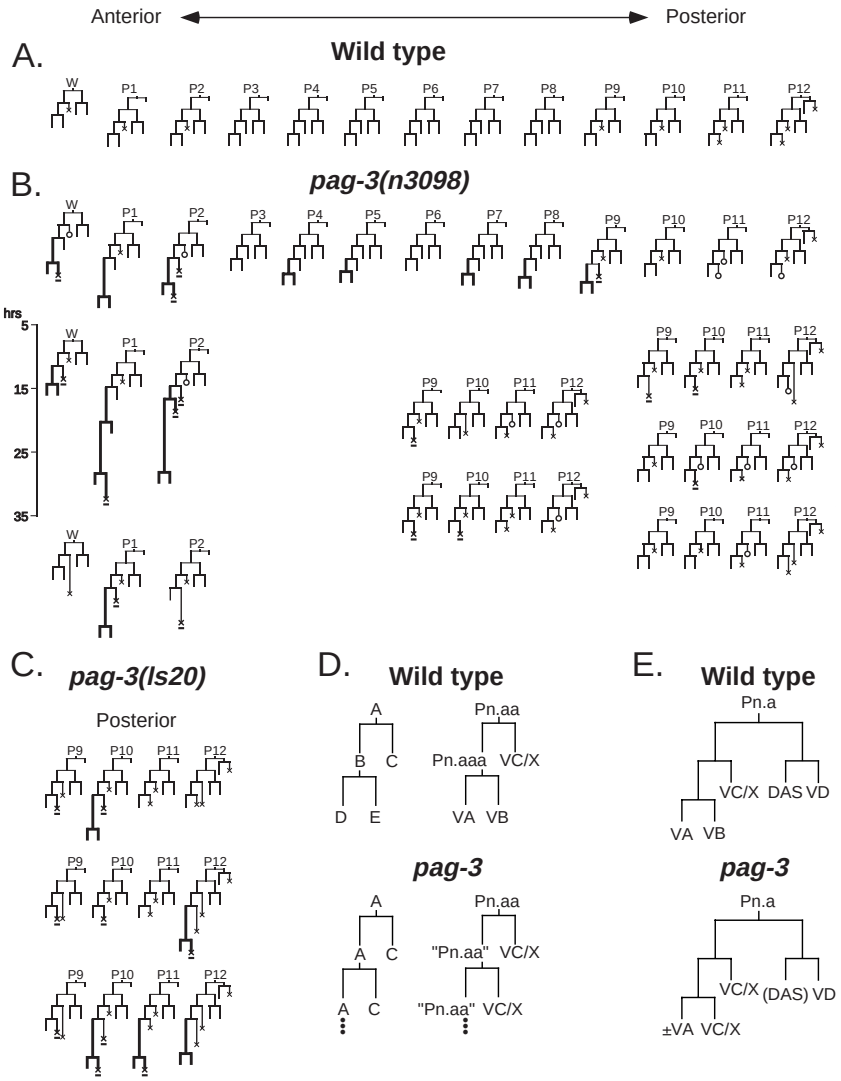

Fig. 3. P cell lineages in *pag-3* mutants. Abnormalities in *pag-3* lineages are indicated by bold lines; surviving cells that die in wild-type lineages are indicated by circles. Cells that die in wild-type animals are indicated by an x; additional cell deaths observed in *pag-3* mutants are indicated by an underlined x. *pag-3* mutants initiated P cell divisions at the same time during development as wild-type animals. The time scale in B applies to all lineages. (A) Lineages of wild-type animals from Sulston and Horvitz (Sulston and Horvitz, 1977). (B) Lineages of *pag-3(n3098)* mutants. (C) P9-P12 cell lineages of three *pag-3(ls20)* mutants. The variability we observed in the division patterns and cell fates in *pag-3* mutants has been observed generally in mutants with cell lineage defects (Sternberg and Horvitz, 1984). In reiterated divisions, the time between mitoses became progressively longer and the morphology during mitosis more abnormal with each round of reiteration, often with what appeared to be aborted attempts at cell division. In those lineages in which the posterior daughter would normally have undergone programmed cell death, cell death often occurred later than observed in wild-type lineages and sometimes did not occur. (D) The cell fates in P cell lineages of wild-type and *pag-3* mutant animals. A,B,C,D,E are distinct cell fates, representing cells that may divide, survive and differentiate, or undergo programmed cell death. VA, VB and VC are motoneuron classes (White et al., 1976). 'Pn.aa' is a neuroblast that divides to generate daughter cells like those normally generated by Pn.aa. (E) Experimentally determined cell fates in the Pn.a lineages of wild-type (Sulston and Horvitz, 1977; White et al., 1976; White et al., 1986) and *pag-3* mutant animals. Cell fates were assessed as described in the text. ±VA is an abnormal VA cell (see text). The fate of the presumptive DAS cell in *pag-3* mutants was not tested experimentally (DAS).

which represented expression in the DA and VA motoneurons, we subtracted the number of fluorescent nuclei in L1 larvae, which represented expression in the juvenile DA motoneurons. The number of VA motoneurons in P*unc-4gfp; pag-3(n3098)* mutants was significantly reduced compared with that in otherwise wild-type animals carrying the P*unc-4gfp* transgene (Table 1). As noted above, the anterior-most daughters generated by the P2-P12 cell lineages of wild-type animals adopt a VA motoneuron fate. Our observations suggest that as assessed by expression of *unc-4*, a characteristic of normal VA motoneurons (Miller and Niemeyer, 1995), the corresponding cells of *pag-3* mutants differentiate abnormally.

