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Expression Patterns of Developmental Control Genes in Normal and *Engrailed-1* Mutant Mouse Spinal Cord Reveal Early Diversity in Developing Interneurons

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The vertebrate spinal cord has long served as a useful system for studying the pattern of cell differentiation along the dorsoventral (d/v) axis. In this paper, we have defined the expression of several classes of genes expressed in restricted d/v domains in the intermediate region (IR) of the mouse spinal cord, in which most interneurons are generated. From this analysis, we have found that spinal cord interneurons and their precursors express unique combinations of transcription factors and Notch ligands at the onset of their differentiation. The domains of expression of a number of different classes of genes share similar boundaries, indicating that there could be a basic subdivision of the ventral IR into four distinct regions. This differential gene expression suggests that spinal cord interneurons acquire unique identities early in their development and that Notch signaling mechanisms may participate in the determination of cell fate along the d/v axis. Gene expression studies in *Engrailed-1* (*En-1*) mutants showed that *En-1*-expressing and other closely positioned classes of neurons do not require the homeodomain protein En-1 for their early pattern of differentiation. Rather, it is suggested that *En-1* may function to distinguish a subset of interneurons during the later maturation of the spinal cord.

Key words: Engrailed-1; *interneurons; Notch ligands; spinal cord; transcription factors; expression patterns*

The vertebrate nervous system is organized along both the anterior–posterior (a/p) and dorsoventral (d/v) axes. How this regionalization occurs during embryogenesis is currently a major focus of developmental neurobiology. Much of our understanding of the early events in d/v pattern formation comes from studies that focus on the development of the spinal cord (for review, see Tanabe and Jessell, 1996). These studies have concentrated primarily on the most ventral and dorsal cells in the spinal cord and the signals that control the specification of these cells. In contrast, despite the fact that the vast majority of neurons in the spinal cord are interneurons, with few exceptions (Shiga and Oppenheim, 1991) the early molecular development of this population of cells is the least well studied.

A growing body of data suggests that cell fates along the d/v axis of the spinal cord are controlled in part by secreted factors emanating from the ventral midline and dorsal non-neural ectoderm. For example, Sonic hedgehog (Shh) has recently been shown to influence floor plate–motoneuron–interneuron cell fate choices in a concentration-dependent manner in an *in vitro* chick explant assay (Ericson et al., 1996). Thus it seems that the establishment of distinct differentiated cell identities along the d/v axis during development is an important mechanism for generating neuronal diversity in the spinal cord.

In the mouse spinal cord, neural differentiation largely takes place between 9 and 15.5 d postcoitum (dpc), beginning rostrally at ~9.0 dpc and progressing caudally (Nornes and Carry, 1978). There is also a ventral to dorsal gradient of cell differentiation, with the majority of ventral cell types being born before 12.5 dpc. To begin to examine the early development of spinal cord interneurons, we have focused on interneurons generated in the intermediate region (IR) of the spinal cord, here defined as the area between the dorsal and ventral horns, during the early stages of cell differentiation at 9.5–11.5 dpc. We have studied the expression of three homeobox-containing transcription factors with restricted domains of expression in post-mitotic cells of the IR: *En-1* (Joyner et al., 1985), *Evx-1* (Bastian and Gruss, 1990), and *Lmx-1* (R. Johnson, unpublished data). We have also analyzed the expression of three genes expressed in the ventricular zone (VZ): the Notch ligands *Jagged-1* (Lindsell et al., 1995) and *Dll-1* (a mouse Delta homolog) (Bettenhausen et al., 1995) and the homeobox gene *Dbx-1* (Shoji et al., 1996). This analysis has revealed that interneurons and their precursors in the early IR express unique combinations of genes in similar d/v domains at the onset of their differentiation. Although it is known that interneurons in the mature spinal cord can be grouped according to similar characteristics, such as morphology, projections, or physiology (Jankowska and Lundberg, 1981), these observations suggest that different classes of interneurons may share early molecular identities and that Notch signaling may participate to generate diversity among interneurons along the d/v axis. Furthermore, the expression patterns of many genes respect similar boundaries, suggesting the existence of fundamental domains of cell differentiation in the developing spinal cord.

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We have also begun to examine the role of *En-1* in spinal cord interneurons by studying in detail the development of *En-1* interneurons at 9.5–15.5 dpc by analyzing gene expression in *En-1* mutants. We have found that *En-1* and other ventral interneurons do not require *En-1* function for their early survival or differentiation. This analysis suggests that the early onset of *En-1* expression in differentiating interneurons may serve a later function in distinguishing this population of interneurons within this broad class of cell type in the spinal cord.

MATERIALS AND METHODS

Generation of mice. The generation of *En-1* ^{lki} (*lacZ* knock-in) embryonic stem (ES) cells and chimeric mice in which the bacterial gene coding for β-galactosidase (β-gal), *lacZ*, is inserted into the *En-1* locus has been described previously (Hanks et al., 1995). Using *En-1* $^{lki/+}$-targeted ES cells, we generated mouse lines using two independently targeted cell lines. Male chimeras were made using morula aggregation as described by Nagy et al. (1993) and bred with CD1 females (Charles River Laboratories, Wilmington, MA) to transmit the allele through the germline.

Homozygote *En-1* $^{lki/lki}$ embryos were obtained by intercrossing heterozygote mice at F2–F6 generations. The lines were maintained on an outbred CD1 background. *En-1* $^{lki/lki}$ embryos were morphologically indistinguishable from *En-1* $^{hd/hd}$ (homeodomain deletion) null mutant mice (Wurst et al., 1994) and were identified by genotyping using yolk sac DNA or by morphological criteria as described previously (Wurst et al., 1994). To obtain *En-1* $^{lki/hd}$ embryos, heterozygous *En-1* $^{lki/+}$ and *En-1* $^{hd/+}$ mice were crossed. Noon on the day of vaginal plug was designated as 0.5 dpc. For mutant analysis, we compared at least 6–10 embryos at all stages with wild-type littermates.

Immunohistochemistry. Embryos were collected in Dulbecco's PBS (D-PBS) containing Mg^{2+} and Ca^{2+} (Cellgro) on ice and then fixed for 30 min to 2 hr in 4% paraformaldehyde (PF)/D-PBS. Tissue was then sunk in 30% sucrose/D-PBS, frozen in Tissue Tek, and sectioned at 12–16 μM in a cryostat. Sections were collected on Fisher Colorfrost-Plus slides, air-dried for ~1 hr, and stored at −20° until used.

For antibody staining, sections were brought to room temperature and then fixed for 5 min in 4% PF. After sections were washed 3 × 5 min in D-PBS, they were blocked with D-PBS containing 10% normal goat serum (NGS), 0.1–0.4% Triton X-100 for 1 hr at room temperature. Antibodies were applied in a buffer containing 1% NGS, 0.1–0.4% Triton X-100 in D-PBS. Primary antibodies used and dilutions were as follows: rabbit anti-Engrailed (α-Enhb-1), 1:500; mouse anti-Lim-1/2 (4F10), 1:2; rabbit anti-Pax-2, 1:200; mouse anti-β-tubulin (TuJ1), 1:5000; rabbit anti-β-gal (5′—3′, Inc.), 1:500; mouse anti-bromodeoxyuridine (BrdU) (Sigma, St. Louis, MO), 1:250. All primary incubations were overnight at 4°C. In double-labeling experiments using monoclonal and polyclonal antibodies, primary antibodies were mixed and incubated together overnight. Secondary antibodies (Jackson ImmunoResearch, West Grove, PA) and dilutions were as follows: fluorescein-conjugated goat anti-rabbit and goat anti-mouse IgG, 1:100; Cy-3-conjugated goat anti-rabbit and goat anti-mouse IgG, 1:200. After staining, sections were dehydrated in graded ethanols, washed in xylene, and coverslipped with Permount (Fisher Scientific, Houston, TX). Fluorescent antibody-labeled sections were coverslipped using Gelmount (biomeda Corp.), and viewed under epifluorescence.

For double-labeling with β-gal and antibodies on sections, β-gal activity was visualized before antibody labeling as follows: sections were washed in PBS containing 0.1% Tween-20 (PBT) and then incubated in 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) (Sigma) solution at 37° for 6–8 hr. After this, sections were washed in PBT twice and then in PBS. Antibody labeling was then performed as described above.

