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Requirement for LIM Homeobox Gene *Isl1* in Motor Neuron Generation Reveals a Motor Neuron–Dependent Step in Interneuron Differentiation

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Summary

Motor neuron differentiation is accompanied by the expression of a LIM homeodomain transcription factor, Islet1 (ISL1). To assess the involvement of ISL1 in the generation of motor neurons, we analyzed cell differentiation in the neural tube of embryos in which ISL1 expression has been eliminated by gene targeting. Motor neurons are not generated without ISL1, although many other aspects of cell differentiation in the neural tube occur normally. A population of interneurons that express Engrailed1 (EN1), however, also fails to differentiate in *Isl1* mutant embryos. The differentiation of EN1$^+$ interneurons can be induced in both wild-type and mutant neural tissue by regions of the neural tube that contain motor neurons. These results show that ISL1 is required for the generation of motor neurons and suggest that motor neuron generation is required for the subsequent differentiation of certain interneurons.

Introduction

The central nervous system (CNS) controls motor function through the activity of motor neurons and their attendant interneurons in the ventral horn of the spinal cord. Motor neurons and ventral interneurons are generated during embryonic development from progenitor cells located in the ventral region of the neural tube (Hamburger, 1948; Langman and Haden, 1970). This process is dependent on an inductive signal from axial mesodermal cells of the notochord that is mediated by the Sonic hedgehog (SHH) protein (Yamada et al., 1991, 1993; Ericson et al., 1992, 1995; Roelink et al., 1994, 1995; Marti et al., 1995; Tanabe et al., 1995). The subsequent steps involved in the generation of motor neurons and ventral interneurons, however, remain to be defined.

The first molecular indicator of motor neuron differentiation is the expression of the transcription factor Islet1 (ISL1) (Karlsson et al., 1990; Ericson et al., 1992; Tsuchida et al., 1994). ISL1 is a member of a family of homeodomain-containing proteins that possess an amino-terminal pair of zinc-binding LIM domains (Dawid et al., 1995). Motor neurons in the embryonic spinal cord express four additional LIM homeobox genes: *Isl2*, *Lim3*, *Gsh4*, and *Lim1* (Tsuchida et al., 1994; Li et al., 1994; Appel et al., 1995; Tokumoto et al., 1995). The combinatorial expression of these genes delineates classes of motor neurons that are organized into discrete columns in the spinal cord and that innervate different groups of target muscles (Tsuchida et al., 1994). *Isl1* is expressed by all classes of motor neurons, and in each class its expression precedes that of other LIM homeobox genes, thus defining an early and common step in motor neuron differentiation.

Vertebrate LIM homeodomain proteins have structural counterparts in Drosophila melanogaster and Caenorhabditis elegans. Genetic studies in these organisms have provided evidence that this family of transcription factors regulates cell fate and axonal projection patterns. In C. elegans, *mec-3* controls the differentiation of mechanosensory neurons and *lin-11* the asymmetric divisions of several cell types (Way and Chalfie, 1988; Freyd et al., 1990). In Drosophila, *apterous* establishes the dorsal fate of cells in the wing imaginal disc (Blair, 1995) and regulates the axonal trajectory of certain interneurons (Lundgren et al., 1995). Inactivation of the mouse *Lim1*, *Lim3* (*Lhx3*), and *Gsh4* genes by targeted mutation leads to perturbations in development (Li et al., 1994; Shawlot and Behringer, 1995; Sheng et al., 1996), suggesting that vertebrate LIM homeobox genes serve functions similar to those of their invertebrate counterparts. The pattern of expression of LIM homeodomain proteins by embryonic motor neurons (Tsuchida et al., 1994) therefore raises the possibility that members of this family of transcription factors are involved in specifying motor neuron identity and connectivity.

To begin to address this issue, we have analyzed the fate of neural cells after elimination of ISL1 function by targeted mutation of the mouse *Isl1* gene and by antisense oligonucleotide-mediated ablation of ISL1 expression in chick neural tube cells in vitro. The generation of motor neurons does not occur in the absence of ISL1 expression, providing evidence that LIM homeodomain proteins are required for the differentiation of subsets of neurons in the vertebrate CNS.

The elimination of ISL1 is not accompanied by a general perturbation in the dorsoventral patterning of the neural tube or in an impairment in the capacity for neuronal differentiation. A subclass of ventral interneurons that express the homeodomain protein Engrailed1 (EN1) is, however, missing from the spinal cord of mice lacking ISL1 function. The differentiation of these EN1$^+$ interneurons can be restored in neural tissue isolated from *Isl1* mutant mice by a signal derived from the region of the neural tube of normal embryos that contains newly generated motor neurons. These findings suggest that a signal provided by motor neurons is required for the differentiation of certain classes of interneurons in the developing spinal cord.

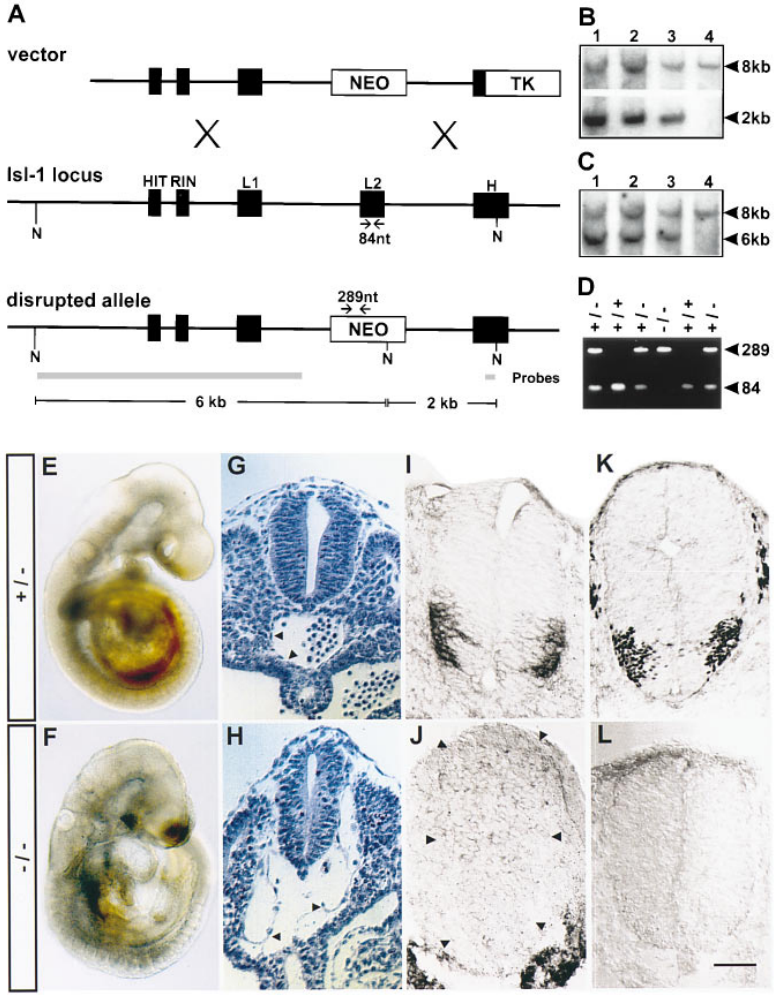

Figure 1. Generation of a Null Allele at the *Isl1* Locus and Analysis of *Isl1* Mutant Embryos

(A) Schematic representation of the targeting vector, the wild-type allele, and the disrupted *Isl1* allele generated by homologous recombination. N, NcoI restriction sites. The HIT, RIN, LIM domains (L1 and L2), and homeodomain (H) exons are shown.

(B and C) Southern blot analysis of recombinant ES clone DNA (lanes 1–3) and wild-type DNA (lane 4) using the two probes shown in (A). Positions of the 8 kb wild-type and 6 kb and 2 kb fragments derived from the disrupted allele are indicated.

(D) Polymerase chain reaction (PCR) analysis of genotype of yolk sac DNA from E9.5 embryos generated from heterozygous intercrosses, using primer locations indicated in (A).

(E and F) Morphology of E9.5 heterozygote (E) and *Isl1*$^{\Delta}$ homozygote (F) embryos.

(G and H) Hematoxylin- and eosin-stained sections showing the neural tube, paraxial mesoderm, and endoderm of heterozygote (G) and *Isl1*$^{\Delta}$ homozygote (H) embryos analyzed at E9.5 (~25 somite stage). Note the disruption of the dorsal aorta (arrowheads) in *Isl1*$^{\Delta}$ homozygote embryos.