More strikingly, the VB motoneurons were essentially entirely absent from the ventral cord of *pag-3(n3098)* mutants, as determined by expression of the P*acr-5gfp* reporter, which is expressed in the DB and VB motoneurons (Winnier et al.,

Table 1. Numbers of VA, VB and VD motoneurons in *pag-3* mutants

	VA and/or DA*		VB and/or DB*		VD and/or DD†	
Stage	P*unc-4gfp*‡	P*unc-4gfp; pag-3*	P*acr-5gfp*§	P*acr-5gfp; pag-3*	+	*pag-3*
L3	11.4±0.5 (VA+DA)	8.5±0.7 (VA+DA)	13.3±0.4 (VB+DB)	6.3±0.2 (VB+DB)	19.0±0.0 (11) (VD+DD)	19.3±0.2 (50) (VD+DD)
L1	3.2±0.3 (DA)	3.5±0.3 (DA)	5.8±0.1 (DB)	5.6±0.2 (DB)	6.0±0.0 (10) (DD)	6.0±0.0 (37) (DD)
L3-L1	8.2±0.8 (VA)	5.0±1.0 (VA)	7.5±0.5 (VB)	0.7±0.4 (VB)	13.0±0.0 (VD)	13.3±0.2 (VD)

*Numbers of fluorescent nuclei in ventral cord; mean±s.e.m. in P3-P10 interval, *n*=35 animals. In this interval in wild-type animals, there are eight VA and eight VB motoneurons (Sulston and Horvitz, 1977).

†Numbers of GABA-staining nuclei; mean±s.e.m. in ventral cord and associated ganglia, *n*=numbers of animals examined. There are 13 VD motoneurons in the complete nervous system (Sulston and Horvitz, 1977).

‡P*unc-4gfp* is expressed in L1 animals in the juvenile DA neurons and in L3 animals in DA neurons and postembryonic VA neurons (Pflugrad et al., 1997). The difference between the number of VA neurons in wild-type animals compared with *pag-3(n3098)* mutants is highly significant ($P<0.0006$; unpaired *t*-test).

§P*acr-5gfp* is expressed in L1 animals in the juvenile DB neurons and in L3 animals in DB neurons and postembryonic VB neurons (Winnier et al., 1999).

1999). By contrast, the number of VD neurons in *pag-3(n3098)* mutants was normal, as determined by staining for the neurotransmitter GABA, which is expressed by the DD and VD motoneurons (McIntire et al., 1993). This observation suggests that the development of the VD motoneurons, which are generated as the posterior daughters of the Pn.ap neuroblasts, was not affected by loss of *pag-3* function. This conclusion was consistent with our observation that the cell divisions of the Pn.ap neuroblasts were normal in *pag-3* mutants. The defects in P cell lineages and the abnormalities in cell fates in *pag-3* mutants are summarized in Fig. 3E.

The absence of VB motoneurons and the abnormality in VA motoneurons of *pag-3* mutants accounts for the uncoordinated movement of these animals.

pag-3 mutants have extra VC-like motoneurons

In the P3-P8 lineages of the midbody, the Pn.aap cells, the normal posterior daughters of the Pn.aa neuroblasts, become VC motoneurons, while in the W, P1, P2 and P9-12 lineages of the anterior and posterior the Pn.aap (and W.ap) cells undergo programmed cell death (Sulston and Horvitz, 1977) (Fig. 1). The cell lineage reiteration defect of *pag-3* mutants completely accounts for the extra programmed cell deaths in the anterior and posterior ventral cord (extra Pn.aap-like daughter cells) and predicts that *pag-3* mutants should also contain extra VC-like motoneurons along the midbody.

To assess the number of VC motoneurons we used a P*lin-11gfp* construct. *lin-11* encodes a LIM homeodomain protein (Freyd et al., 1990) required for vulval morphogenesis (Ferguson et al., 1987) and is expressed in the VC motoneurons, vulval cells, gonadal and uterine cells, and several head neurons (Newman et al., 1999; Freyd, 1991) (this work). We generated an integrated reporter construct containing a portion of the *lin-11* promoter fused to *gfp* (Reddien et al., 2001) and used it to detect VC and VC-like cells in *pag-3* mutants. *pag-3* mutants had about three times as many P*lin-11gfp*-expressing neurons as did wild-type animals (Fig. 4). To test whether the Pn.aap-like cells (i.e. Pn.aaap, Pn.aaaap, etc.) in *pag-3* mutants showed other characteristics of VC motoneurons, we stained *pag-3* mutants with antiserum directed against the FMRF-amide neuropeptide expressed by the VC motoneurons (Schinkmann and Li, 1992) and observed many extra FMRF-amide-positive neurons in the ventral cords of *pag-3* mutants (Fig. 4).

