Topographic Organization of Embryonic Motor Neurons Defined by Expression of LIM Homeobox Genes

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Summary

Motor neurons located at different positions in the embryonic spinal cord innervate distinct targets in the periphery, establishing a topographic neural map. The topographic organization of motor projections depends on the generation of subclasses of motor neurons that select specific paths to their targets. We have cloned a family of LIM homeobox genes in chick and show here that the combinatorial expression of four of these genes, Islet-1, Islet-2, Lim-1, and Lim-3, defines subclasses of motor neurons that segregate into columns in the spinal cord and select distinct axonal pathways. These genes are expressed prior to the formation of distinct motor axon pathways and before motor columns appear. Our results suggest that LIM homeobox genes contribute to the generation of motor neuron diversity and may confer subclasses of motor neurons with the ability to select specific axon pathways, thereby initiating the topographic organization of motor projections.

Introduction

The formation of neuronal connections during development depends on the generation of distinct classes of neurons and on the extension of axons to their targets along specific pathways. In many regions of the vertebrate nervous system, neurons extend axons and innervate target cells in a systematic manner that reflects their position of origin, creating topographic neural maps (Sperry, 1963; Udin and Fawcett, 1986; Hunt and Cowan, 1990). The identity of molecules that distinguish neurons on the basis of their position and control the formation of topographic projections has, however, remained elusive (Sanes, 1993).

The pattern of innervation of skeletal muscles by spinal motor neurons exhibits a high degree of spatial order and represents one of the better-studied topographic neural projections. The topographic organization of motor projections is a consequence of the generation of subclasses of motor neurons early in spinal cord development. Motor neuron subclasses become evident as their axons select distinct pathways to their targets and as their cell bodies segregate into longitudinally aligned columns within the spinal cord (Figure 1). The precise relationship between the formation of a motor neuron in the spinal cord and the identity of its target in the periphery, therefore, emerges during embryonic development and contributes to the topographic organization of motor projections apparent in the adult animal.

The selection of distinct axonal pathways by subclasses of motor neurons occurs in response to guidance cues in their local environment and determines their eventual targets (Tosney, 1991; Landmesser, 1992; Eisen, 1994). The cellular basis of motor axon guidance has been analyzed in detail in the chick embryo. Motor neurons that innervate axial muscles appear to respond to cues that are provided by the precursors of their eventual muscle targets within the dermomyotome (Tosney, 1987, 1988). By contrast, the axons of motor neurons that innervate limb muscles appear to ignore the dermomyotome and instead are guided by cues associated with cells of the limb mesenchyme (Lance-Jones and Landmesser, 1980a, 1980b, 1981a, 1981b; Ferguson, 1983; Whitelaw and Holliday, 1983a; Tosney and Landmesser, 1984; Phelan and Holliday, 1990; Lance-Jones and Dias, 1991). These embryological studies have provided persuasive but indirect evidence that spinal motor neurons possess intrinsic differences that permit them to respond selectively to cues that guide their axons to appropriate targets. The identification of molecules that distinguish embryonic motor neurons on the basis of their axonal projections and their position in the spinal cord might, therefore, provide insight into the mechanisms that establish the topographic organization of motor connections.

Embryonic motor neurons express the transcription factor Islet-1 soon after they leave the cell cycle (Ericson et al., 1992; Yamada et al., 1993). Islet-1 is a member of a family of homeobox genes found in vertebrates and invertebrates that encode proteins with a homeodomain and cysteine–histidine-rich LIM domains (Way and Chalfie, 1988; Karlsson et al., 1990; Freyd et al., 1990). Genetic studies in Caenorhabditis elegans and Drosophila melanogaster have shown that LIM homeobox genes are required for the asymmetrical divisions of precursor cells and control the fates of many cell types, including neurons (Way and Chalfie, 1988; Bourguin et al., 1992; Cohen et al., 1992; Freyd et al., 1990). For example, the C. elegans mec-3 gene controls the differentiation of mechanosensory neurons (Way and Chalfie, 1988), and the Drosophila apterous gene is required for the fasciculation and pathfinding of a subset of interneurons (Bourguin et al., 1992; J. Thomas, personal communication). These findings raise the possibility that LIM homeobox genes also control neuronal identity and axonal pathfinding in vertebrates.

We have found that Islet-1 mRNA expression is restricted to a subset of embryonic motor neurons during the period that motor axons select specific pathways. This observation prompted us to determine whether sub-
classes of motor neurons that select different axonal pathways and occupy different positions in the spinal cord might be distinguished by expression of members of the LIM homeobox gene family. To address this issue, we cloned six additional chick LIM homeobox genes, Islet-2, Lim-1, Lim-2, Lim-3, LH-2, and Lmx-1 (Karlsson et al., 1990; Taira et al., 1992, 1993; German et al., 1992; Xu et al., 1993) and determined their patterns of expression within the embryonic spinal cord. The combinatorial expression of four of these genes, Islet-1, Islet-2, Lim-1, and Lim-3, distinguishes subclasses of motor neurons that select distinct axonal pathways in the periphery and that occupy different columns in the spinal cord. The expression of these genes by motor neurons is evident prior to the formation of distinct motor axon pathways and before the segregation of motor neurons into columns. These findings raise the possibility that the combinatorial expression of LIM homeobox genes confers embryonic motor neurons with the ability to select distinct axon pathways and to segregate into columns, thus initiating the topographic organization of motor connections with their target muscles.