(I and J) In situ localization of *Isl1* transcript in heterozygote embryos (I) and the disrupted *Isl1*$^{\Delta}$ transcript in homozygote embryos (J). *Isl1* transcript is detectable in the ventral neural tube and in ventral mesoderm and endoderm (data not shown) of heterozygotes. The disrupted *Isl1*$^{\Delta}$ transcript is not detected in the neural tube of homozygote embryos at E9.5 but is expressed in the mesoderm ventral to the neural tube.

(K and L) ISL1 protein expression in heterozygote (K) and *Isl1*$^{\Delta}$ homozygote (L) embryos at E9.5. No ISL1 protein is detected in any tissue in *Isl1*$^{\Delta}$ homozygote embryos. Images in (G)–(L) are representative of 11–15 embryos examined.

Scale bar in (E), 450 μm; in (F), 250 μm; in (G), 80 μm; in (H), (I), and (K), 50 μm; in (J) and (L), 30 μm.

Results

Generation of Mice Lacking *Isl1* Function

To generate a targeted mutation in the mouse *Isl1* gene, we used DNA from an *Isl1* genomic clone to construct a targeting vector that included 4.2 kb of 5′ DNA and 1.6 kb of 3′ DNA flanking a neomycin resistance (*neo*) gene (Figure 1A). The targeting vector was electroporated into mouse embryonic stem (ES) cells and DNA was isolated from G418-resistant, FIAU-insensitive ES colonies and analyzed by Southern blotting. The wild-type locus generated an 8 kb fragment and the disrupted locus, 6 kb and 2 kb fragments (Figures 1B and 1C). Of 72 ES cell colonies screened, eight contained the mutant allele and had undergone a single homologous integration at the *Isl1* locus (Figure 1C; data not shown).

Four chimeric mice were generated from two independent ES cell clones that transmitted the disrupted *Isl1* allele (termed *Isl1*$^{\Delta}$) through the germline. One line was backcrossed to C57BL/6J mice for two to four generations, and the other was maintained in a 129/Sv background. Heterozygote mice were bred to produce homozygous mutant offspring (Figure 1D), and similar phenotypes were obtained with lines generated from the two clones, independent of strain background.

Mice Lacking ISL1 Die during Embryogenesis

The *Isl1*$^{\Delta}$ allele exhibited Mendelian segregation in embryos examined from E8.5 to E11.5 (Table 1). At E8.5, *Isl1*$^{\Delta}$ homozygote embryos appeared similar in size and morphology to their heterozygote and wild-type littermates (data not shown). By E9.0–E9.5, however, *Isl1*$^{\Delta}$ homozygote embryos were markedly smaller than heterozygote or wild-type embryos (Figures 1E and 1F). *Isl1*$^{\Delta}$ homozygote embryos examined at E10.5 showed no advance in development compared with E9.5 (data not shown). At E11.5, *Isl1*$^{\Delta}$ homozygote embryos were necrotic, and embryos were not recovered at later stages (Table 1; data not shown). Thus, *Isl1*$^{\Delta}$ homozygote embryos are arrested in their development soon after E9.5. Despite this, mutant embryos exhibited an overtly normal organization of neural, mesodermal, and endodermal tissues (Figures 1E and 1F; data not shown).

Table 1. Analysis of Genotype of F2 Embryos at Different Stages of Development

Genotype	E8.5 (%)	E9.5 (%)	E10.5 (%)	E11.5 (%)	P10 (%)
+/+	9 (43)	35 (26)	6 (19)	4 (31)	52 (50)
+/−	8 (38)	69 (51)	20 (61)	6 (46)	53 (50)
−/−	4 (19)	32 (24)	7 (21)	3 (23)	0

Note that the number of wild-type and heterozygote embryos obtained at P10 does not conform to Mendelian frequencies. The reason for this has not been determined. These numbers are representative of >300 embryos examined.

Histological analysis of *Isl1*$^{\Delta}$ homozygote embryos at E9.5–E10.5, however, revealed abnormalities in the organization of the vascular endothelium and its surrounding mesenchyme, notably a disruption in the formation of the dorsal aorta (Figures 1G and 1H). An impairment in vascular development is therefore a possible cause of the embryonic lethality in *Isl1*$^{\Delta}$ homozygotes.

Isl1$^{\Delta}$ homozygote embryos analyzed at E9.5 expressed the *Isl1*$^{\Delta}$ transcript in mesodermal cells, but no expression was detected in neural tissues (Figures 1I and 1J; data not shown). No ISL1 immunoreactivity was detected in any tissues in *Isl1*$^{\Delta}$ homozygote embryos (Figures 1K and 1L), indicating that the targeted mutation eliminates expression of ISL1 protein. The loss of ISL1 perturbs the differentiation of many of the cell types that normally express the protein, including sensory neurons of dorsal root and cranial sensory ganglia, cells of the endocrine pancreas and of the splanchnic mesenchyme (S. L. P., T. M. J., T. E., and H. Edlund, unpublished data). The present study, however, is confined to an analysis of the involvement of ISL1 in the development of motor neurons and other cell types generated in the neural tube.

Motor Neuron Differentiation Does Not Occur in the Absence of ISL1

To determine whether ISL1 is a necessary component in the pathway leading to motor neuron differentiation, we examined, in *Isl1*$^{\Delta}$ homozygote and heterozygote embryos, several markers that are expressed by newly differentiated motor neurons: the homeobox genes *Isl2*, *HB9*, *Lim3*, and *Gsh4*; the surface membrane proteins TAG1, polysialylated neural cell adhesion molecule (N-CAM), and Neu differentiation factor (*NDF*); and the enzyme choline acetyltransferase (*ChAT*). In E9.5 ($\sim$25 somite stage) heterozygote embryos, each marker was expressed by motor neurons in the ventral region of the hindbrain and cervical spinal cord (Figures 2A–2F; data not shown). In somite-matched *Isl1*$^{\Delta}$ homozygote embryos, none of these markers was expressed by cells in the hindbrain or spinal cord (Figures 2G–2L; data not shown). These results indicate that motor neuron differentiation has not occurred at E9.5 in the hindbrain and spinal cord of mouse embryos lacking ISL1.

We next determined whether there is a more widespread perturbation in neural tube differentiation in *Isl1*$^{\Delta}$ homozygote embryos examined at E9.5. The dorsoventral pattern of the neural tube appeared similar in heterozygote and mutant embryos, as assessed by the dorsal restriction in expression of *Pax3* and MSX1/MSX2 and by the ventral restriction in expression of *Nkx2.2* (Figures 2M–2O and 2S–2U). Notochord and floor plate differentiation also appeared normal in *Isl1*$^{\Delta}$ homozygote embryos as judged by expression of HNF3β (Figures 2P and 2V). Moreover, *Shh* expression was detected at similar levels in the notochord and floor plate of heterozygote and *Isl1*$^{\Delta}$ homozygote embryos (Figures 2Q and 2W), providing evidence that the signal that triggers motor neuron differentiation is intact in mice lacking ISL1. In addition, the ability of cells in the neural tube to differentiate into LIM1^{+}/LIM2^{+} interneurons was not impaired (Figures 2R and 2X). These results show that there is not a widespread perturbation of neural tube development in mice lacking ISL1.

Since *Isl1*$^{\Delta}$ homozygote embryos failed to develop normally beyond E9.5, we were concerned that motor neuron differentiation was not blocked but only delayed. To permit neuronal differentiation to occur over a longer period than was possible in vivo, we isolated segments of the neural tube from thoracic and lumbar levels of 20–25 somite stage *Isl1*$^{\Delta}$ homozygote and heterozygote embryos and grew them in vitro for 72 hr. This additional period of time in vivo is sufficient for the generation of all motor neurons (Nornes and Carry, 1978).

At the time of isolation, neural tube explants derived from heterozygote embryos expressed few, if any, ISL2^{+} motor neurons and low levels of *HB9* mRNA (data not shown). The number of ISL2^{+} motor neurons and the expression of *HB9* mRNA increased markedly over the 72 hr culture period (Figures 3B and 3E). In contrast, explants from *Isl1*$^{\Delta}$ homozygote embryos did not contain ISL2^{+} neurons or express *HB9* mRNA after 72 hr in vitro (Figures 3B, 3D, and 3E). The extent of differentiation of HNF3β^{+} floor plate cells, LIM1^{+}/LIM2^{+} interneurons, and neurofilament expression was, however, similar in neural tube explants derived from *Isl1*$^{\Delta}$ homozygote and heterozygote embryos (Figures 3A–3E; data not shown). These results provide evidence that the absence of motor neurons in mice lacking ISL1 is not simply the result of a delay in motor neuron differentiation and reflects the inability of neural progenitors to generate motor neurons.