RNA in situ *hybridization.* Whole-mount RNA *in situ* hybridization of embryos was performed as described (Parr et al., 1993), with modifications (Knecht et al., 1995). Antisense riboprobes used were *Pax-2* (Dressler et al., 1990), *Evx-1* (Dush and Martin, 1992), *Dbx-1* (Shoji et al., 1996), *Lmx-1* (R. Johnson, unpublished), *Jagged-1* (Lindsell et al., 1995), and *Dll-1* (Bettenhausen et al., 1995). At least three to four normal and 3–4 *En-1* mutants embryos were examined at 9.5, 10.5, and 11.5 dpc for each probe. For whole-mount double-labeling with β-gal and *in situ* hybridization, β-gal activity was visualized before *in situ* hybridization. Embryos were collected and fixed as described above, washed in PBT, and incubated in X-gal for 6–8 hr at 37°. After this, embryos were washed 3 × 10 min in PBT and refixed overnight in 4% PF. Embryos were stored in PBS at 4°C until whole-mount *in situ* hybridization was performed, as described above, except that the methanol dehydration and peroxide bleaching steps were omitted.

For sectioning after whole-mount staining, embryos were embedded in 4% agarose and sectioned on a Leica VT-1000E Vibratome at 50–75 μM, mounted on Fisher Colorfrost Plus slides, and air-dried. The entire spinal cord was sectioned in all cases. Sections were counterstained with Nuclear Fast Red, dehydrated, and coverslipped.

Retrograde axon labeling. Wild-type CD1 embryos were collected in cold PBS and then transferred to L-15 medium (Leibovitz's, without glutamine, from Specialty Medium) on ice. Embryos were decapitated and eviscerated, and ventral laminectomies were performed. Fluorescein- or rhodamine-conjugated, lysinated dextran, 3 kDa molecular weight (Molecular Probes, Eugene, OR), dissolved in PBS containing 1% Triton X-100, was pressure-injected into the ventral midline or ventrolateral marginal zone–white matter using a glass microcapillary pipette (tip diameter of ~50 μM). Spinal cords were incubated for 4–6 hr at room temperature in L-15 medium, after which they were fixed in 4% PF on ice for 30 min to 1 hr. Embryos were then processed for cryosectioning and antibody detection as described above.

BrdU labeling and detection. Two pregnant CD1 females were injected intraperitoneally with a 20 mg/ml solution of BrdU (Sigma) in PBS, 2 and 4 hr before they were killed. Litters (12–15 embryos each) were collected and processed for antibody detection as described above. For detection of BrdU, a mouse monoclonal antibody to BrdU (Sigma) was used at 1:250 after sections were incubated for 40 min in 2N HCl. For double-labeling, incubation with X-gal was performed before antibody detection of BrdU.

Image analysis and processing. Fluorescent images were visualized on a Zeiss Axioskop microscope equipped with epifluorescence and a Princeton Instruments cooled-CCD camera. Double-labeled sections were collected using different filter sets, and color encoding and image superimposition were performed using Metamorph image processing software (Universal Imaging Corporation, West Chester, PA). Raw digital images were processed in Adobe Photoshop.

Histological sections were photographed on a Leitz DMRXE compound microscope with Kodak Ektachrome 64T or 160T slide film. Digital images were made by scanning slides on a Nikon LS3510AF film scanner and processed in Adobe Photoshop. Most images were corrected for color balance, contrast, brightness, or cropping using Adobe Photoshop, but no other modifications were made.

RESULTS

Interneurons express unique transcription factors at the early stages of their differentiation

Previous studies have shown that *En-1* (Davis et al., 1991) and *Evx-1* (Bastian and Gruss, 1990) are expressed in the developing spinal cord. In this report, we have analyzed the expression of these genes in relation to one another, and to a third gene expressed in the spinal cord, *Lmx-1*, a member of the LIM-domain transcription factor family.

The expression of *En-1, Evx-1*, and *Lmx-1* was first seen in the spinal cord beginning at 9.5–10.5 dpc in the IR, a region that lies between the dorsal and ventral horns and contains primarily interneurons. At this stage of development, spinal cord cells are beginning to differentiate, moving from the VZ to the intermediate zone (IZ) in the process. To directly compare the early expression domains of these genes, we used a targeted mouse line, *En-1* $^{lki/+}$, in which the bacterial reporter gene *lacZ* was inserted into the first exon of *En-1* (Hanks et al., 1995) (also see Materials and Methods). The expression of β-gal in heterozygous *En-1* $^{lki/+}$ mice was found to be almost identical to normal *En-1* expression in the spinal cord, as determined by double-labeling using X-gal to detect β-gal and En antibodies to detect En-1 protein, and thus serves as a faithful reporter of *En-1* expression (data not shown). By using double-labeling with X-gal to detect β-gal (*En-1*) and RNA *in situ* analysis to detect *Evx-1* or *Lmx-1* in the same embryos, we were able to accurately establish the domains of gene expression, using the domain of *En-1* expression as a reference point along the d/v axis.

At 10.5 dpc, expression of *Evx-1* was detected in a cluster of cells located dorsal to and not overlapping β-gal (*En-1*)-expressing cells in the IZ (Fig. 1*a*). This relationship was seen at all rostrocaudal levels of the spinal cord and hindbrain at 10.5 and 11.5 dpc. The detection of *Evx-1* at the lateral margins of the VZ strongly suggests that it is expressed in postmitotic cells. Thus, *En-1* and *Evx-1* are expressed in nonoverlapping, adjacent populations of early neurons in the ventral IR. In addition, the rostral boundary of expression of *Evx-1* at the border of rhombomeres (r) 1 and 2 in the hindbrain corresponds precisely with the rostral boundary of *En-1* expression (data not shown). These complementary but similar patterns suggest a related mechanism for regulating the early expression of *En-1* and *Evx-1* in postmitotic cells of the ventral spinal cord and hindbrain.

The domain of *Lmx-1* expression was also found in cells of the IZ, located immediately dorsal to the developing sulcus limitans at 10.5 dpc (Fig. 1*b*). *Lmx-1* expression is separated from *En-1*-expressing cells by a gap, only a portion of which is occupied by *Evx-1*-expressing cells (Fig. 1*c*). In contrast to *En-1* and *Evx-1*, the rostral boundary of *Lmx-1* expression in this domain does not end at the r1–r2 border in the hindbrain but instead continues through r1 into the midbrain (data not shown).

These observations show that differentiating interneurons in the ventral two thirds of the IR express unique domains of gene expression along the d/v axis: *En-1* the most ventral, followed by *Evx-1* and then *Lmx-1*. The presence of gaps in expression reveals the existence of two additional domains in the ventral IR, located between *Evx-1* and *Lmx-1* (Fig. 1*c*) and *En-1* and *Islet-1* (data not shown).

The *En-1* and *Evx-1* expression domains correspond to ventral domains of *Jagged-1* and *Dll-1* expression

The expression patterns of the Notch ligands Jagged-1/Serrate and Delta-1/Dll-1 in the vertebrate spinal cord have been described in several recent studies (Lindsell et al., 1995, 1996; Myat et al., 1996). To compare the expression domains of *Jagged-1* and *Dll-1* in the VZ with domains of gene expression in the IZ, we used double X-gal/*in situ* analysis in *En-1* $^{\mathrm{lki}/+}$ mice. This analysis revealed that the region of the neuroepithelium from which *En-1*-expressing cells derive corresponds precisely to the ventral *Jagged-1* stripe in the VZ throughout the spinal cord and hindbrain at 10.5 and 11.5 dpc (Fig. 1*d*). In addition, the rostral limit of this ventral *Jagged-1* stripe was at the r1–r2 border, corresponding to the rostral limit of *En-1* expression (data not shown). The dorsal *Jagged-1* stripe was also located in the VZ just ventral to the sulcus limitans and to the *Lmx-1* domain, which is located dorsal to the sulcus limitans (Fig. 1*e*), in the region that does not correspond to the domains of expression of *En-1, Evx-1*, or *Lmx-1*. This dorsal *Jagged-1* stripe extends rostrally through r1 into the midbrain (data not shown).