Most of the extra VC-like neurons were in the midbody, as

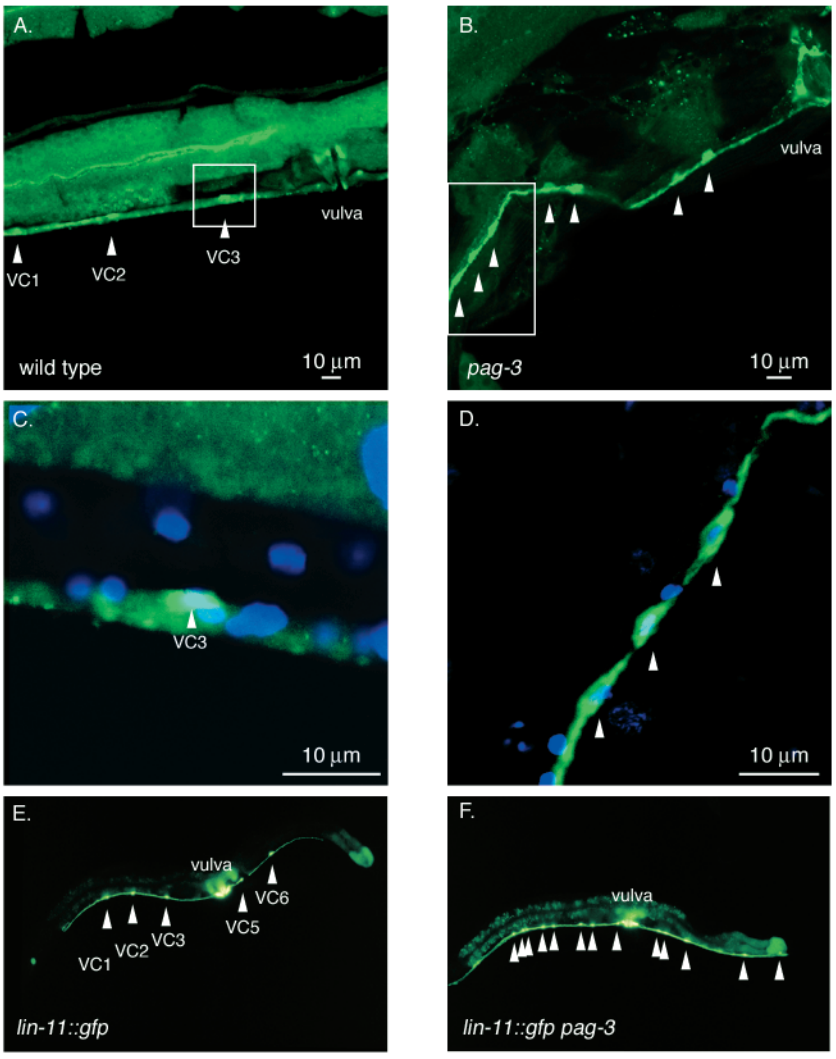

Fig. 4. VC and VC-like neurons in *pag-3* mutants. (A-F) Anterior is leftwards, and ventral is downwards. (A) Wild-type hermaphrodite stained with antiserum against the neuropeptide FMRFamide, which is expressed by the VC motoneurons. The three VC motoneurons anterior to the vulva are indicated, with a detailed view of the inset containing VC3 shown in C. In C, FMRFamide staining is visible in the cytoplasm surrounding the VC3 nucleus; nuclei are stained with DAPI. (B) *pag-3(n3098)* hermaphrodite stained with antiserum against the neuropeptide FMRFamide. Seven FMRFamide-positive nuclei anterior to the vulva are shown, with a detailed view of the inset shown in (D). (E-G) A chromosomally integrated P*lin-11gfp* reporter, *nIs106*, was used to determine the number of VC and VC-like neurons in wild-type and *pag-3* mutant animals. In wild-type animals, the P*lin-11gfp* reporter is expressed in vulval cells and some head neurons, as well as in the six VC motoneurons (Freyd, 1991). (E) Adult *nIs106* hermaphrodite. VC motoneuron nuclei are indicated. Vulval fluorescence obscures one and often two of the VC nuclei that flank the vulva (VC4 and VC5) (White et al., 1976), as in this image of a wild-type animal in which the VC4 nucleus is not visible. (F) Adult *nIs106 pag-3(n3098)* hermaphrodite. (G) Number of fluorescent nuclei seen in the ventral cords of adult hermaphrodites. *pag-3(ls64)* is R115opal (Jia et al., 1997). No attempt was made to count nuclei obscured by vulval fluorescence.

G. Number of VC or VC-like cells in the ventral nerve cord of *pag-3* mutants

Genotype	No. fluorescent nuclei	n
nIs106	4.3 ± 0.4	50
nIs106 pag-3(n3098)	13.9 ± 0.4	33
nIs106 pag-3(ls64)	12.9 ± 0.3	50

visualized by expression of the $P_{lin\text{-}11}gfp$ construct (Fig. 4). However, *pag-3* mutants also had some fluorescent nuclei in the anterior and posterior cord, where the Pn.aap cells in wild-type animals normally undergo programmed cell death. As seen in the cell lineages diagrammed in Fig. 3, not all Pn.aap and 'Pn.aap-like' cells generated by the anterior and posterior P lineages died in *pag-3* mutants. For example, eight of 24 P9-12.aap survived in the six *pag-3(n3098)* mutants we observed. Some of the surviving Pn.aap cells may differentiate to become VC-like cells and express $P_{lin\text{-}11}gfp$ [as has been directly confirmed in *ced-3* mutants or mutants defective in engulfment of cell corpses (Reddien et al., 2001)] accounting for the fluorescent nuclei in these regions of the ventral cords of *pag-3* mutants.

We conclude that the number of corpses in the ventral cords of *pag-3* mutants was determined by two factors: generation by reiterated cell lineages of extra posterior daughter cells (Pn.aap-like cells) that can undergo programmed cell death and a failure of some of these cells to die in *pag-3* mutants.