Motor Neuron Differentiation Depends on ISL1 Function in Neural Cells

The results described above leave open the possibility that the absence of motor neurons in mice lacking ISL1 is secondary to a defect in other cell types. For example, an early signal from ventral mesodermal cells that normally express ISL1 (see Figure 1J; data not shown) could confer neural plate cells with the competence to generate motor neurons, and this signal might be lost under conditions in which ISL1 function is eliminated from mesodermal cells.

To address this issue, we examined whether motor neuron differentiation depends on *Isl1* function in neural cells. Antisense oligonucleotides directed against *Isl1* transcripts were used in an attempt to eliminate ISL1 expression in isolated neural tube explants grown in

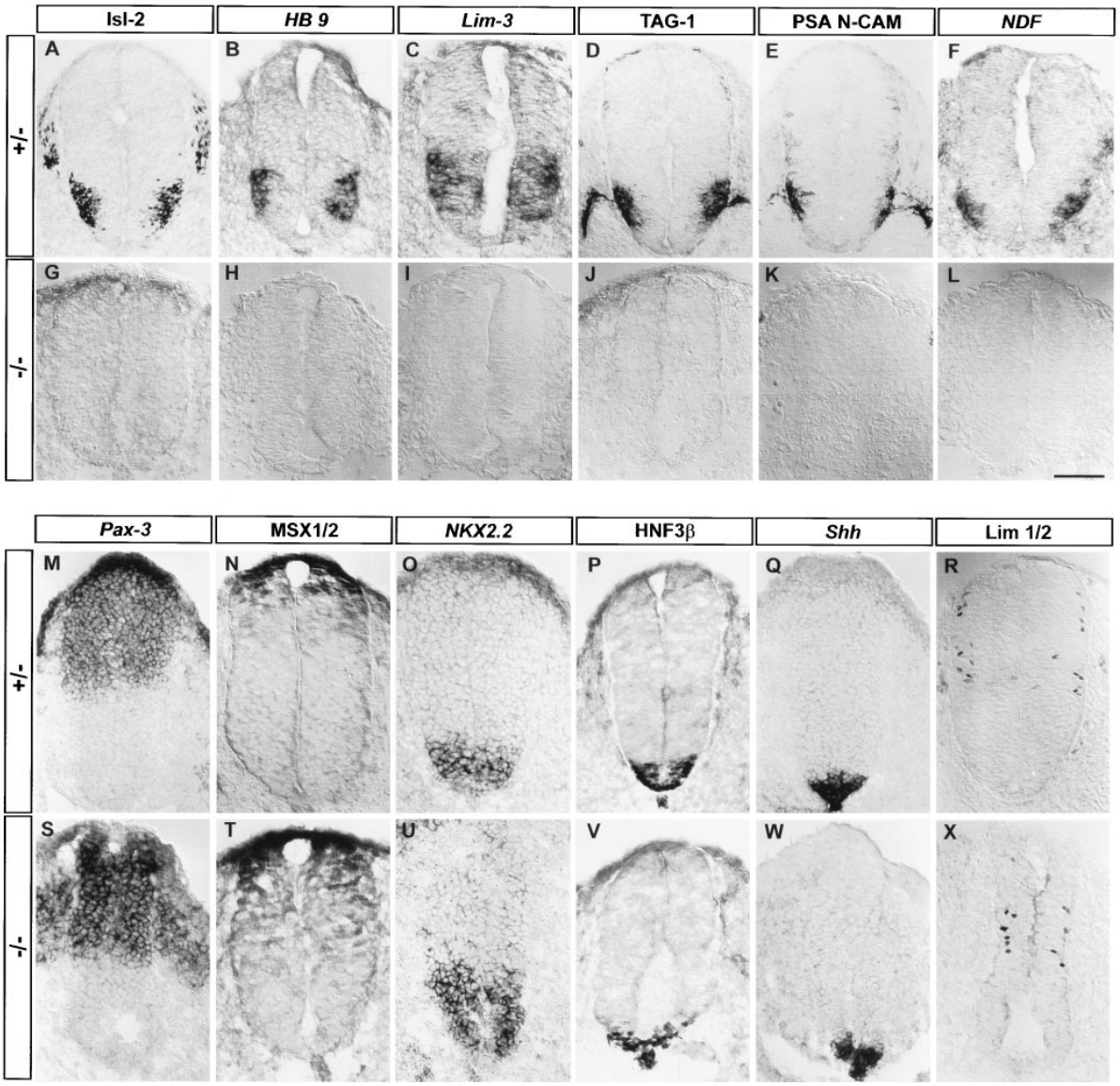

Figure 2. Gene Expression in the Neural Tube of Mice Lacking ISL1

(A–L) Expression of motor neuron markers in the cervical region of the neural tube of ~25 somite stage heterozygote (A–F) and *Isl1*$^{\Delta}$ homozygote (G–L) mouse embryos.

(M–X) Expression of markers of cell pattern in the cervical neural tube of ~25 somite stage heterozygote (M–R) and *Isl1*$^{\Delta}$ homozygote (S–X) embryos. (+/−), heterozygote embryos; (−/−), *Isl1*$^{\Delta}$ homozygote embryos. Images are representative of 10–12 embryos examined.

Scale bar in (A)–(F) and (M)–(R), 75 μm; in (G)–(L) and (S)–(X), 50 μm.

vitro. These experiments were performed on chick neural tissue since the early differentiation of motor neurons in vitro has been studied most extensively in this species (Yamada et al., 1993). Stage 10 cervical neural tube explants were grown in vitro for 36 hr in the presence of *Isl1* or control antisense oligonucleotides. Explants grown with control oligonucleotides generated a ventrolateral column of motor neurons as assessed by ISL1^{+}/ISL2^{+}/SC1^{+} cells (Figures 4A and 4K) and by expression of *Isl1* and *ChAT* mRNAs (Figure 4J; data not shown). Explants grown with *Isl1* antisense oligonucleotides showed a concentration-dependent decrease in the number of ISL1^{+}/ISL2^{+} cells (Figures 4E and 4I), ISL1^{+}/ISL2^{+}/SC1^{+} cells (Figure 4L), and *Isl1* and *ChAT* mRNAs (Figure 4J; data not shown). The differentiation of floor plate cells as assessed by ventral midline expression of SC1 (Figures 4K and 4L), interneurons as assessed by 3A10 (Figures 4B and 4F) and LIM1/LIM2 expression (Figures 4C, 4G, and 4I), and neural crest cells (Figures 4D and 4H) was similar in explants grown with *Isl1* or control antisense oligonucleotides.

The selective loss of ISL2 and *ChAT* and the absence of SC1 expression in ventrolateral regions of neural tube explants grown with *Isl1* antisense olignucleotides provide evidence that motor neuron differentiation fails to occur in chick neural tube explants in which ISL1 expression has been eliminated. These results support the idea that the loss of ISL1 function in neural cells is sufficient

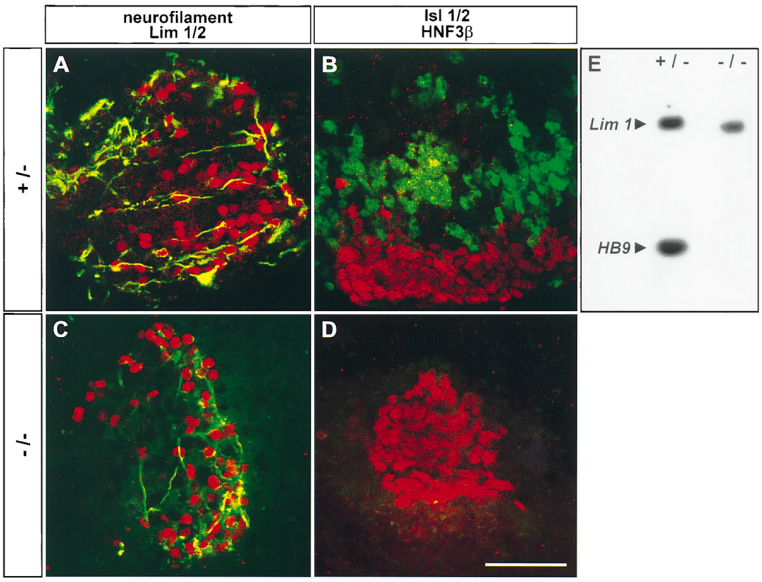

Figure 3. Failure of Motor Neuron Generation in Cultured Neural Tube Explants Derived from *Isl1*$^{\Delta}$ Homozygote Embryos

Neural tube explants were isolated from thoracic and lumbar regions of 20–25 somite stage *Isl1*$^{\Delta}$ homozygote and heterozygote embryos and grown in vitro for 72 hr. Explants from heterozygote embryos contain numerous LIM1^{+}/LIM2^{+} (red) and neurofilament-positive (green) interneurons (A), HNF3β^{+} (red) floor plate cells, and ISL2^{+} (green) motor neurons (B) and express the motor neuron–specific *HB9* mRNA (E). Explants derived from *Isl1*$^{\Delta}$ homozygote embryos contain LIM1^{+}/LIM2^{+} (red) and neurofilament-positive (green) interneurons (C) and HNF3β^{+} (red) floor plate cells, but do not give rise to ISL2^{+} (green) cells (D) and do not express *HB9* mRNA (E). Similar results were obtained in explants derived from six to eight embryos of each genotype. Scale bar in (A)–(D), 30 μm.

to account for the absence of motor neurons in *Isl1*$^{\Delta}$ homozygote mouse embryos.