We have also compared the expression domains of *Dll-1* and *En-1* in the spinal cord. As reported previously, there is a gap in the expression of *Delta-1* in the ventral VZ of the rat (Lindsell et al., 1996). Comparison of *En-1* and *Dll-1* expression revealed that this gap in *Dll-1* expression corresponds to the region where *En-1* expression is seen (Fig. 1*f*). *Dll-1* expression dorsal to this domain is continuous up to the roof plate. Because we have shown above that the *Evx-1* and *Lmx-1* expression domains lie dorsal to the *En-1* domain, and that *Dll-1* expression in the VZ is also immediately dorsal to *En-1*, it can be inferred that *Evx-1* and *Lmx-1* derive from a region of the neuroepithelium in which *Dll-1* is expressed. Taken together, these results suggest a potential role for *Jagged-1* and *Dll-1* in establishing and/or maintaining the pattern of cell differentiation along the d/v axis in postmitotic interneurons of the mouse IR.

Dbx-1 is expressed in the intermediate zone in a domain dorsal to *En-1*

The expression of *Dbx-1* has recently been described in the spinal cord using both RNA *in situ* analysis (Shoji et al., 1996) and *Dbx-1*-enhancer-element-driven *lacZ* expression (Lu et al., 1996). These studies show that *Dbx-1* expression can be seen as early as 9.5 dpc extending in continuous, bilateral stripes in the VZ along the length of the spinal cord and hindbrain. To compare the domains of *Dbx-1* and *En-1* expression, we again made use of X-gal/*in situ* double-labeling. Analysis of expression at 9.5–11.5 dpc revealed that *Dbx-1* was expressed in a domain located immediately dorsal to *En-1* at all rostrocaudal levels, similar in position along the d/v axis to the *Evx-1* expression domain but extending into the VZ (Fig. 1*g*). At 9.5 dpc, *Dbx-1* expression was seen along the entire length of the neural tube, whereas *En-1* expression was detected only in more rostral regions (midthoracic and higher) (Davis et al., 1991) (data not shown). Because of the rostrocaudal progression of maturation in the spinal cord, this observation shows that *Dbx-1* expression precedes *En-1* expression in the spinal cord. In addition, *Dbx-1* expression in this domain spans both the VZ and IZ (Fig. 1*g*). These observations suggest that *Dbx-1* may be expressed in both dividing and postmitotic cells and that *Dbx-1* may play a role in establishing a position along the d/v axis at stages preceding cell differentiation.

En-1 is expressed in postmitotic ventral interneurons

To begin to examine the role of transcription factors in spinal cord interneurons, we have analyzed the cells expressing *En-1* in more detail in normal and *En-1* mutant mice. We have also analyzed gene expression in other populations of spinal cord interneurons in the *En-1* mutant.

Previous studies showing *En-1* expression in the IR at the lateral margins of the VZ and its progression from rostral to caudal as cell differentiation is occurring in the spinal cord suggest that *En-1* is first expressed as cells become postmitotic (Davis and Joyner, 1988; Davis et al., 1991). To confirm this, and to determine whether *En-1* expressing cells are neurons, two markers were used; a neuron-specific form of tubulin, β-III-tubulin, which identifies postmitotic neurons (Lee et al., 1990), and BrdU, which identifies proliferating cells (Gratzner, 1982; Nowakowski et al., 1989). In the following analyses, we have used a polyclonal antiserum that detects both En-1 and En-2 proteins (Davis et al., 1991); however, *En-2* expression is never detected in the embryonic mouse spinal cord (Davis and Joyner, 1988), and thus this antibody reveals only *En-1* expression. At 9.5–10.5 dpc, En protein was detected in the nucleus of cells located at the lateral margins of the VZ, as well as in a few cells located close to the lumenal surface. Most, if not all, En-expressing cells also expressed β-tubulin (Fig. 2*a*), indicating that they are postmitotic neurons. To confirm that they are postmitotic, short pulses (2–4 hr before mice were killed) of BrdU labeling were used to mark mitotically active neuroepithelial cells. Of four embryos examined from two separate litters, En expression was not detected in cells that had incorporated BrdU, confirming that the expression of *En-1* is initiated in postmitotic neurons (Fig. 2*b*). En immunoreactivity was detected occasionally in cells close to the lumenal surface; however, these cells seemed to have just completed their final division close to the ventricle, because En staining was not