The HOM-C genes do not encode the positional information that influences the *pag-3* phenotype

As noted above, the likelihood that a W.aaa or Pn.aaaa cell would divide and thereby generate extra posterior daughter cells (which could undergo programmed cell death) varied with position along the anteroposterior body axis and differed between the two *pag-3* alleles. We tested the hypothesis that genes of the *C. elegans* homeotic gene cluster either promote extra divisions in the anterior ventral cord or prevent extra divisions in the posterior ventral cord. These genes – *ceh-13*, *lin-39*, *mab-5* and *egl-5* – specify spatial patterning along the anteroposterior body axis (Brunschwig et al., 1999; Burglin and Ruvkun, 1993; Chisholm, 1991; Clark et al., 1993; Kenyon, 1986; Salser et al., 1993). We determined the numbers of cell corpses in the ventral cords of *ced-1; pag-3(n3098)* mutants defective in *lin-39*, *mab-5*, or *egl-5* [*ceh-13* mutants die during embryogenesis; (Brunschwig et al., 1999)]. We found that loss of the spatial information controlled by *lin-39*, *mab-5* and *egl-5* did not affect the likelihood of extra divisions (Table 2). How positional information influences the *pag-3* phenotype remains unknown.

Table 2. Effect of loss of Hox gene function on the number of cell corpses in the ventral nerve cords of *pag-3* mutants

Genotype	Number of corpses*		
	Anterior†	Midbody‡	Posterior§
ced-1¶	2.6±0.1	0	6.1±0.1
ced-1; pag-3¶	3.3±0.2	0	4.8±0.2
ced-1; lin-39	2.9±0.1**	4.2±0.1**,††	5.7±0.1
ced-1; lin-39; pag-3	4.3±0.2**	4.1±0.3**,††,‡‡	5.2±0.2
ced-1; mab-5	*2.2*±0.1	0	3.2±0.1§§
ced-1; mab-5; pag-3	3.5±0.2	0	3.5±0.3§§
ced-1; egl-5	2.6±0.1	0	5.0±0.2¶¶
ced-1; egl-5; pag-3	3.7±0.2	0	4.4±0.3¶¶

*Values are mean±s.e.m. Sixty mutants were examined for the *lin-39* strains, 30 for the *mab-5* and *egl-5* strains.

†Generated by W, P1 and P2.

‡Generated by P3-P8.

§Generated by P9-P12

¶Data from Fig. 2.

**In *lin-39* mutants, nuclei generated by P3-P6 cell lineages migrate to the region of the ventral cord around the P2 descendants (Clark et al., 1993). As a consequence, corpses derived from P3-P6 may be counted as P2 corpses, increasing the number of cell corpses in the anterior ventral cord and reducing the number of cell corpses in the midbody.

††In *lin-39* mutants, the six VC motoneurons undergo programmed cell death; the null allele *lin-39(n1760)* (Clark et al., 1993) and the *pag-3(n3098)* allele were used for the studies reported in this table.

‡‡In *ced-1; lin-39; pag-3* mutants, as many as 10 cell corpses were observed in the midbody in single animals, suggesting that *lin-39* is required for survival of the Pn.aap and Pn.aap-like cells in *pag-3* mutants, as *lin-39* is for the Pn.aap cells in otherwise wild-type animals (Clark et al., 1993).

§§*mab-5* is required for deaths of the P11.aaap and P12.aaap cells (Kenyon, 1986), reducing by two the number of cell corpses in the posterior ventral cord of *mab-5* mutants. The null allele *e1239* (Kenyon, 1986) was used for the studies reported in this table.

¶¶In *egl-5* mutants, P12 is transformed to a P11-like fate, reducing by one the number of cell corpses in the posterior ventral cord of *egl-5* mutants. The null allele *n945* (Chisholm, 1991) was used for the studies reported in this table.

PAG-3 is expressed in the Pn.aa neuroblasts and their descendants

To characterize the PAG-3 expression pattern, we raised a rabbit polyclonal antiserum that specifically recognized PAG-3 and used it to stain wild-type animals of various stages (see Materials and Methods). Previous studies have indicated that a transgene reporter construct with β-galactosidase under the control of *pag-3* promoter sequences is expressed in the BDU neurons, the mechanosensory neurons, the two AVF interneurons, and the VA and VB motoneurons in the ventral cord (Jia et al., 1997). We found that PAG-3 protein was expressed more widely in the nervous system than had been observed using the $P_{pag\text{-}3}lacZ$ reporter. We detected PAG-3 during embryonic development in many nuclei ~280 minutes after fertilization (data not shown). At this developmental stage, the cells derived from the AB cell lineages, which generate most of the neuronal cells, are beginning to terminally differentiate (Sulston et al., 1983). In late L2 larvae anti-PAG-3 staining was seen in approximately two dozen cells in the head, all six mechanosensory neurons, the BDU neurons, approximately ten cells in the tail as well as in the ventral cord. PAG-3 staining of many cells in the head and tail remained detectable in adult animals (data not shown).

In the ventral cord, PAG-3 was first detected in the Pn.aa neuroblasts (Fig. 5), the daughters of which are abnormal in *pag-3* mutants. PAG-3 was not detected in the Pn.ap neuroblasts or their descendants, consistent with our observation that these cells appear to be unaffected by loss of *pag-3* function. PAG-3 was present equally in each of the descendant cells of Pn.aa after subsequent rounds of division (i.e. the Pn.aaa, Pn.aap, Pn.aaaa and Pn.aaap cells), including the three differentiating neurons generated by each Pn.aa neuroblast. In most cells, PAG-3 protein became undetectable shortly after the cells had been generated in the L1, but PAG-3 was present in six cells in the ventral cords of adults (see below). Pn.aap undergoes programmed cell death in the W, P1, P2 and P9-12 lineages (Fig. 1). PAG-3 protein was observed in the P2.aap and P9-P12.aap cells that died, as well as in the homologous P3-P8.aap cells that survived (Fig. 5 and data not shown), indicating that the simple presence or absence of PAG-3 as these cells are being generated does not determine the life versus death decision. The precise expression pattern of PAG-3 in the W and P1 descendants was difficult to define unambiguously, as there are many nuclei in the retrovesicular ganglion at the anterior end of the ventral cord (in which W

and P1.a divide) and as two embryonic cells in this region expressed PAG-3. Taken together these data suggest that in the ventral cord PAG-3 protein is first produced precisely in those neuroblasts abnormal in *pag-3* mutants, persists through the divisions of the Pn.aaa cells, and is present at least transiently in the three differentiating cells generated by each Pn.aa neuroblast.