Fate of Prospective Motor Neurons in the Absence of ISL1

We next examined the fate of cells destined to give rise to motor neurons. In wild-type embryos, dividing progenitor cells in the ventral neural tube are multipotential, giving rise both to motor neurons and to interneurons (Leber et al., 1990). It seemed possible, therefore, that cells destined to give rise to motor neurons might, in the absence of ISL1, differentiate into interneurons. In this case the ventral neural tube of *Isl1*$^{\Delta}$ homozygote embryos might be expected to contain large numbers of interneurons, despite the absence of motor neurons. To test this possibility, we examined the expression of three general neuronal markers: *SCG10*, the 155 kDa neurofilament subunit, and a neuronal β-tubulin isoform. The ventral neural tube of *Isl1*$^{\Delta}$ homozygote embryos was markedly depleted of *SCG10*$^{+}$, 155 kDa neurofilament-positive subunit, and β-tubulin-positive neurons (Figures 5A–5F). Thus, the absence of motor neurons in *Isl1*$^{\Delta}$ homozygote embryos is not accompanied by the differentiation of large numbers of ventral interneurons, suggesting that motor neurons do not convert to interneurons in the absence of ISL1.

There was a marked thinning of the neuroepithelium in the ventral neural tube of *Isl1*$^{\Delta}$ homozygote embryos (see Figures 2V and 2X), suggesting that cells destined to generate motor neurons do not remain undifferentiated. To test whether cells might instead be eliminated, we analyzed the neural tube of *Isl1*$^{\Delta}$ homozygote and heterozygote embryos for signs of apoptotic cell death. Heterozygote embryos examined at E9.5 showed a very low incidence of cell death in the ventral neural tube at any rostrocaudal level as assessed by the presence of condensed nuclei (data not shown) and by end labeling of fragmented DNA (Figures 5G, 5I, and 5K). The neural tube of *Isl1*$^{\Delta}$ homozygote embryos showed a similarly low incidence of apoptotic cell death at caudal (prospective lumbar) levels, which normally have not begun to generate motor neurons (Figure 5H). More rostrally, at cervical spinal and hindbrain levels in which motor neurons normally are generated, there was a marked increase in the incidence of apoptotic cell death, and this increase was restricted to the ventral neural tube (Figures 5J and 5L; Table 2). These results provide evidence that cells destined to differentiate into motor neurons die by apoptosis in the absence of ISL1.

To examine whether a decrease in cell proliferation might also contribute to the depletion of cells in the ventral neural tube of *Isl1*$^{\Delta}$ homozygote embryos, we counted the number of mitotic cells, assessed by mpm-2 immunoreactivity. There was an ~73% decrease in the number of mpm-2^{+} cells in the ventral neural tube of *Isl1*$^{\Delta}$ homozygote embryos (Figures 5M and 5N; Table 3). This decrease in ventral mitotic cells was apparent only at levels at which motor neurons are normally generated (Table 3). The loss of motor neurons may therefore have an indirect effect on cell proliferation in the ventral neural tube (see Discussion).

EN1^{+} Interneurons Fail to Differentiate in Mice Lacking ISL1

Although some interneurons are generated in the neural tube of *Isl1*$^{\Delta}$ homozygote embryos, it seemed possible that certain classes of interneurons, in particular, those located close to motor neurons, might be affected. One such class of interneurons expresses the homeodomain protein EN1 (Davis and Joyner, 1988; Davidson et al., 1988). We therefore determined whether the absence of motor neurons influences the differentiation of EN1 interneurons.

In wild-type embryos, EN1^{+} interneurons were first detected at E9.5, shortly after the onset of motor neuron differentiation, and were found in a restricted region close to the dorsal border of the motor neuron population (Figures 6A and 6B). The number of EN1^{+} interneurons increased steadily from E10.5, and these neurons

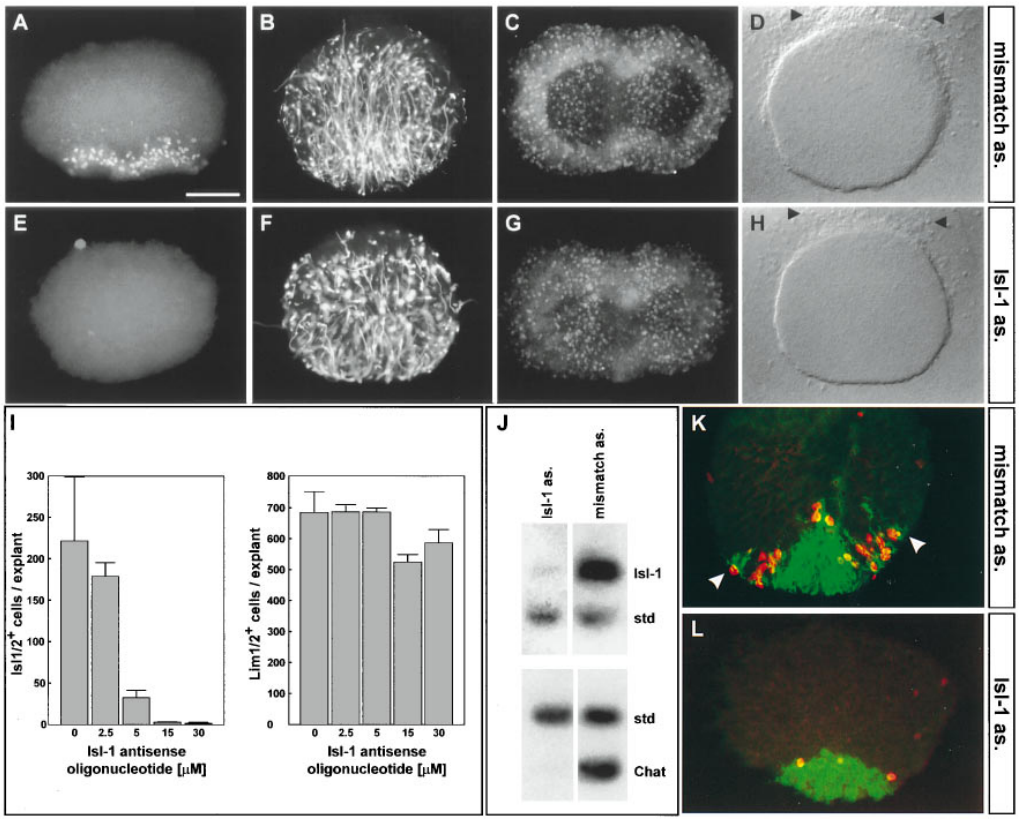

Figure 4. Antisense *Isl1* Oligonucleotides Block Motor Neuron Differentiation In Vitro

(A–H) Lateral views of 1–2 somite-length cervical neural tube explants of stage 10 chick neural tube grown in vitro for 36 hr in the presence of mismatch (control) (A–D) or *Isl1* antisense (E–H) oligonucleotide. In all examples shown, control and *Isl1* oligonucleotide was added to a final concentration of 10 μM.

(A and E) ISL1$^+$/ISL2$^+$ cells are detected in the ventral region of neural tube explants grown in control (A), but not *Isl1* antisense (E), oligonucleotides.

(B and F) Expression of the 3A10 neuronal antigen in control (B) and *Isl1* antisense (F) oligonucleotides.

(C and G) Detection of LIM1$^+$/LIM2$^+$ interneurons in neural tube explants grown in control (C) and *Isl1* antisense (G) oligonucleotides. In these explants, the neural tube has been opened at the dorsal midline.

(D and H) Interference contrast micrograph showing presumed neural crest cells (arrowheads) that have migrated from the dorsal region of neural tube explants grown in control (D) or *Isl1* antisense (H) oligonucleotides.

(I) Quantitation of ISL1$^+$/ISL2$^+$ and LIM1$^+$/LIM2$^+$ neurons in neural tube explants grown in different concentrations of *Isl1* antisense oligonucleotides. Bars show mean ± SEM obtained from at least eight explants at each concentration.