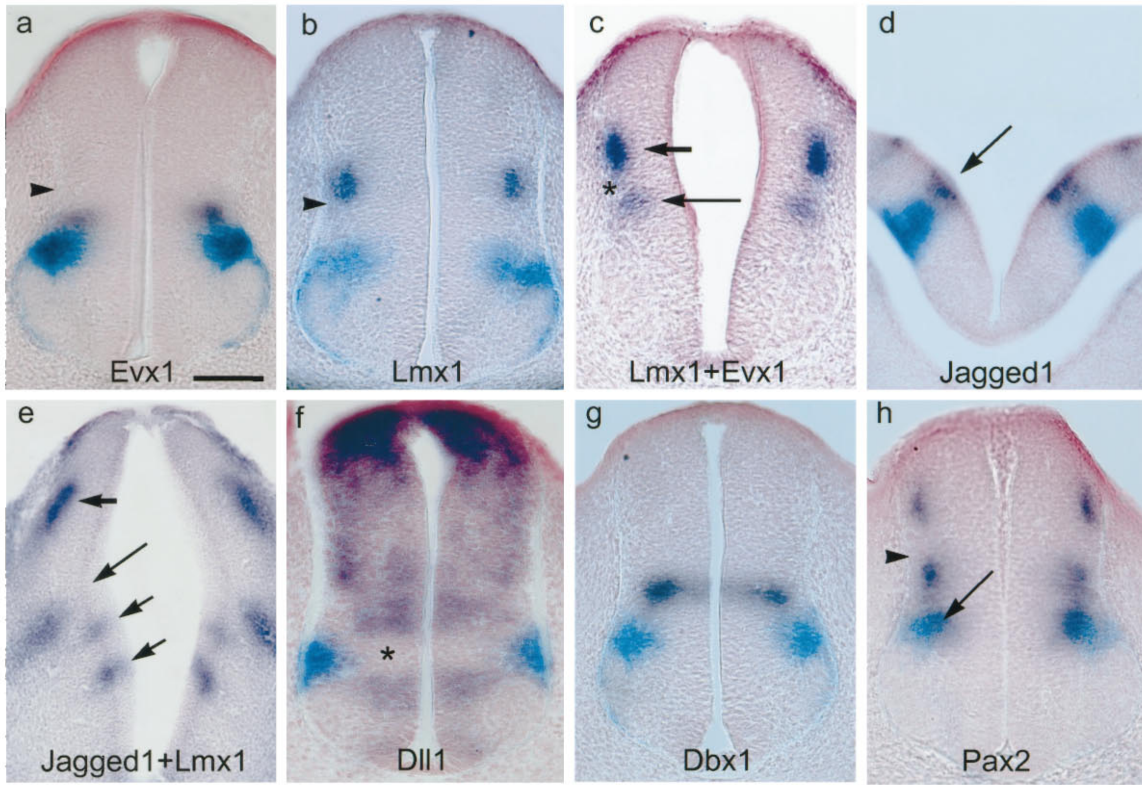


FIGURE 1

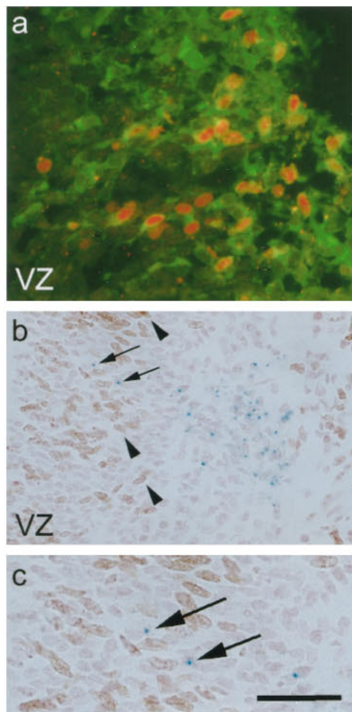
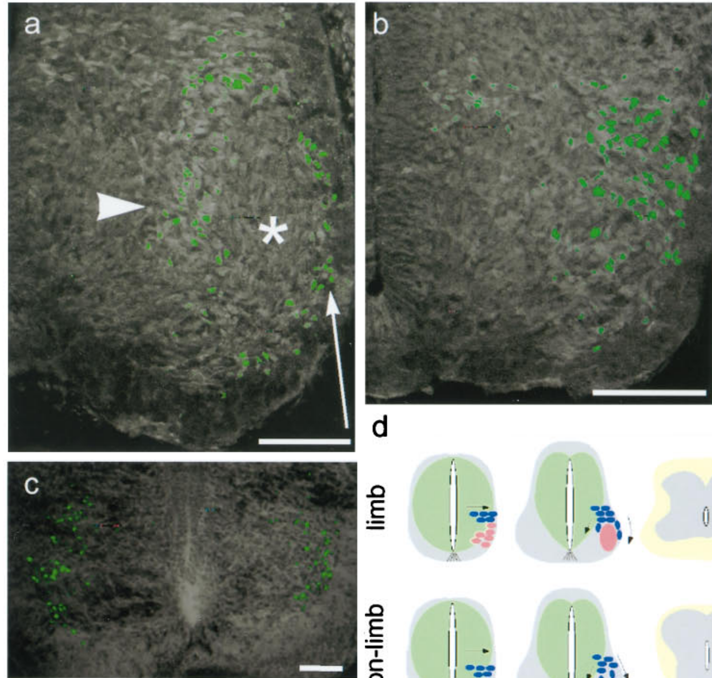


FIGURE 2



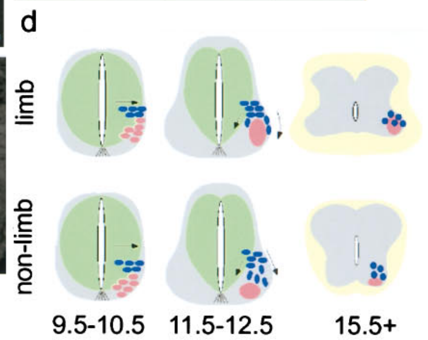


FIGURE 3

observed independent of β-tubulin or in conjunction with BrdU labeling.

The expression pattern of *En-1* was also analyzed at later stages of spinal cord development. At 10.5–15.5 dpc, En immunoreactivity was detected in a group of cells located at roughly the same d/v position as at earlier stages (Fig. 3). In addition, many En-expressing interneurons were located more ventrally. Cells in the IR of the spinal cord are generated in an outside-to-inside manner, with lateral cells being born largely before medial ones (Nornes and Carry, 1978). Analysis of the expression of *En-1* in ventral cells over time showed that the more ventrally located, *En-1*-expressing cells were first detected at the lateral margins of the spinal cord at ~10.5 dpc, whereas more medially, ventrally located *En-1*-expressing cells only began to appear at ~11.5 dpc. These observations suggest that as *En-1* cells are generated, many begin a ventral migration as they mature. In the regions of the spinal cord containing a lateral motor column (LMC), the majority of ventrally migrating *En-1*-expressing interneurons were lateral and medial to motoneurons, whereas some were found in the LMC among motoneurons (Fig. 3*a*). In contrast, in non-LMC-containing regions (e.g., midthoracic), *En-1* cells instead were more diffusely situated in the ventral horn (Fig. 3*b*). After the majority of motoneuron cell death has occurred in the mouse spinal cord, at 15.5 dpc (Lance-Jones, 1982), these rostrocaudal differences in *En-1* expression are much less pronounced; many *En-1* interneurons were found at the same d/v position as where they were generated, as well as ventrolaterally in the ventral horns (Fig. 3*c*). These observations might be explained by the lateral migration of some medially located *En-1* cells before 15.5 dpc. It is also possible that *En-1* is upregulated in some ventral horn interneurons at these stages. In either case, it seems that there are differences in the migration patterns of *En-1* interneurons along the a/p axis of the spinal cord, and that these early region-specific migration patterns may be influenced by the number and kind of motoneurons. These early patterns are summarized in Figure 3*d*.

To identify the projections of *En-1* interneurons, a fluorescent-dextran tracer (3 kDa dextran) was injected into the ventral commissure at the midline and ventrolateral funiculus to label commissural and ipsilateral/association interneurons, respectively (Silos-Santiago and Snider, 1992, 1994). These injected embryos were then processed for En immunoreactivity. In the vast majority of injections, we were unable to detect any *En-1*-expressing cell that clearly co-labeled after retrograde tracing of these two major classes of interneurons (Fig. 4) ($n = 4-8$ embryos each at 11.5, 12.5, and 13.5 dpc). In a few cases we observed 3 kDa dextran labeling of *En-1*-expressing interneurons after injection into the ventrolateral funiculus; however, it is likely that such labeling resulted from the uptake of tracer by cells from their leading process during their migration out of the VZ, because they were very few in number and were only found near the injection site (Fig. 4*b, arrow*). In addition, *En-1* expression was detected in some cells that had been labeled by 3 kDa dextran injections directly into the ventrolateral gray matter (data not shown). These observations taken together suggest that *En-1* interneurons are more likely to be locally projecting, or to project in the ventral white matter in the vicinity of the ventral roots or wholly within the gray matter.

En-1 marks a subset of *Lim-1/2*, and *Pax-2*-expressing interneurons

To further characterize the class of neurons that express *En-1*, we compared its expression with other markers of IR cell types. At 9.5 dpc, the expression of the LIM-domain genes *Lim-1* and *Lim-2* are confined to cells in the IR (Fujii et al., 1994; Tsuchida et al., 1994). We compared the expression of *En-1* with *Lim-1* and *Lim-2* using both double-label X-gal/RNA *in situ* and antibody analysis. Using *in situ* probes specific for *Lim-1* or *Lim-2*, we found that *En-1* expression was detected at the ventral limit of both the *Lim-1* and *Lim-2* expression domains (data not shown). At this stage *Lim-1* and *Lim-2* expression overlap completely in this domain. Using an antibody that detects both *Lim-1* and *Lim-2* proteins (Tsuchida et al., 1994), we found that En-1 was expressed in a subset of *Lim-1/2*-expressing cells at all stages examined and that all *En-1* interneurons also express Lim-1/2 proteins (Fig. 5).

At the onset of expression at 9.5 dpc, *En-1* expression marked the ventral limit of Lim-1/2 expression (Fig. 5*c*). At 10.5–11.5 dpc, both En-1 and Lim-1/2 expression became more widespread. The ventrally located, likely migrating *En-1*-expressing cells continued

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Figure 1. Gene expression in spinal cord and hindbrain interneurons and their precursors defines distinct d/v domains at 10.5–11.5 dpc in wild-type and *En-1* $^{lki/+}$ embryos. *En-1* expression is shown in *blue*. *a–c, f–h*, 10.5 dpc spinal cord; *d, e*, 11.5 dpc hindbrain. *a*, *Evx-1* (*purple*) is expressed in a domain immediately dorsal to *En-1* (*blue*) and ventral to the sulcus limitans (*arrowhead*). *b*, *Lmx-1* expression (*purple*) is detected in a domain just dorsal to the sulcus limitans (*arrowhead*). *c*, *Evx-1* expression (*thin arrow*) is separated from the *Lmx-1* domain (*thick arrow*) by a gap (*asterisk*) located ventral to the sulcus limitans. *d*, Expression of the ventral *Jagged-1* stripe (*arrow*, *angle* also indicating mediolateral plane) in the VZ of the posterior hindbrain corresponds to the domain of *En-1* expression along the hindbrain and spinal cord. *e*, Both *Jagged-1* domains (*short arrows*) lie ventral to the *Lmx-1* expression domain (*long arrow*) and the sulcus limitans. The *angle of arrows* indicates mediolateral plane. *Thick arrow* indicates additional dorsal *Lmx-1* domain in posterior hindbrain. *f*, *Dll-1* expression (*purple*) is widespread in the VZ except for a region corresponding to the domain of *En-1* expression (*asterisk*). *g*, *Dbx-1* expression marks a domain that spans the VZ and IZ and is immediately dorsal to *En-1*. *h*, *En-1* expression overlaps the ventral limit of the *Pax-2* domain (*purple; arrow*), which extends up to the sulcus limitans (*arrowhead*). Scale bar (shown in *a*): *a–d, f, g*, 100 μM; *e*, 120 μM; *h*, 87 μM.