PAG-3 expression persisted throughout the life of the animal in four cells in the retrovesicular ganglion at the anterior end of the ventral cord and in two cells in the posterior ventral cord. In newly hatched L1-stage larvae, before the initiation of the postembryonic W and P cell lineages, two cells in the retrovesicular ganglion expressed PAG-3. Based on position, these cells were most likely the RIG interneurons (Fig. 6 and data not shown). After completion of the W and P cell lineages, two additional cells in the retrovesicular ganglion and two cells in the posterior ventral cord contained detectable PAG-3 protein. Based on the locations of the nuclei and their appearance only after completion of the W and P cell lineages, we thought these nuclei might be the two AVF and the VA11 and VA12 neurons, respectively. We confirmed this hypothesis by staining animals carrying an integrated $P_{unc\text{-}4}lacZ$ reporter, which is expressed in the AVF and VA as well as other neurons (Miller and Niemeyer, 1995), with PAG-3 antiserum and monoclonal antibody against β-galactosidase (Fig. 6). The four postembryonically generated neurons that expressed PAG-3 in adults – AVFR/L, VA11 and VA12 – are lineally related as the anterior-most descendants of W, P1, P11 and P12, respectively.

pag-3 probably affects differentiation of the PVQ neurons

pag-3 was initially identified in mutants that misexpressed mechanosensory genes in the BDU interneurons and was proposed to affect specification or differentiation of the BDU cell fate (Jia et al., 1996). We found that *pag-3* similarly affects the PVQ interneurons. The PVQs are a pair of interneurons (PVQL and PVQR) located in the tail lumbar ganglia and can be visualized using an $P_{sra\text{-}6}gfp$ reporter (Troemel et al., 1995). In 30 adult wild-type animals, a total of 60 fluorescent nuclei were observed, whereas *pag-3(n3098)* mutants failed to express the reporter (one fluorescent nucleus was observed in 30 adult animals). In those rare *pag-3* mutants in which the PVQ neurons expressed $P_{sra\text{-}6}gfp$, the axons appeared normal (data not shown). Expression of $P_{sra\text{-}6}gfp$ in the ASH and ASI neurons, which also express this reporter (Troemel et al., 1995), was normal in *pag-3* mutants, suggesting that the PVQ neurons were missing or failed to express the $P_{sra\text{-}6}gfp$ reporter. Using Nomarski optics, we examined the tail region of wild-type animals carrying the $P_{sra\text{-}6}gfp$ reporter and in blinded assays identified the PVQ neurons, which have slightly larger nuclei than adjacent neurons and occupy characteristic positions at the anterior margins of the lumbar ganglia adjacent to the rectum (White et al., 1986). In similar assays of $P_{sra\text{-}6}gfp$; *pag-3(n3098)* mutants, we identified nuclei with the positions and morphology expected of PVQ neurons, but, unlike in wild-type animals, these nuclei did not express the reporter. The abnormalities in the PVQ neurons could reflect a defect