(J) Reverse transcription–PCR assay for *Isl1* and *ChAT* transcripts. Expression of *Isl1* and *ChAT* transcript is greatly reduced in neural tube explants grown in *Isl1* antisense when compared with control oligonucleotides. Amplification of an internal standard template (std) is shown.

(K and L) Transverse sections of the ventral region of neural tube explants grown in control (K) or *Isl1* (L) antisense oligonucleotide for 36 hr and labeled with antibodies directed against ISL1/ISL2 (red) and SC1 (green). In the presence of the mismatch oligonucleotide (K), a ventral midline group of ISL1$^-$/ISL2$^-$/SC1$^+$ (floor plate) cells is present, together with a more lateral group of ISL1$^+$/ISL2$^+$/SC1$^+$ motor neurons (arrowheads). In the presence of *Isl1* antisense oligonucleotide (L), the ventral midline group of ISL1$^-$/ISL2$^-$/SC1$^+$ cells is maintained, but there are no SC1$^+$ cells in the ventrolateral region that normally contains motor neurons. Similar results were obtained in eight explants for each assay condition.

Scale bar in (A)–(H), 40 μm; in (K) and (L), 18 μm.

migrated ventrally from E12, settling close to motor neurons (Figures 6C and 6D). EN1 and ISL1/ISL2 were not coexpressed by single neurons in the spinal cord at any stage examined (Figures 6B–6D; data not shown). The temporal and spatial relationship between ISL1$^+$/ISL2$^+$ motor neurons and EN1$^+$ interneurons in the spinal cord was conserved in chick (data not shown).

Strikingly, EN1$^+$ interneurons were not detected in the neural tube of 25 somite stage *Isl1*$^\Delta$ homozygote embryos (Figure 6E). To exclude that there was simply a delay in the differentiation of EN1$^+$ interneurons, we cultured cervical neural tube explants isolated from 20–25 somite stage heterozygote and *Isl1*$^\Delta$ homozygote embryos for an additional 48 hr in vitro. EN1$^+$ interneurons were detected in neural tube explants derived from heterozygote but not from *Isl1*$^\Delta$ homozygote embryos (Figures 6F and 6G).

One possible explanation for the absence of EN1$^+$ interneurons in *Isl1*$^\Delta$ homozygote embryos is that ISL1 is expressed by and required in dividing progenitor cells that give rise both to motor neurons and to EN1$^+$ interneurons. We therefore examined whether dividing cells in the ventral neural tube of wild-type embryos normally express ISL1. E9.5 embryos were incubated in

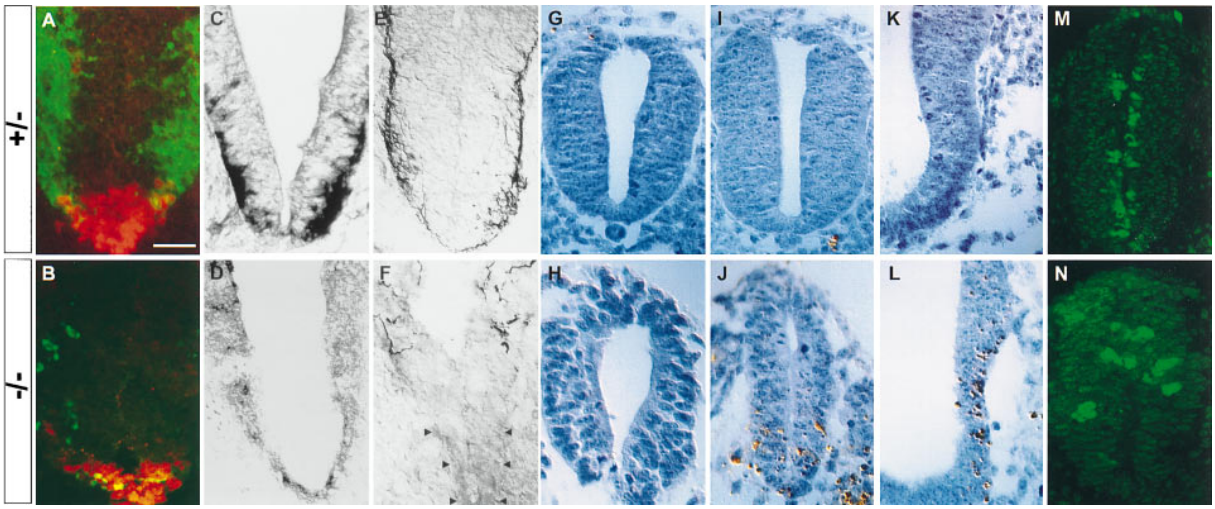

Figure 5. Differentiation and Fate of Ventral Neurons in Heterozygote and *Isl1*$^{\Delta}$ Homozygote Embryos

(A and B) Ventral neural tube of E9.5 heterozygote (A) and *Isl1*$^{\Delta}$ homozygote (B) embryos labeled to detect HNF3β^{+} (red) floor plate cells and neuronal β-tubulin-positive cells (green). In heterozygote embryos, most β-tubulin-positive cells correspond to motor neurons, and there is a marked depletion of β-tubulin-positive cells in the ventral neural tube of *Isl1*$^{\Delta}$ homozygote embryos.

(C and D) Localization of *SCG10* transcript in the hindbrain of E9.5 heterozygote (C) and *Isl1*$^{\Delta}$ homozygote (D) embryos. In heterozygote embryos, *SCG10*$^{+}$ cells, primarily motor neurons, are detected in ventral regions of the hindbrain (C), but few *SCG10*$^{+}$ cells are detected ventrally in *Isl1*$^{\Delta}$ homozygote embryos (D).

(E and F) Localization of neurofilament protein in the hindbrain of E9.5 heterozygote (E) and *Isl1*$^{\Delta}$ homozygote (F) embryos. Most neurofilament-positive cells in heterozygote embryos correspond to motor neurons, and few neurofilament-positive cells are detected in the ventral region of *Isl1*$^{\Delta}$ homozygote embryos.

(G, I, and K) Sections of E9.5 heterozygote embryos showing apoptotic cells, detected by end labeling of fragmented DNA. In heterozygote embryos, few apoptotic cells are detected in the caudal neural tube at a level at which motor neurons have not differentiated (G), at a spinal cord level at which differentiated motor neurons are present (I), or at a hindbrain level at which extensive motor neuron differentiation has occurred (K).

(H, J, and L) Sections through *Isl1*$^{\Delta}$ homozygote embryos. At a caudal level at which motor neuron differentiation would not normally have occurred (H), few apoptotic cells are detected. At a spinal cord level at which motor neuron differentiation normally has occurred (J), there are many apoptotic profiles in the ventral spinal cord. At a hindbrain level that normally contains many motor neurons (L), there is also a high incidence of apoptotic profiles in the ventral region.

(M) Labeling of M phase mpm-2^{+} cells in the lumenal region of the neural tube of an E9.5 heterozygote embryo.

(N) Neural tube of an *Isl1*$^{\Delta}$ homozygote embryo showing a depletion of mpm-2^{+} cells in the ventral half of the neural tube.

Scale bar in (A)–(F), 30 μm; in (G)–(J) and (M), 60 μm; in (K) and (L), 25 μm; in (N), 34 μm.

vitro with bromodeoxyuridine (BrdU) (5 μM) for 45 min to detect cells in S phase and those that have recently progressed into G2 phase. No cells that incorporated BrdU expressed ISL1 (Figure 6H). Similarly, ISL1 was not detected in any mpm-2^{+} M phase cells (Figure 6I). These results argue against the possibility that ISL1 is expressed in dividing progenitors within the ventral neural tube. Thus, the loss of EN1^{+} interneurons does not appear to result from a transient requirement for ISL1 in the progenitors of EN1 interneurons. Experiments described below provide independent evidence that there is no cell-intrinsic requirement for ISL1 in EN1^{+} interneurons at any stage in their differentiation.

Induction of EN1^{+} Interneurons by Regions of the Neural Tube That Contain Motor Neurons

The results described above raise the possibility that the differentiation of EN1^{+} interneurons depends on the prior generation of motor neurons. This possibility predicts several features of EN1^{+} interneuron differentiation in wild-type neural tissue: first, the differentiation of

Table 2. Distribution of Apoptotic Cells in the Neural Tube of Heterozygote and *Isl1*$^{\Delta}$ Homozygote Embryos

		Heterozygote		*Is1*$^{\Delta}$ Homozygote	
Tissue	Position	Cells/Section	V/D Ratio	Cells/Section	V/D Ratio
Hindbrain	D	3.8 ± 0.8	0.9	2.5 ± 0.8	9.2
	V	3.4 ± 0.9		23 ± 6.3	
Cervical spinal cord	D	1.1 ± 0.7	0.82	0.4 ± 0.2	23.7
	V	0.9 ± 0.3		9.5 ± 1.6	

Numbers are mean ± SEM; n = 10 sections analyzed at each level. The V/D ratio indicates ratio of labeled cells in the ventral (V) and dorsal (D) halves of the neural tube. The decrease in the number of apoptotic (fragmented DNA labeled) cells in the dorsal neural tube of *Isl1*$^{\Delta}$ homozygote embryos compared with heterozygotes probably results from the overall decrease in size of mutant embryos.