Figure 2. En-1 expression is detected in postmitotic neurons. *a*, In posterior regions of the spinal cord at 10.5 dpc, En-1 protein (*red*) is detected in the nuclei of cells that are migrating out of the VZ. En-1-expressing cells also express β-tubulin (*green*), identifying them as differentiating neurons. *b*, *En-1* interneurons (*blue* β-gal staining in *En-1* $^{lki/+}$ embryos) do not incorporate BrdU (*brown* nuclei, *arrowheads*), indicating that they are postmitotic. *c*, Enlargement of section shown in *b*. *Arrows* in *b* and *c* indicate *En-1* interneurons migrating out of the VZ. Section through the midlumbar region of a 10.5 dpc embryo. Lateral is to the *right* in both images. *VZ*, Ventricular zone. Scale bar (shown in *c*): *a*, 100 μM; *b*, 50 μM; *c*, 25 μM.

Figure 3. The expression of *En-1* over time suggests that *En-1*-expressing interneurons undergo a region-specific ventral migration. *a*, In the lumbar spinal cord at 11.5 dpc, many *En-1*-expressing cells (*green*) are detected lateral (*arrow*) and medial (*arrowhead*) to motoneurons (*asterisk*). *b*, At the same stage in midthoracic regions, *En-1*-expressing cells are found throughout the ventrolateral ventral horn. *c*, At 15.5 dpc, *En-1*-expressing interneurons (*green*) are widely dispersed in the ventrolateral gray matter of the spinal cord. Shown are transverse sections through the lower thoracic spinal cord. *d*, Summary of the predicted early migration paths of *En-1* interneurons. In this schematic, the migration of *En-1* cells (*blue*) is depicted in relation to motoneurons (*pink*) at thoracic (*bottom*) and lumbar (*top*) levels. *Arrows* indicate possible migratory routes of *En-1* interneurons. Lateral is to the *right* in *a* and *b*. Scale bars: *a, b*, 50 μM; *c*, 100 μM.

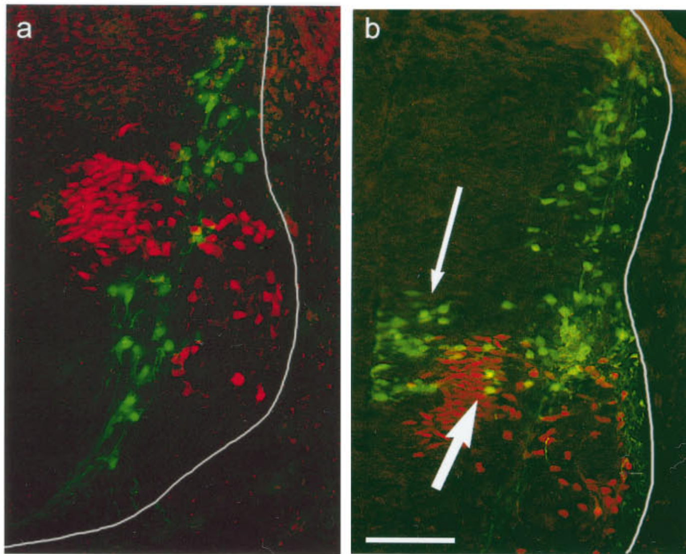

FIGURE 4

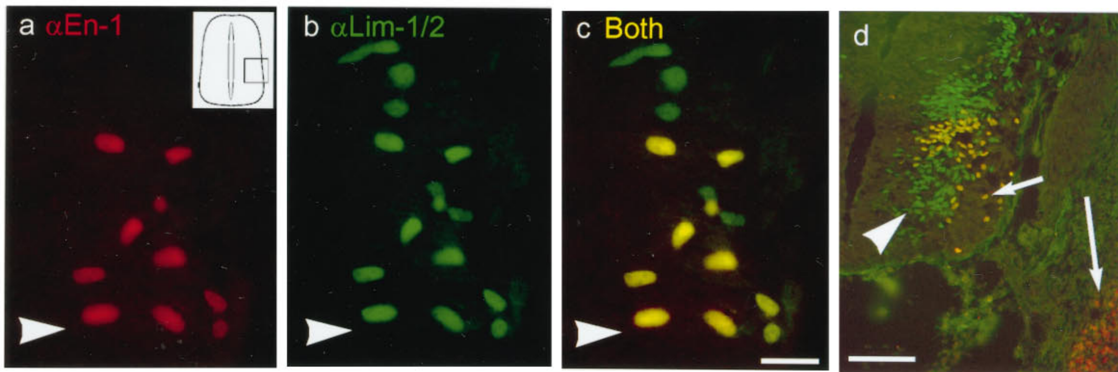

FIGURE 5

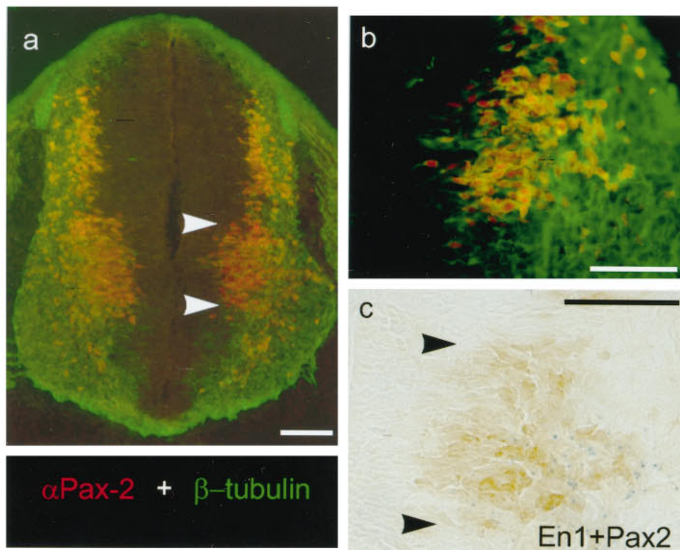

FIGURE 6

to express Lim-1/2 (Fig. 5*d*). These ventrally located *En-1*-expressing cells did not, however, express motoneuron markers such as Islet-1/2 proteins at any stage examined (data not shown), in agreement with previously published findings (Pfaff et al., 1996). At 11.5 dpc, many additional Lim-1/2-expressing cells were detected ventral to the region where En-1 and Lim-1/2 initially overlapped. These Lim-1/2-expressing cells did not express En-1 (Fig. 5*d, arrowhead*). By analogy to the chick, these cells may be *Lim-1*-expressing motoneurons that are migrating through earlier born motoneurons to take up a more superficial position in the lateral motor column (Tsuchida et al., 1994).

The paired box-containing gene *Pax-2* has also been shown to be expressed broadly in the IR of the spinal cord during the early stages (9.5–11.5 dpc) of cell differentiation (Fig. 6*a*) (Nornes et al., 1990). At the onset of its expression, Pax-2 protein is detected at the margins of the VZ in two domains: beginning dorsal to the ventral horn motor column and extending up to the sulcus limitans, and in a second domain in the developing dorsal horn (Fig. 6*a*). *Pax-2*-expressing cells also expressed β-tubulin and are thus postmitotic neurons (Fig. 6*a,b*). Using double-labeling in whole embryos, we found that β-gal (*En-1*) expression overlapped the ventral limit of the region of the IR in which *Pax-2* RNA was expressed (Fig. 1*h*). To determine whether *En-1* and *Pax-2* are expressed in the same cells, we used two different methods. First, we examined sections from *En-1* $^{1ki/+}$ embryos stained with X-gal and Pax-2 antibody. This analysis reveals that there is extensive overlap between *En-1* (β-gal) and Pax-2-expressing cells in the ventral IR (Fig. 6*c*). Second, we compared the expression of Pax-2 and Lim-1/2 proteins in wild-type sections at 10.5 dpc. We found that Pax-2 and Lim-1/2 proteins are coexpressed in cells of the ventral IR at the onset of their expression at this stage (Fig. 7*a–c*). Because we have shown above that all *En-1* interneurons express Lim-1/2 proteins in the ventral IR, we can infer that *En-1* interneurons also express Pax-2 proteins in this same domain.