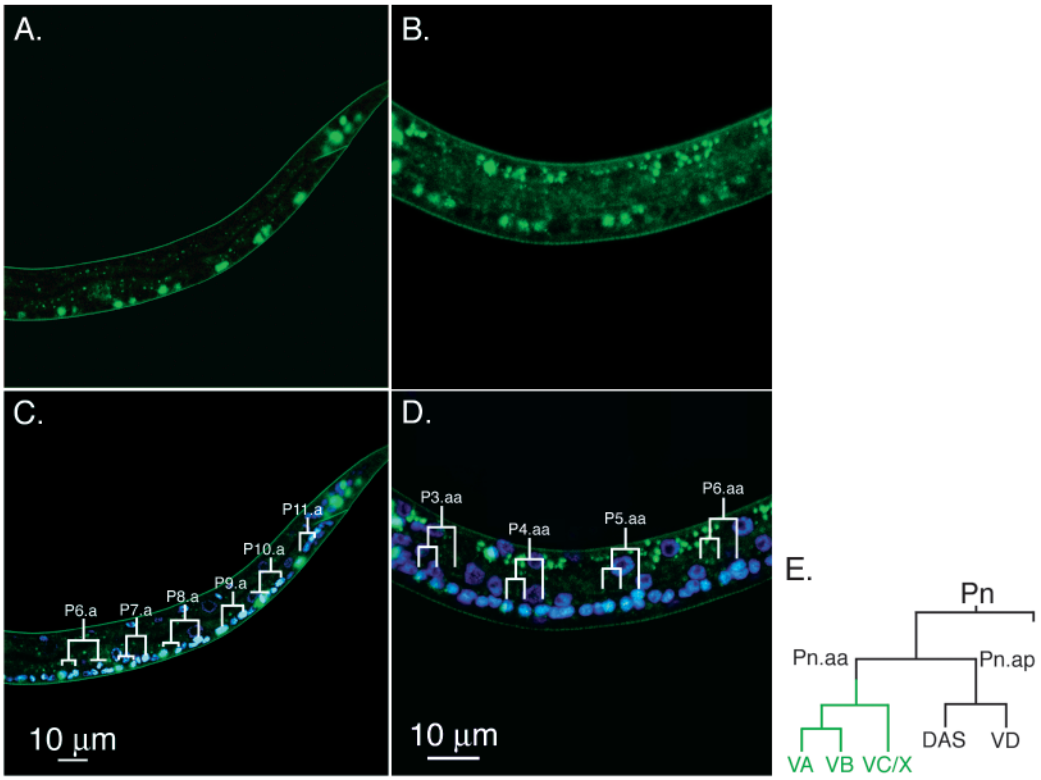

Fig. 5. Expression pattern of PAG-3 during postembryonic development of the ventral nerve cord. (A,B) PAG-3 antibody staining of wild-type L1/L2 larvae. In all panels, anterior is leftwards and ventral is downwards. (C,D) DAPI co-staining of animals in A,B. In the postembryonic ventral cord, anterior lineages generally divide earlier than posterior lineages (Sulston and Horvitz, 1977). (A,C) In this animal the P6.aa, P7.aa and P8.aa neuroblasts have divided, while P10.aa and P6.ap are in the process of mitosis. The P9.aa and P11.aa neuroblasts have not yet divided. P12.aa and P12.ap are out of the focal plane in the preanal ganglion. PAG-3 protein was detected in the Pn.aa, but not in the Pn.ap, neuroblasts. By examining the staining of many animals at various stages of development, we could trace PAG-3 protein continuously from the Pn.aa neuroblasts to all three neurons generated by them. In A,C, the Pn.aaa neuroblasts were larger and appeared brighter than their posterior sister cells, the Pn.aap cells, which terminally differentiate (P3-P8.aap) or undergo programmed cell death (P1-P2.aap, P9-P12.aap). (B,D) PAG-3 protein in each of the three terminally differentiating neurons generated by the Pn.aa neuroblasts. (E) Diagram of P cell lineage with cells containing PAG-3 protein indicated by green lines. Pn.aa neuroblasts did not contain detectable PAG-3 when they were generated, but became positive a short time later.

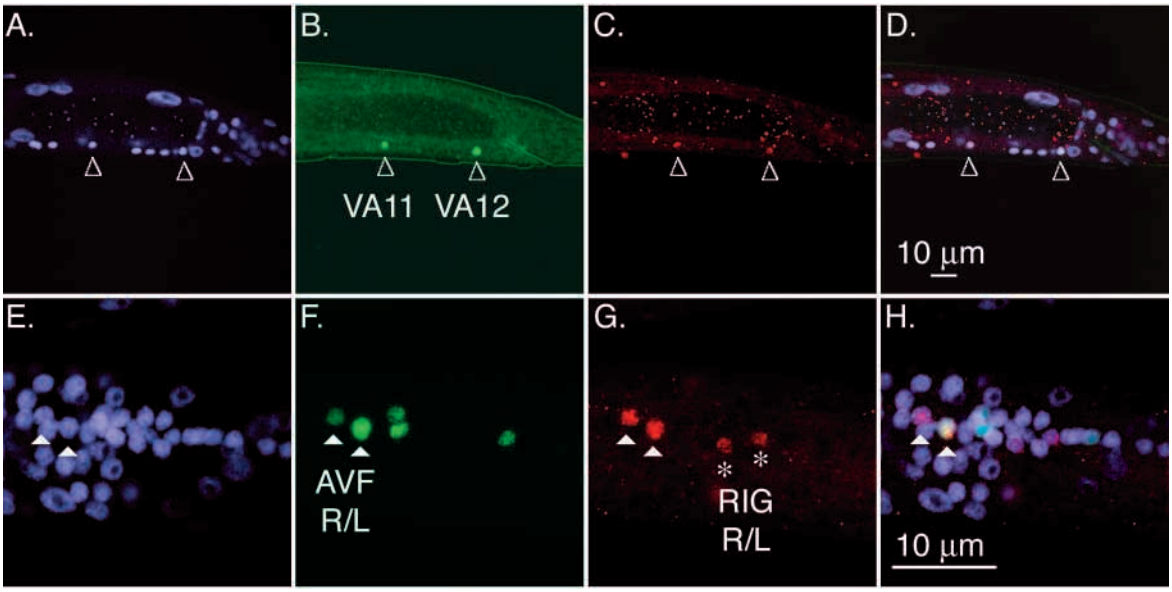

Fig. 6. Identification of PAG-3-staining ventral cord neurons in adult animals. Otherwise wild-type adult *C. elegans* carrying an integrated $P_{unc\text{-}4}$*lacZ* transgene were co-stained with PAG-3 antiserum and monoclonal antibody directed against β-galactosidase. DAPI was used to identify nuclei. (A-D) The posterior ventral cord; anterior is leftwards and ventral is downwards. VA11 and VA12 are marked with open triangles. (E-H) The retrovesicular ganglion and anterior ventral cord; ventral view, anterior is leftwards. AVFR and AVFL are marked with filled triangles. (A) DAPI, (B) β-galactosidase expression in VA11 and VA12 (Miller and Niemeyer, 1995). (C) PAG-3 staining. (D) Merged image. (E) DAPI staining of nuclei in the retrovesicular ganglion. (F) β-galactosidase expression in five cells in the retrovesicular ganglion. $P_{unc\text{-}4}$*lacZ* is expressed in the AVF interneurons, the VA motoneurons, and additional cells in the retrovesicular ganglion (Miller and Niemeyer, 1995). (G) PAG-3 staining. By several criteria (see text), the two anterior-most PAG-3 expressing cells in the retrovesicular ganglion are the right and left AVF interneurons. The two more posterior PAG-3-positive cells in the retrovesicular ganglion are the embryonically generated right and left RIG interneurons; they are marked with asterisks. (H) Merged image.