Table 3. Distribution of Mitotic Cells in the Neural Tube of Heterozygote and *Isl1*$^{\Delta}$ Homozygote Embryos

		Heterozygote		*Isl1*$^{\Delta}$ Homozygote	
Tissue	Position	Cells/Section	V/D Ratio	Cells/Section	V/D Ratio
Cervical spinal cord	D	18 ± 2.6	0.83	6.1 ± 1.0	0.27
	V	15 ± 3.0		1.6 ± 0.4	
Lumbar spinal cord	D	6.6 ± 2.1	0.91	5.7 ± 1.8	1.0
	V	6.0 ± 1.9		5.7 ± 2.8	

Numbers are mean ± SEM; n = 8–11 sections analyzed at each level. The V/D ratio indicates ratio of labeled cells in the ventral (V) and dorsal (D) halves of the neural tube. The decrease in the number of mitotic (mpm-2^{+}) cells in the dorsal neural tube is *Isl1*$^{\Delta}$ homozygote embryos compared with heterozygotes probably results from the overall decrease in size of mutant embryos. No motor neurons have differentiated at prospective lumbar spinal cord levels at the 25 somite stage examined.

EN1^{+} interneurons will not occur in the absence of motor neurons; second, the generation of motor neurons will be accompanied by the differentiation of EN1 interneurons; and, third, a signal from motor neurons will induce EN1^{+} interneurons in competent neural tissue.

To test these predictions, we examined the relationship between the differentiation of EN1^{+} interneurons and ISL1^{+}/ISL2^{+} motor neurons in chick neural plate explants in vitro. Previous studies have shown that motor neurons do not differentiate in explants derived from intermediate regions of the chick neural plate grown alone in vitro (Yamada et al., 1993; Tanabe et al., 1995). We therefore examined whether EN1^{+} interneurons differentiate independently of motor neurons in such explants. Intermediate neural plate explants grown alone in vitro for 48 hr did not give rise either to EN1^{+} interneurons or to ISL1^{+}/ISL2^{+} motor neurons (Figure 7A; data not shown). We next examined whether the notochord-mediated induction of motor neurons in intermediate neural plate explants is accompanied by the differentiation of EN1^{+} interneurons. Intermediate neural plate explants grown in contact with the notochord for 48 hr contained both EN1^{+} interneurons and ISL1^{+}/ISL2^{+} motor neurons (Figure 7B). In these explants, EN1^{+} interneurons were located farther from the notochord than were ISL1^{+}/ISL2^{+} motor neurons (Figure 7B).

We have also begun to address whether the differentiation of EN1^{+} interneurons depends on a signal provided by motor neurons. To provide a source of a potential motor neuron–derived signal, we isolated the ventrolateral region of the neural tube, devoid of ventral midline floor plate tissue, from stage 10–18 quail embryos. When grown in vitro for 24 hr, these ventrolateral explants contained motor neurons but not floor plate cells (Figure 7D; data not shown). Such quail ventrolateral explants induced EN1^{+} interneurons (Figure 7C), but not ISL1^{+}/ISL2^{+} motor neurons (Figure 7D), in chick intermediate neural plate explants. Quail dorsal neural tube tissue and somitic tissue did not induce EN1^{+} interneurons (Figures 7E and 7F) or ISL1^{+}/ISL2^{+} motor neurons (data not shown). Thus, neural tissue that contains newly differentiated motor neurons provides a signal that induces EN1^{+} neurons in intermediate neural plate explants without the accompanying differentiation of motor neurons.

Rescue of EN1^{+} Interneurons in the Neural Tube of Mice Lacking ISL1

These results led us to examine whether this neurally derived signal can also restore the differentiation of EN1^{+} interneurons in neural tube explants isolated from *Isl1*$^{\Delta}$ homozygote embryos. Quail ventrolateral neural tube explants induced EN1^{+} interneurons (Figure 7G), but not ISL1^{+}/ISL2^{+} motor neurons (data not shown), in such neural tube explants. Quail dorsal neural tube or somite explants did not possess this inductive activity (Figure 7H; data not shown).

These results show that a signal derived from regions of the neural tube that contain newly differentiated motor neurons restores the differentiation of EN1^{+} interneurons in neural tissue isolated from *Isl1*$^{\Delta}$ homozygote embryos. They also establish that ISL1 function is not required in a cell-autonomous manner at any stage in the differentiation of EN1^{+} interneurons.

Discussion

A Requirement for ISL1 in the Generation of Motor Neurons

In vertebrates, the early differentiation of motor neurons is associated with the expression of members of a family of LIM homeodomain transcription factors. One of these proteins, ISL1, is expressed by both somatic and visceral motor neurons, and its expression precedes that of other LIM homeodomain proteins (Tsuchida et al., 1994). The present study establishes that ISL1 is required for the generation of somatic and visceral motor neurons. Expression of the LIM homeobox genes *Isl2*, *Lim3*, *Gsh4*, and *Lim1* further delineates functional subclasses of motor neurons. (Tsuchida et al., 1994; Li et al., 1994; our unpublished data). Targeted mutation of mouse *Gsh4*, *Lim3*, and *Lim1* has indicated that these genes also have essential functions in development (Li et al., 1994; Shawlot and Behringer, 1995; Sheng et al., 1996), but their role in the diversification of motor neuron subclasses remains to be defined.

Although the present study establishes that ISL1 is required for motor neuron generation, the exact role of ISL1 in this process remains unclear. In part, this reflects uncertainty about the precise point at which cells commit to a motor neuron fate. Lineage analyses have shown that progenitor cells in the spinal cord are not committed to a motor neuron fate until close to their final cell division (Leber et al., 1990). Thus, motor neuron fate might be determined immediately before the final division of motor neuron progenitors, at a stage similar to that at which specific neuronal fates in the cerebral cortex appear to be determined (McConnell and Kaznowski, 1991). In the hindbrain, however, similar lineage studies have suggested that a restriction of progenitor cells to

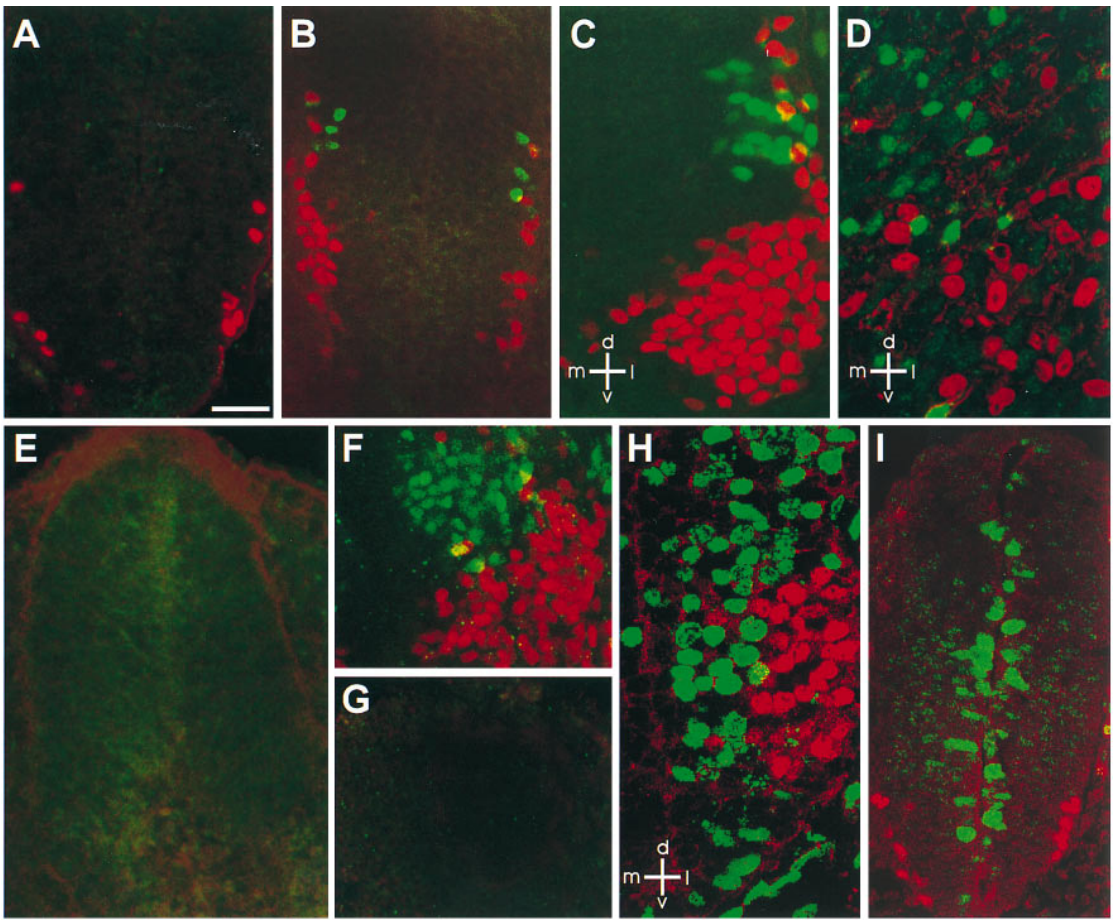

Figure 6. Differentiation of Motor Neurons and EN1$^+$ Interneurons in Embryonic Mouse Spinal Cord