We have also found that the dorsal limit of expression of Pax-2 and Lim-1/2 is coincident at 9.5–10.5 dpc, extending up to the sulcus limitans (Fig. 7*d–f*) and abutting the *Lmx-1* domain (data not shown). At 11.5 dpc, the expression of Lim-1/2 and Pax-2 diverges significantly; in the ventral horn many cells appear that express only Pax-2 or Lim-1/2 proteins, but not both, although many other cells in the IR continue to express both proteins. In addition, the domains of expression of Pax-2 and Lim-1/2 only partially overlap in the dorsal horns (data not shown). Thus it seems that the early expression of Pax-2 and Lim-1/2 overlaps in the IR but not at later times in more ventral or dorsal regions of the spinal cord. Furthermore, En-1 expression marks the ventral boundary of this region of overlapping Pax-2 and Lim-1/2 expression in the IR.

En-1 is not required for the survival or early differentiation of *En-1*-expressing cells

We have shown that interneurons in the spinal cord express unique transcription factors along the d/v axis, dividing the ventral IR into at least four ventral domains. As a first step in exploring the role of these genes in interneuron identity, we have analyzed the pattern of gene expression in the ventral spinal cord in the absence of *En-1* gene function.

En-1 is expressed in the midbrain–hindbrain region beginning at ~8.5 dpc, in addition to its expression in the spinal cord and hindbrain (Davis et al., 1991). Mice lacking *En-1* function have a loss of midbrain and cerebellar structures that derive from the *En-1*-expressing brain region, suggesting that *En-1* is required for the specification, survival, and differentiation of these neural precursors (Wurst et al., 1994). In contrast, loss of *En-1* function in the ventral ectoderm of the developing limb does not lead to loss of *En-1*-expressing ectodermal cells (C. Loomis and A. Joyner, unpublished observations), but instead results in an alteration of ventral ectoderm and mesoderm cell fate and limb patterning (Loomis et al., 1996).

To study the fate of *En-1*-expressing cells in the spinal cord of *En-1* mutants, we analyzed β-gal expression in homozygous *En-1* $^{1ki/1ki}$ and compound heterozygous null mutant *En-1* $^{1ki/hd}$ embryos at various stages. In these mice, the targeting of *lacZ* into the first exon of *En-1* results in the disruption of *En-1* while simultaneously providing a marker for *En-1*-expressing cells. This allele results in a total loss of *En-1* function, because the mutant seems to be morphologically the same as null *En-1* $^{hd/hd}$ mutants and En-1 protein expression is not detected in the spinal cord with anti-En antibodies (data not shown).

At 9.5–10.5 dpc, expression of β-gal in *En-1* $^{1ki/1ki}$ mice was detected in the same region of the ventral IZ as in normal heterozygous littermates at all rostrocaudal levels (Fig. 8*a*). Thus, *En-1* expression is initiated normally during the early phase of its

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Figure 4. En-1 interneurons do not project in the ventral commissure or ventrolateral funiculus. *a*, Commissural interneurons are labeled with fluorescein-3 kDa dextran (*green*) after injection into the ventral midline at 11.5 dpc. *En-1* expression (*red*) does not colocalize with the retrograde tracer. *b*, Injections into the ventrolateral funiculus at 11.5 dpc labels ipsilaterally projecting association neurons (*green*) and some neuroepithelial cells, the endfeet of which are located near the injection site (*thin arrow*). The vast majority of *En-1* cells (*red*) do not label after these injections. Occasionally, some *En-1* cells are labeled (*thick arrow; yellow–green* cells) but are likely migrating cells that have taken up dye from their leading process. Shown are transverse sections through lumbar spinal cord. Lateral is to the *right*. Approximate margin of the spinal cord is outlined in *white*. Scale bar: *a*, 42 μM; *b*, 50 μM.

Figure 5. En-1 interneurons comprise a subset of Lim-1/2 interneurons. At the onset of its expression at 9.5 dpc, *En-1* (*a, red*) is detected in a ventral subset of cells expressing Lim-1/2 (*b, green*) proteins. All *En-1*-expressing interneurons express Lim-1/2 (*c*). Overlapping expression is seen in *yellow*. *Arrowheads* mark the early ventral boundary of En/Lim expression. *d*, At 11.5 dpc, some *En-1* interneurons have begun a ventral migration (*short arrow*). These cells continue to express Lim-1/2 protein (*yellow–orange*). In this image, double-labeled cells are *yellow–orange*, whereas *En-1* expression in the sclerotome, which does not overlap with Lim-1/2 expression, is *red* (*long arrow*). Shown are transverse sections through the lumbar spinal cord. In both images, lateral is to the *right* and dorsal to the *top*. *Inset* indicates region of spinal cord shown in *a–c*. Scale bars: *a–c*, 25 μM; *d*, 100 μM.

Figure 6. Pax-2 is a broad marker of early differentiating interneurons. *a*, At 11.5 dpc, *Pax-2* protein (*red*) is detected in the nucleus of many cells at the lateral margins of the VZ. In the ventral spinal cord, this expression is dorsal to the ventral horn and extending up to the sulcus limitans (region between *arrowheads*). *Pax-2* cells also express cytoplasmic β-tubulin (*green*) and are thus differentiating neurons. *b*, High-power view showing individual Pax-2 expressing cells coexpressing β-tubulin. *c*, *En-1* expression (*blue* cells from an *En-1* $^{1ki/+}$ embryo) overlaps *Pax-2* (*brown*) in the ventral IZ. All En-1 cells express Pax-2. Boundaries of the ventral Pax-2 domain are indicated by *arrowheads*. Scale bars: *a*, 100 μM; *b, c*, 50 μM.