in the cell lineages generating these neurons. The PVQ neurons are generated during embryogenesis, making direct analysis of cell lineages difficult. The sister cells of the PVQR and PVQL neurons undergo programmed cell death (Sulston et al., 1983). A defect in the cell lineages that generate the PVQ neurons might generate an abnormal number of cell corpses, as we have observed in the ventral nerve cord lineages of *pag-3* mutants. We compared the number of cell corpses in the lumbar ganglia of *ced-1; pag-3(n3098)* and *ced-1* mutants, and found them to be similar (0.7±0.1 and 0.6±0.1, respectively, n=70 animals). PAG-3 was expressed in the PVQ neurons throughout the life of wild-type animals, as demonstrated by co-staining with PAG-3 antiserum of animals carrying the $P_{sra\text{-}6}$*gfp* reporter (data not shown). These data suggest that *pag-3* is required for differentiation of the PVQ interneurons.

DISCUSSION

pag-3 determines neuroblast fate

Prior studies of the *pag-3* gene established that loss of *pag-3* function results in abnormal gene expression in the BDU interneurons and a behavioral defect in locomotion (Jia et al., 1996). Mosaic analysis suggests that *pag-3* is required within the nervous system to prevent the locomotory defects, although how *pag-3* acts has remained unclear (Jia et al., 1997). Through an analysis of cell lineages in the ventral nerve cord and of PAG-3 protein expression in individual cells of the developing nervous system, we have established that *pag-3* determines the fate of specific anterior neuroblasts in the ventral cord of *C. elegans.* Our data suggest that *pag-3* may also act in neuronal differentiation, as discussed below.

We observed that in *pag-3* mutants the Pn.aaa neuroblasts of the ventral nerve cord reiterated the fate of their Pn.aa mothers. The posterior daughter cells produced by the reiterated divisions (e.g. Pn.aap cells) generally adopted fates appropriate for the corresponding wild-type lineages. However, some of the Pn.aap cells in *pag-3* mutants, none of which divided, were variably abnormal. Some Pn.aap and Pn.aap-like cells derived from P cell lineages in the anterior and posterior ventral cord, all of which die in wild-type lineages, instead survived and differentiated. These observations suggest that both daughter cells of the Pn.aa neuroblasts were abnormal: the Pn.aaa cells failed to determine their proper fate as neuroblasts and the Pn.aap cells did not always undergo programmed cell death. These abnormalities presumably reflect a requirement for *pag-3* in the Pn.aa mother cells to establish the Pn.aaa and Pn.aap cell fates prior to cell division, separate functions for *pag-3* in the two daughter cells after this division has been completed, or both. The expression pattern of PAG-3 protein was consistent with all three of these possibilities, as protein was present in the parental neuroblast Pn.aa and in both daughter cells.

pag-3 may also affect neuronal differentiation

The PVQ and BDU neurons in *pag-3* mutants are abnormal, despite being generated by apparently normal cell lineages, suggesting that these neurons fail to differentiate properly in *pag-3* mutants. The VA motoneurons also differentiate

abnormally in *pag-3* mutants, as demonstrated by the defective backwards movement of these animals and by abnormal expression of an *unc-4* reporter gene. A role for *pag-3* in differentiating and differentiated neurons is supported by the expression pattern of the protein. In the Q neuroblast lineages PAG-3 protein was detected only after the generation of the terminally differentiating AVM and PVM mechanosensory neurons (data not shown). In many neurons, including the PVQ, BDU and touch neurons, PAG-3 protein was present throughout the life of the animal. Some of these cells are abnormal in *pag-3* mutants (the BDU and PVQ neurons), while others have no obvious defect [the touch neurons (Jia et al., 1996)]. These data suggest that *pag-3* functions in diverse contexts within the developing nervous system, with an essential role in the development of some neuroblasts and neurons and a currently unapparent role in other neurons.

PAG-3 does not appear to be associated with any obvious single characteristic of differentiated neurons. In the ventral nerve cord, PAG-3 was expressed in cells that live and in cells that undergo programmed cell death; in cells that express *lin-11* and in cells that do not; and transiently in the VA2-10 motoneurons but continuously in VA11 and VA12. PAG-3 is required for cell-fate determination by the Pn.aa neuroblasts but not by the Pn.ap neuroblasts. PAG-3 was expressed in adult animals in neurons of several different types, including motoneurons (VA11, VA12), sensory neurons (ALM, PLM, AVM and PVM) and interneurons (BDU, RIG and AVF). Given its extensive similarity to mammalian Gfi-1 transcription factors, PAG-3 most probably affects cell fates by regulating transcription. Rather than affecting expression of a single common set of genes in all neuronal lineages, our finding that *pag-3* is expressed in many neuronal subtypes at different points in neuronal development suggests that *pag-3* cooperates with other factors to regulate the expression of cell type- and developmental stage-specific sets of genes to generate the complex pattern of neuronal subtypes seen in *C. elegans*.

pag-3 expression was specifically activated in the Pn.aa neuroblasts, the descendants of which were abnormal in *pag-3* mutants. By contrast, expression was not activated to a detectable level in the sisters of these cells, the Pn.ap neuroblasts, the descendants of which appeared normal in *pag-3* mutants. These observations suggest that *pag-3* acts specifically in the Pn.aa neuroblasts and their descendants. PAG-3 protein was present at the time of generation of neurons descended from each Pn.aa cell. It is shortly after the generation of the terminal cells in these lineages that these neurons begin to adopt identifying characteristics, such as class-specific patterns of axonal projections or the morphological characteristics of a dying cell (Sulston, 1976; Sulston and Horvitz, 1977; White et al., 1992; Knobel et al., 1999). PAG-3 thus may well be expressed in response to Pn.aa lineage-specific signals to determine blast cell fates and then act later to induce distinctive differentiated features characteristic of the neurons produced by those lineages.