(A–D) Detection of EN1$^+$ interneurons (green) and ISL1$^+$/ISL2$^+$ motor neurons (red) in the ventral spinal cord of mouse embryos.

(A) At caudal levels of E9.5 embryos, ISL1$^+$/ISL2$^+$ motor neurons, but not EN1$^+$ interneurons, are detected.

(B) At rostral levels of E9.5 embryos, EN1$^+$ interneurons are detected close to the dorsal-most group of ISL1$^+$/ISL2$^+$ motor neurons. Of 308 EN1$^+$ neurons, none expressed ISL1$^+$/ISL2$^+$, and of 1837 ISL1$^+$/ISL2$^+$ neurons examined, none expressed EN1.

(C) At E10.5, EN1$^+$ interneurons are located close to a dorsal cluster of ISL1$^+$/ISL2$^+$ motor neurons.

(D) At E15.5, EN1$^+$ interneurons have migrated ventrally and are intermingled with motor neurons in the ventral spinal cord.

(E) Section through E9.5 *Isl1*$^\Delta$ homozygote embryos showing that neither EN1$^+$ interneurons (green) nor ISL1$^+$/ISL2$^+$ motor neurons (red) are present.

(F) Section through the neural tube of a heterozygote embryo that had been grown in vitro for 48 hr. Both ISL1$^+$/ISL2$^+$ motor neurons (red) and EN1$^+$ interneurons (green) are detected.

(G) Section through E9.5 *Isl1*$^\Delta$ homozygote embryo grown in vitro for 48 hr, showing that no EN1$^+$ interneurons (green) or ISL1$^+$/ISL2$^+$ motor neurons (red) are detected.

(H) Ventral cervical neural tube of a wild-type E9.5 embryo labeled with BrdU for 45 min to detect cells in S and G2 phases of the cell cycle. No ISL1$^+$/ISL2$^+$ cells (red) have incorporated BrdU (green) (n = 2748 BrdU$^+$ cells; n = 2921 ISL1$^+$/ISL2$^+$ cells analyzed; data from four embryos).

(I) Thoracic neural tube of a wild-type E9.5 embryo labeled with mpm-2 (green) and ISL1/ISL2 (red) antibodies. No double-labeled cells are detected (n = 259 mpm-2$^+$ cells; n = 776 ISL1$^+$/ISL2$^+$ cells analyzed; data from three embryos).

Scale bar in (A), (B), (F), and (G), 25 μm; in (E), 15 μm; in (C) and (E), 30 μm; in (D) and (H), 10 μm.

a motor neuron fate may occur several divisions before the generation of a postmitotic motor neuron (Lumsden et al., 1994).

The present study indicates that ISL1 is expressed in prospective motor neurons only after they have undergone their final mitotic division. Thus, if motor neuron fate is determined prior to the final division of the progenitor cell, ISL1 could have a role in the developmental progression of a previously committed motor neuron. It cannot be excluded, however, that motor neuron fate in the spinal cord is determined only after the final division of the progenitor cell. If this is the case, ISL1 could be involved in the initial determination of motor neuron identity. However, misexpression of ISL1 in neural cells in vivo and in vitro does not result in the expression of later motor neuron markers (H. Roelink, M. Ensini, S. L. P., and T. M. J., unpublished data), and thus, even if ISL1 is involved in this commitment process, it is unlikely to be the sole determinant of motor neuron fate.

A Motor Neuron–Dependent Step in the Differentiation of Ventral Interneurons

The differentiation of a specific class of interneurons in the embryonic spinal cord, defined by expression of

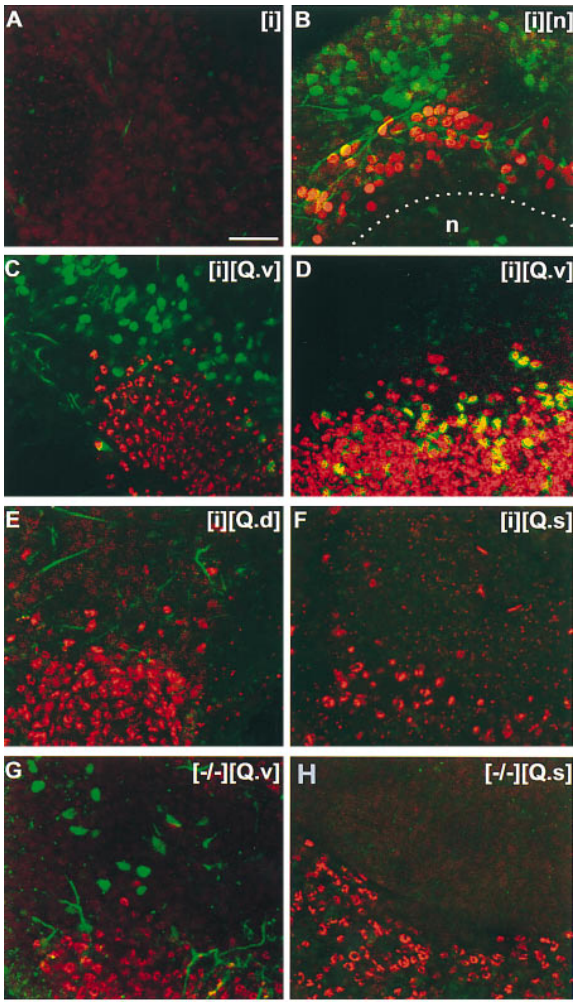

Figure 7. Induction of EN1$^+$ Interneuron Differentiation

(A–F) Differentiation of EN1$^+$ interneurons and ISL1$^+$/ISL2$^+$ motor neurons in stage 10 chick intermediate neural plate (i) explants grown in vitro for 48 hr

(A) No EN1$^+$ interneurons (green) or ISL1$^+$/ISL2$^+$ motor neurons (red) are detected in intermediate neural plate explants grown alone. Green wisps reflect a nonspecific cross reactivity of the anti-EN antibody with chick epitopes in axonal processes.

(B) Notochord (n) induces both ISL1$^+$/ISL2$^+$ motor neurons (red) and EN1$^+$ interneurons (green) in intermediate neural plate explants. ISL1$^+$/ISL2$^+$ motor neurons are consistently located closer to the notochord than are EN1$^+$ interneurons.

(C) Quail ventrolateral (Qv) neural tube tissue (QCPN$^+$; red) induces EN1$^+$ interneurons (green) in chick intermediate neural plate explants.

(D) Quail ventrolateral neural tube tissue (QCPN$^+$; red) does not induce ISL1$^+$/ISL2$^+$ motor neurons (green) in chick intermediate neural plate explants. ISL1$^+$/ISL2$^+$ motor neurons (yellow cells) are detected in the quail tissue close to the junction with chick tissue.

(E) Quail dorsal (Qd) neural tube tissue (QCPN$^+$; red) does not induce EN1$^+$ interneurons (green) in chick intermediate neural plate explants.

(F) Quail somitic (Qs) tissue (QCPN$^+$; red) does not induce EN1$^+$ interneurons (green) in chick intermediate neural plate explants.

(G and H) Differentiation of EN1$^+$ interneurons in neural tube tissue derived from *Isl1*$^\Delta$ homozygote mouse embryos grown in vitro for 48 hr.

(G) Quail ventral (Qv) neural tissue (QCPN$^+$; red) induces the differentiation of EN1$^+$ interneurons (green) in neural tube tissue derived from *Isl1*$^\Delta$ homozygote embryos.