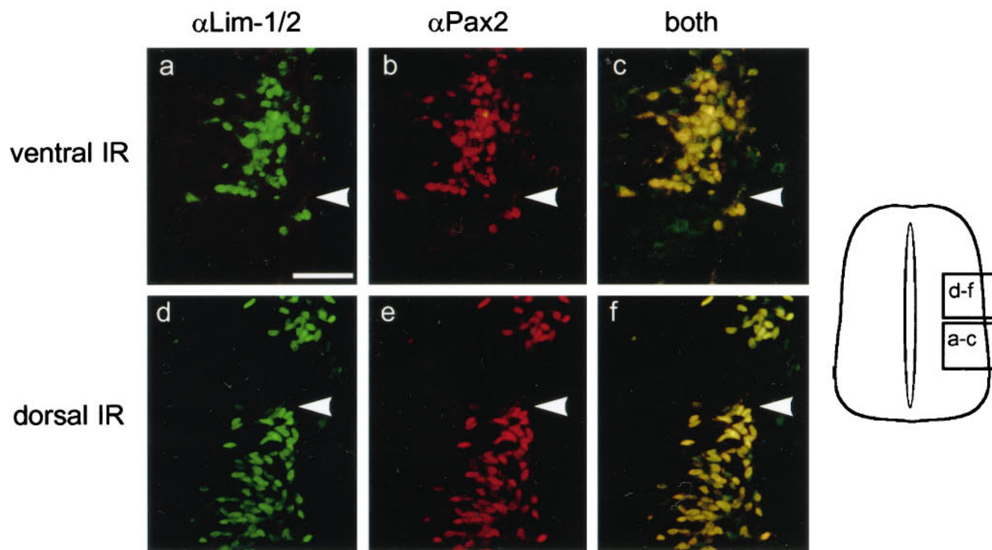

FIGURE 7

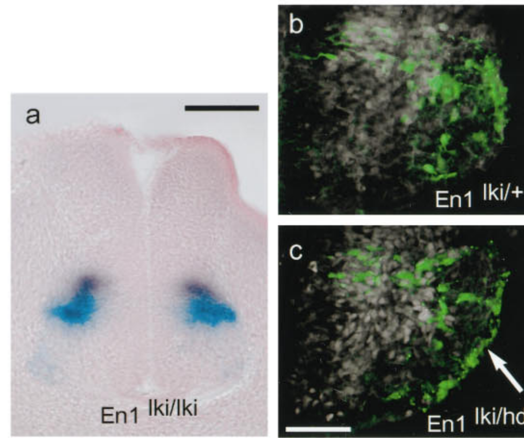

FIGURE 8

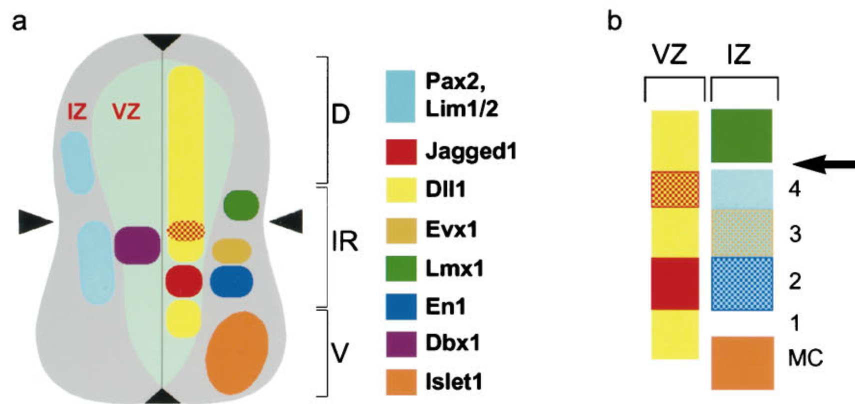

FIGURE 9

expression in the absence of *En-1* gene function in the spinal cord.

The persistence of the *En-1*-expressing cells in *En-1* mutants has allowed for an analysis of their fate in the absence of *En-1* function. To control for the expression levels of β-gal in *En-1* $^{1ki/+}$ heterozygotes and homozygotes, we analyzed staining patterns in *En-1* $^{1ki/+}$ embryos in comparison with *En-1* $^{1ki/hd}$, both of which contain a single copy of *lacZ*. At 11.5–15.5 dpc, the expression of β-gal in *En-1* $^{1ki/hd}$ embryos was seen in cells located near the marginal zone lateral to LMC motoneurons (Fig. 8*b,c*). β-gal expression in the spinal cord of these embryos seemed to be indistinguishable from *En-1* $^{1ki/+}$ littermates at all stages and rostrocaudal levels examined. Thus, *En-1*-expressing interneurons are able to undergo a normal primary migration out of the VZ zone, as well as a secondary migration ventrally, in the absence of *En-1* gene function.

We then set out to determine whether *En-1* expression was required for the normal development and differentiation of interneurons in *En-1* $^{1ki/1ki}$ embryos. One possibility is that *En-1* is required to specify the identity of *En-1* interneurons or their neighbors; in its absence, these cells may adopt other fates. To test this possibility we analyzed the expression of markers of nearby cell types in *En-1* $^{1ki/1ki}$ mice. Using double-labeling, we found that both *Evx-1* (Fig. 8*a*) and Islet-1/2 (data not shown) staining patterns in *En-1* $^{1ki/1ki}$ embryos were similar to controls. These findings revealed that *En-1* interneurons do not adopt the fates of more ventral or dorsal cell types, as marked by the expression of two genes specifically found in populations of cells close to *En-1*-expressing interneurons. We have also examined the expression of *Dbx-1*, *Jagged-1*, and *Lmx-1* in *En-1* mutants and found them to be similar to normal embryos at 10.5–12.5 dpc (data not shown). Together, these results show that the function of *En-1* is not required non-cell-autonomously to initiate normal gene expression in nearby cells as assayed by the expression of genes that mark distinct cell types or domains in the IR, nor is it required cell-autonomously to prevent *En-1*-expressing interneurons from adopting neighboring phenotypes.

We also analyzed the expression of markers that are expressed in *En-1* interneurons in *En-1* $^{1ki/1ki}$ mice. Both Lim-1/2 and Pax-2 expression were detected in the cells expressing β-gal in *En-1* $^{1ki/1ki}$ embryos, as in their heterozygous littermates (data not shown). These findings suggest that *Lim-1/2* and *Pax-2* may be upstream or in an independent pathway to *En-1* in ventral interneurons.

Finally, it has been shown in the grasshopper that ventral nerve cord neuroblasts require *En* function to undergo a switch between generating glial and neuronal daughters (Condron et al., 1994). We were able to detect the neuron-specific intermediate filament β-tubulin in *En-1* $^{1ki/1ki}$ embryos in β-gal-expressing cells (data not shown); therefore, it is not likely that *En-1* serves a similar function to determine glial versus neuronal fates in the mouse.

The persistence of *En-1*-expressing interneurons in *En-1* mutant mice reveals that *En-1* function is not required for several facets of early interneuron differentiation, namely cell migration and gene expression, nor is it required for patterning of adjacent cell types. These findings point to a later primary role for *En-1* in determining interneuron function in the mouse spinal cord. We have also examined β-gal expression in *En-1* $^{1ki/1ki}$ embryos at 15.5 and 17.5 dpc and found that *En-1* interneurons persist even at these later embryonic stages (data not shown). Thus it will be possible to study the later development of these cells and of the spinal cord in general in the absence of *En-1* function.

DISCUSSION

In this study, we have focused on the early development of the most abundant population of cells in the vertebrate spinal cord: interneurons. We have found that differentiating interneurons express unique transcription factors in distinct domains along the d/v axis. This analysis shows that differentiating interneurons in the spinal cord are organized into at least four d/v domains, based on differential gene expression, ventral to the sulcus limitans. The correspondence of these four domains to the expression domains of the Notch ligands *Jagged-1* and *Dll-1* in the VZ raises the possibility that Notch-mediated lateral inhibition could play a role in patterning differentiation of neurons along this axis. We have summarized the expression patterns studied in this paper in Figure 9. Finally, we have shown that early interneuron development in the *En-1* mutant seems normal in many respects, including d/v gene expression patterns, *En-1* cell migration and projections, and overall spinal cord morphology. We cannot conclude that loss of *En-1* does not have any adverse effect on spinal cord development, because of the lack of specific independent markers for *En-1*-expressing interneurons or identified target genes or analysis of later interneuron function. Taken together, our results suggest that the early onset of transcription factor expression in differentiating interneurons may serve a later function in distinguishing like groups of cells in this broad class of neuron.

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Figure 7. Lim-1/2 and Pax-2 proteins are coexpressed in early differentiating cells of the ventral IR. Sections through midthoracic spinal cord at 9.5 dpc stained with antibodies to Lim-1/2 (*a, d, green*) and Pax-2 (*b, e, red*). *a–c*, In the ventral IR, Lim-1/2 and Pax-2 expression overlaps in cells located dorsal to the motor column (boundary indicated by *arrowheads*). *d–f*, In the region of the sulcus limitans (*arrowhead*), the expression of Lim-1/2 and Pax-2 is coincident in the ventral IR ventral to the sulcus. The dorsal domain of Lim-1/2 and Pax-2 expression is seen at the *top* of these figures. Lateral is to the *right*, dorsal to the *top* in all figures. *Schematic* at *right* indicates areas shown in corresponding figure rows. Scale bar, 25 μM.

Figure 8. *En-1* and *Evx-1* interneurons do not require *En-1* function for their survival or early differentiation. *a*, Both *En-1* (*blue* β-gal staining) and *Evx-1* (*purple*) expression is initiated normally in *En-1* $^{1ki/1ki}$ embryos at 10.5 dpc. *b*, In a wild-type *En-1* $^{1ki/+}$ embryo at 11.5 dpc, β-gal expression (*green*) (detected with antibodies to β-gal) is seen in *En-1* interneurons migrating out of the VZ and ventrally. *c*, In *En-1* $^{1ki/hd}$ embryos at the same stage, β-gal expression (*green*) is virtually identical to that of wild-type embryos. Shown are transverse sections through the lower thoracic cords. Lateral is to the *right* in *b* and *c*. Scale bars: *a*, 100 μM; *b, c*, 50 μM.