The function of PAG-3 may be similar to that of UNC-86, a POU-homeodomain protein that couples cell lineage cues to aspects of terminal differentiation (Finney and Ruvkun, 1990; Finney et al., 1988). Like mutations in *pag-3*, mutations in *unc-86* result in the reiteration of some neuroblast lineages (Chalfie et al., 1981). *unc-86* is the only other *C. elegans* gene known to be able to mutate to cause reiterative cell lineage defects that specifically affect development of the nervous system. In addition to being required for neuroblast determination, *unc-86* also specifies characteristics of the mechanosensory neurons generated by those neuroblasts (Chalfie et al., 1981; Duggan et al., 1998; Finney and Ruvkun, 1990; Sze et al., 1997). *pag-3* may function similarly in the ventral nerve cord lineages. In these lineages, *pag-3* determines Pn.aa neuroblast fate and may also establish the fate of the VA and VB motoneurons generated by those neuroblasts.

Does *pag-3* have a direct role in regulating programmed cell death?

We identified *pag-3* in a screen for mutations in genes that affect whether individual cells in the ventral nerve cord survive or undergo programmed cell death. Does *pag-3* function during cell-death specification? Two observations intrigued us. First, the protein most similar to PAG-3 encoded by the *C. elegans* genome is CES-1, which specifies the fate of programmed cell death for particular neurons (Ellis and Horvitz, 1991; Metzstein and Horvitz, 1999). Second, Gfi-1, a mammalian protein structurally very similar to PAG-3, can directly repress transcription of the proapoptotic gene *Bax* in cultured lymphocytes and prevent apoptosis (Grimes et al., 1996). These observations suggested that in *C. elegans*, *pag-3* might act to prevent programmed cell death and were consistent with our observation that *pag-3* mutants contained extra cell deaths. However, our studies established that the extra cell deaths in the ventral cord of *pag-3* mutants are instead explained by a defect in the cell-fate determination of the Pn.aa neuroblasts.

Nonetheless, it remains possible that PAG-3 directly regulates the programmed cell death pathway in ventral cord neurons or in other cells in which PAG-3 is expressed. Specifically, loss-of-function mutations in *ces-1* have normal patterns of cell deaths; the role of *ces-1* in regulating programmed cell deaths of individual cells was identified by a gain-of-function mutation (Ellis and Horvitz, 1991). The current model for *ces-1* function is that *ces-1* expression must be repressed in cells that are to undergo programmed cell death (Metzstein and Horvitz, 1999). No similar gain-of-function mutations in *pag-3* are currently available. We tested whether overexpression of *pag-3* using the broadly expressed heat-shock promoter could prevent programmed cell deaths, but found *pag-3* overexpression to be lethal (data not shown). Further work will be required to assess whether PAG-3 directly regulates the transcription of genes that control programmed cell death.

pag-3 counterparts may determine blast cell identity in other organisms

The *Drosophila* protein Senseless is very similar in sequence to PAG-3 and is necessary for the development of sensory organs in the peripheral nervous system (Nolo et al., 2000). In *senseless* mutant flies, there is a severe loss of neurons in the peripheral nervous system and an excess of programmed cell deaths (Salzberg et al., 1997). It is unclear at present whether the defects in *senseless* mutants result from abnormal cell-fate determination by peripheral nervous system neuroblasts, abnormalities in neuronal survival and differentiation, or both. *pag-3* mutants differ from *senseless* mutants in that neuronal loss was not a prominent aspect of the *pag-3* mutant phenotype and was noted in the ventral cord only after analysis of the cell

lineages. However, this difference may be only quantitative. There may well be equivalent underlying effects on neuroblast determination. The excess of programmed cell deaths in *senseless* mutants could result from cell-lineage reiterations that generate extra dying cells, as in *pag-3* mutants. Alternatively, if *senseless* acts in the neuroblasts of the peripheral nervous system of *Drosophila* to determine neuroblast fate in a manner analogous to the way *pag-3* acts in the ventral nerve cord of *C. elegans*, the abnormal neuronal cells generated may undergo programmed cell death, as do abnormal neurons in other *Drosophila* mutants defective in development (Steller and Grether, 1994).

Two mammalian genes closely related to *pag-3*, *Gfi-1* and *Gfi-1B*, are expressed in hematopoietic cells of adult rats and mice (Gilks et al., 1993; Tong et al., 1998). Proviral activation of *Gfi-1* expression by Moloney murine leukemia virus in lymphocytes promotes the development of lymphomas (Gilks et al., 1993; Scheijen et al., 1997; van Lohuizen et al., 1991). The ectopic expression of *Gfi-1* using the *Lck* promoter disturbs early T lymphocyte development (Schmidt et al., 1998). We report here that in the absence of *pag-3* function, specific blast cells, the Pn.aa neuroblasts, adopt a stem cell-like pattern of division. Our finding of a required role for *pag-3* in this determination of blast cell fates leads us to suggest that in mammals mutations in the *Gfi-1* genes may similarly affect cell lineage determination and lead to cellular proliferation. Perhaps, independently or together with an effect of *Gfi-1* on apoptosis, such a defect may lead to a loss of particular cell types or a pool of abnormal cells prone to the development of malignancy.

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