(H) Quail somite (Qs) tissue (QCPN$^+$; red) does not induce the differentiation of EN1$^+$ interneurons (green) in neural tube tissue derived from *Isl1*$^\Delta$ homozygote embryos. Quail dorsal neural tube tissue is also without inductive activity (data not shown).

Scale bar, 25 μm.

the homeodomain protein EN1, fails to occur in *Isl1*$^\Delta$ homozygote embryos. This observation raises the possibility that the differentiation of EN1 interneurons depends on the prior generation of motor neurons (Figure 8). Since ISL1 is expressed selectively by motor neurons in the ventral spinal cord (Ericson et al., 1992; Tsuchida et al., 1994), the absence of EN1$^+$ interneurons in *Isl1*$^\Delta$ homozygote embryos appears to be a secondary consequence of the loss of motor neurons. Consistent with this idea, neural tissue containing motor neurons is able to restore the differentiation of EN1$^+$ interneurons in neural tube explants derived from *Isl1*$^\Delta$ homozygote embryos and to induce EN1$^+$ interneurons in chick neural plate explants. Motor neurons themselves represent a likely cellular source of the signal that induces EN1$^+$ interneurons (Figure 8). It is possible, however, that the immediate source of this signal is another ventral cell type that is itself dependent on motor neurons.

Motor neurons may have additional influences on the differentiation of neighboring cells. One line of evidence for this derives from the detection of a decrease in the ratio of mitotic cells in the ventral compared to the dorsal neural tube of *Isl1*$^\Delta$ homozygote embryos. We cannot exclude that the disproportionate decrease in ventral mitotic cells results from defects in nonneural tissues or is a byproduct of the high density of dying cells in the ventral neural tube. However, studies in the developing CNS have provided evidence that differentiating neurons provide feedback or lateral signals to nearby progenitor cells (Chitnis et al., 1995). In some regions of the CNS, such signals appear to restrict the ability of progentors to assume equivalent neuronal fates (Negishi et al., 1982; Reh and Tully, 1986; Reh, 1987). It is possible, therefore, that motor neurons normally signal to adjacent progenitor cells, biasing them against the selection of a motor neuron fate and permitting the differentiation of other ventral cell types. In *Isl1*$^\Delta$ homozygote embryos, the absence of motor neurons could eliminate such a feedback signal and permit additional progenitor cells to embark on a nonproductive pathway of motor neuron differentiation that culminates in cell death. The continuation of this futile cycle might account for the observed depletion of progenitor cells and the marked thinning of the neuroepithelium in the ventral neural tube.

Such a feedback mechanism could, in principle, contribute to the loss of EN1$^+$ interneurons in *Isl1*$^\Delta$ homozygote embryos, for example, by depleting the progenitors of these neurons. The restoration of EN1$^+$ interneurons in the neural tube of *Isl1*$^\Delta$ homozygote embryos could therefore reflect, in part, the reinstatement of this motor neuron–dependent feedback pathway. However, the induction of EN1$^+$ interneurons in wild-type chick neural tissue cannot obviously be explained by the operation of this feedback pathway, since the progenitors of EN1$^+$ interneurons have not been depleted. Thus, in normal neural tissue, the differentiation of EN1$^+$ interneurons

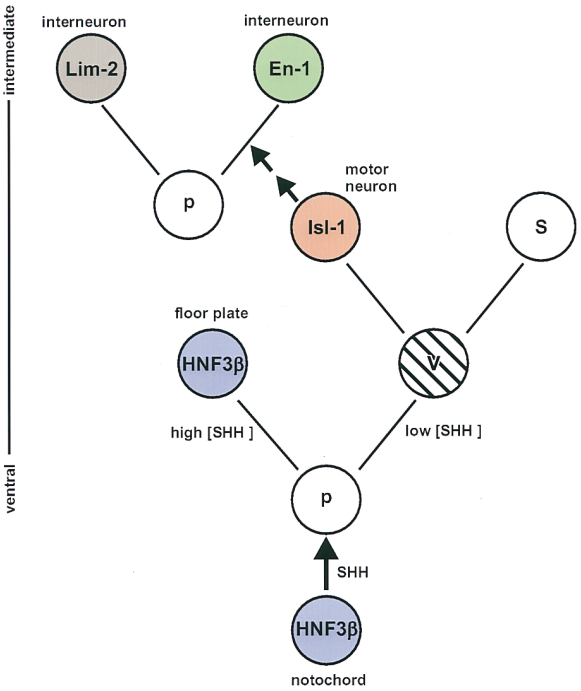

Figure 8. Model of Sequential Interactions that Control the Differentiation of Motor Neurons and EN1 Interneurons

The model outlines the possible steps involved in the generation of floor plate cells, motor neurons, and EN1$^+$ interneurons in the ventral neural tube. Floor plate differentiation results from the exposure of naive (*Nkx2.2*$^-$, *Msx1*$^+$, and *Pax3*$^+$; Barth and Wilson, 1995; Liem et al., 1995) neural plate progenitor cells (p) to a high concentration of SHH, inducing the expression of HNF3β and consequent floor plate differentiation (for details see Roelink et al., 1995). Exposure of progenitor cells to lower concentrations of SHH results in the generation of one or more ventralized (v) (*Nkx2.2*$^{+/-}$, *Msx1*$^-$, and *Pax3*$^-$) progenitor cell types, which retain the capacity for further cell division. The progeny of ventralized cells include motor neurons and other cell types (sibling cell [s]; see Leber et al., 1990). The present study shows that motor neuron differentiation is accompanied by and requires the expression of ISL1. Motor neurons may then be the source of a secondary inductive signal (double arrows) that acts directly or indirectly on nearby progenitor cells to induce the differentiation of EN1$^+$ interneurons. The differentiation of EN1$^+$ interneurons could involve a signal from an intervening cell type that is itself dependent on motor neurons. Naive neural plate progenitor cells give rise to LIM1$^+$/LIM2$^+$ interneurons in the absence of SHH-mediated and motor neuron–dependent signals (T. Tsuchida and T. M. J., unpublished data). The model predicts that ventralized cells do not give rise to EN1$^+$ interneurons in response to the motor neuron–derived signal, but they may respond to this signal with the generation of other classes of ventral interneurons.

can most easily be explained through the action of a motor neuron–dependent inductive signal.

Motor Neuron–Dependent Signals and Neural Tube Patterning

The apparent requirement for motor neurons in the differentiation of EN1$^+$ interneurons suggests that the patterning of distinct neuronal cell types in the ventral neural tube involves a series of inductive interactions (Figure 8). An early inductive signal mediated by SHH appears to confer progenitor cells in the ventral neural tube with ventralized properties, as defined by expression of the homeobox genes *Nkx2.1* and *Nkx2.2* (Barth and Wilson, 1995; Ericson et al., 1995; Y. Tanabe, personal communication) and the extinction of expression of *Msx1* and *Pax3* (Liem et al., 1995). The ventralization of progenitor cells by SHH appears to be required for the subsequent generation of motor neurons (Yamada et al., 1993; Roelink et al., 1995; Tanabe et al., 1995). In contrast, EN1$^+$ interneurons can be generated in chick intermediate neural plate explants that appear to contain naive progenitors that have not been exposed to SHH (Liem et al., 1995). Moreover, progenitor cells that have been ventralized by SHH lose the ability to generate EN1 interneurons in response to motor neuron–dependent signals (data not shown). This might explain why EN1$^+$ interneurons are positioned distal to motor neurons in neural plate–notochord conjugates and are located dorsal to the motor neuron population in vivo.

Progenitor cells that have been ventralized by SHH may, however, still respond to motor neuron–derived signals but with the generation of other classes of ventral interneurons. Several classes of interneurons distinct from those that express EN1 have been shown to differentiate in the ventral spinal cord close to the position of motor neurons. One class expresses the homeobox gene *Evx1* (Bastian and Gruss, 1990) and another, the *Gsh4* and *Lim3* genes (in the absence of *Isl1* and *Isl2* expression) (Tsuchida et al., 1994; Zhadanov et al., 1995). Neither *Gsh4* nor *Lim3* are expressed in the neural tube of *Isl1*$^\Delta$ homozygote embryos (Figure 2; data not shown), raising the possibility that the generation of this class of ventral interneuron also depends on motor neurons. Motor neurons might therefore be required for the subsequent differentiation of classes of ventral interneurons that function to coordinate motor neuron activity (Burke and Rudomin, 1977; Brown, 1981). The ability of early-born projection neurons to regulate the subsequent differentiation of neighboring interneurons could provide an effective means of organizing local relay circuits throughout the vertebrate CNS.

Experimental Procedures

Experimental Procedures are available on request and on the World Wide Web at http://cpmcnet.columbia.edu/dept/neurobeh/jessell.

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