Figure 9. *a*, Summary of the expression patterns of genes in the intermediate region of the spinal cord at 10.5–11.5 dpc. *Green shading* denotes the ventricular zone (*VZ*), *gray shading* the intermediate zone (*IZ*). *Arrowheads* indicate the position of the developing sulcus limitans. *b*, Schematic highlighting overlapping expression patterns in differentiating neurons of the IZ and the corresponding domains of *Jagged-1* and *Dll-1* in the VZ. Four distinct regions of gene expression are seen dorsal to the motor column (*MC*) and extending up to the sulcus limitans (*arrow*). These domains are numbered *1–4* from ventral to dorsal. Overlapping expression domains are shown by *checkerboard patterns*.

Territories of gene expression in differentiating interneurons

Our analysis has revealed the existence of a number of distinct domains of gene expression in differentiating interneurons, primarily in the ventral spinal cord. Lim-1/2 and Pax-2 are expressed the most widely, initially in a region beginning dorsal to the motor column and extending to the sulcus limitans. Within this domain, *En-1* and *Evx-1* are expressed, with *En-1* marking the early ventral boundary of Lim/Pax expression, whereas *Evx-1* is immediately dorsal to *En-1* but not extending to the sulcus limitans. Together, with two domains located between *En-1* and *Isl-1* and *Evx-1* and *Lmx-1*, at least four distinct domains of gene expression exist in the IR ventral to the sulcus limitans (Fig. 9*b*). *Lmx-1* expression marks a population of cells located immediately dorsal to the sulcus limitans. This gene may mark the ventral limit of the dorsal territory of cell differentiation in the spinal cord and indicates that the dorsal IR is also divided into discrete domains of cell differentiation.

The organization of domains of gene expression along the d/v axis suggests that they may arise as a result of mechanisms that are known to influence cell fates along this axis: Shh and bone morphogenetic protein signaling from ventral and dorsal regions, respectively. Shh has been shown to be an important determinant of ventral cell fates in vertebrates (Echelard et al., 1993; Roelink et al., 1994; Marti et al., 1995; Chiang et al., 1996; Ericson et al., 1996). Thus, the domains of expression of genes such as *En-1*, *Evx-1, Jagged-1*, and *Dbx-1* that are restricted to the ventral spinal cord may be established by Shh signaling. In contrast, the domains of more broadly expressed genes, such as Lim-1/2, Pax-2, and *Dll-1*, which are also expressed dorsally, may not be determined solely by either ventral or dorsal signaling mechanisms alone, or they may be independent of them. These genes instead may play a role in determining local responses to these signals.

Finally, we have found that the expression domains of all genes studied in this paper extend rostrally through the spinal cord into the hindbrain. *En-1*, *Evx-1*, and the ventral *Jagged-1* domain all share a common rostral boundary of expression at r1–r2. Although subtle differences do exist in the expression of some of these genes in the hindbrain as compared with the spinal cord, for example additional domains of expression, these observations suggest that many or most ventrally derived hindbrain interneurons share common features with interneurons of the spinal cord.

Notch signaling

Does Notch-mediated lateral inhibition play a role in establishing domains of cell differentiation along the d/v axis in the mouse spinal cord? Our results show that *Jagged-1* and *Dll-1* are expressed in patterns in the ventral VZ, which corresponds to regions defined by postmitotic gene expression. In particular, the domain of *En-1* expression seems to correlate precisely with the ventral *Jagged-1* stripe and a *Dll-1*-negative region throughout the spinal cord and hindbrain, whereas the dorsal *Jagged-1* stripe corresponds to the region immediately ventral to the *Lmx-1* expression domain and the sulcus limitans. *En-1*, *Evx-1*, and the ventral *Jagged-1* stripe also share a rostral expression boundary at the r1–r2 border. The existence of fundamental d/v domains of gene expression in differentiating interneurons and the possible role of Notch signaling in generating these patterns is suggested further by recent findings that several mammalian *fringe* genes are expressed in patterns similar to *Dll-1* and complementary to *Jagged-1* (and *En-1*) expression domains (Johnston et al., 1997; Laufer et al., 1997).

Although their expression initially is confined to the VZ, it has been shown that the Notch ligands Serrate/Jagged and Delta-1 are expressed in postmitotic cells in the chick spinal cord (Myat et al., 1996). The striking correlation of expression domains of *En-1* and *Jagged-1* suggests that *Jagged-1* may be expressed in immature *En-1* interneurons as they differentiate. The expression of these genes may not overlap temporally, however, because *Jagged-1* expression is rapidly downregulated as cells move out of the VZ (Lindsell et al., 1995), whereas *En-1* expression is primarily seen in lateral regions of the VZ. It has been proposed that Notch signaling in vertebrates serves to maintain and allow expansion of precursor cell populations during neurogenesis (Morrison et al., 1997). It remains unclear, however, why Notch ligands are expressed in distinct d/v domains in the vertebrate spinal cord if their primary function is to inhibit differentiation by activating widely expressed Notch receptors. Because three Notch genes have been shown to be expressed in the vertebrate spinal cord (Lindsell et al., 1996; Myat et al., 1996), one possibility is that Jagged-1 and Dll-1 may activate Notch receptors with different affinities, resulting in a differential effect on genes downstream of Notch and a patterned effect on cell differentiation.

Role of transcription factors in interneuron development

What function does the differential expression of transcription factors play in subgroups of developing interneurons? Our results demonstrate that many or most interneurons acquire unique molecular identities during their early differentiation. These identities may be important for specifying and maintaining aspects of interneuron function at later stages of development. In vertebrates, many spinal cord interneurons have been shown to undergo extensive secondary migration in the gray matter (Leber and Sanes, 1995; Lu et al., 1996) (this study). Thus, it may be crucial for groups of neurons in the IR to establish common identities at early developmental stages, when spatially influenced mechanisms of fate determination may function, to maintain these identities during the widespread mixing brought about by migration in the maturing spinal cord.

Our analysis of the expression of genes in closely related cells along the d/v axis in the *En-1* mutant shows that *En-1* is not required to generate the pattern of interneuron cell differentiation along this axis, including *En-1* interneurons. This conclusion is based on the observation that adjacent cells express appropriate markers in the *En-1* mutant and that *En-1* cells do not require *En-1* function for their differentiation, nor do they adopt the fate of adjacent cell types. Although we have not identified markers specific to *En-1* interneurons, we have determined that *En-1* cells express the more general ventral interneuron markers Lim-1/2 and Pax-2 in both normal and *En-1* mutant spinal cords. Thus, it seems that *En-1* expression is downstream of the mechanisms that control early d/v patterning in the ventral spinal cord.

It is likely that many or all phenotypic characteristics of interneurons are ultimately controlled by different transcription factors that are expressed in subgroups of interneurons in the spinal cord. In the mature vertebrate spinal cord, functionally similar classes of interneurons are generally found in localized regions of the gray matter (Thomas and Wilson, 1965; Jankowska and Lindstrom, 1972). It has been shown that subsets of motoneurons express unique combinations of LIM-domain genes, which corre-

late with their initial axon projection patterns (Tsuchida et al., 1994). Because in general neuronal projections are initiated early and are similar for closely related groups of cells (Yaginuma et al., 1990; Silos-Santiago and Snider, 1992, 1994; Eide and Glover, 1996), it is possible that the expression of some transcription factors in groups of interneurons may be important in specifying early projection patterns. Consistent with this is the observation that *En-1*-expressing interneurons seem to share similar projections in the spinal cord. Although we have not systematically studied the projections of *En-1* interneurons in *En-1* mutants, we have observed that they do not alter their normal projections to become commissural or ipsilateral/association interneurons in the absence of *En-1* (data not shown). Thus, if *En-1* specifies the connectivity or projections of these neurons it likely does so on a much finer scale than studied here. It is also possible that *En-1* or other region-specific patterns of gene expression play a role in organizing afferent inputs to groups of related interneurons.

Taken together, these studies provide a set of marker genes for studying early d/v patterning at a fine scale in normal and mutant embryos, and for determining the influence of secreted inducing factors on this process. Furthermore, this analysis suggests that some of these genes may play critical roles in early or late interneuron development.

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