Results

Isolation of LIM Homeobox Genes Expressed in Embryonic Spinal Cord

In the embryonic chick spinal cord, antibodies directed against Islet-1 label motor neurons that innervate skeletal muscles (somatic motor neurons) and motor neurons that innervate sympathetic neurons (visceral motor neurons) (Ericson et al., 1992). To define the expression of Islet-1 in chick spinal cord in more detail, we isolated a full-length chick Islet-1 cDNA (see Experimental Procedures) and determined the distribution of Islet-1 mRNA by in situ hybridization. Between stages 14 and 22, the pattern of expression of Islet-1 was similar to that defined by anti-Islet-1 antibodies (Ericson et al., 1992). From stages 23 onward, however, Islet-1 was not expressed by all motor neurons, as defined independently by expression of Islet-1 immunoreactivity and choline acetyltransferase (ChAT) mRNA (Figure 2; data not shown). The detection of Islet-1 immunoreactivity in motor neurons that do not express Islet-1 mRNA suggested that these neurons express a gene related to Islet-1 that encodes a protein that is recognized by anti-Islet-1 antibodies.

We have isolated a chick LIM homeobox gene, Islet-2, that encodes a protein with 68% identity to chick Islet-1 and has an almost identical homeodomain (see Experimental Procedures). The expression of Islet-2 in the ventral spinal cord is congruent with that of Islet-1 immunoreactivity and ChAT (see below), suggesting that it occupies the entire somatic motor pool. These observations indicate that existing rabbit antisera and monoclonal antibodies raised against Islet-1 recognize both Islet-1 and Islet-2.

The patterns of expression of Islet-1 and Islet-2 led us to examine whether other LIM homeobox genes are also expressed by motor neurons. To assess this, we cloned chick homologs of the LIM homeobox genes Lim-1, Lim-2, Lim-3, LH-2, and Lmx-1 (see Experimental Procedures; T. T. et al., unpublished data; G. Tremml and T. M. J., unpublished data) and determined their patterns of expression in embryonic chick spinal cord by in situ hybridization. Two of these genes, Lim-1 and Lim-3, are expressed by motor neurons, Lim-1, however, is also expressed by interneurons throughout the spinal cord and Lim-3 by cells located just dorsal to motor neurons (Figure 1).
LIM Homeobox Genes and Motor Neuron Topography

Figure 2. Expression of LIM Homeobox Genes in Chick Spinal Cord
Transverse sections of stage 34-35 chick spinal cord, showing patterns of expression of Islet-1, Islet-2, Lim-1, and Lim-3, determined by in situ hybridization. Sections are obtained at approximately the levels shown in Figure 1b. (e), (j), (o), and (t) show tracings of the outline of the spinal cord and the extent of motor pools defined independently by expression of ChAT mRNA (data not shown). Lim-1 mRNA expression in a thin arc lateral to the LMC in (c), (h), (m), and (r) (arrowheads in [c] and [m]) is located in cells of Hofmann's nucleus major (Huber, 1936; Dubey et al., 1966). Overlapping domains of LIM homeobox gene expression in motor columns are encoded by colors in (e), (j), (o), and (t). Blue, Islet-1, Islet-2, Lim-3; red, Islet-1, Islet-2; green, Islet-2, Lim-1; brown, Islet-1. This color code is consistent with that used in Figure 1 to delineate motor columns. This analysis derives from studies on six to twelve embryos. Scale bar represents 270 μm in (a)-(e), 300 μm in (f)-(j), 265 μm in (k)-(o), and 375 μm in (p)-(t).

2). Lim-2, LH 2, and Lmx 1 are not expressed by motor neurons but define other distinct subsets of neurons in the embryonic spinal cord (T. T. et al., unpublished data; G. Tremml et al., unpublished data). Virtually all neurons generated in the spinal cord from stages 14 to 35 express one or a combination of LIM homeobox genes.

LIM Homeobox Genes Reveal the Organization of Motor Columns in the Spinal Cord
Motor neurons in the embryonic chick spinal cord can be subdivided into five major subclasses on the basis of their columnar organization and the position of their targets in the periphery (see Figure 1): first, motor neurons located in the medial subdivision of the median motor column (MMC) project to axial muscles that differentiate near the vertebral column; second, motor neurons in the lateral subdivision of the median motor column (MMC) project to body wall muscles that differentiate within the ventral lateral plate myotome; third, motor neurons in the medial subdivision of the lateral motor column (LMC) project to limb muscles that derive from the ventral region of the embryonic muscle mass; fourth, motor neurons in the lateral subdivision of the lateral motor column (LMC) project to limb muscles derived from the dorsal muscle mass; and fifth, visceral motor neurons, located in the column of Terni (CT), project to sympathetic neurons. The columns that contain these five subclasses of motor neurons occupy discrete rostrocaudal domains (see Figure 1a) and at a particular segmental level occupy different transverse positions within the spinal cord (Figure 1b).

The subdivision of motor neurons into columns is apparent by stage 35, after newly generated motor neurons have migrated laterally to their final positions (Hamburger, 1948; Langman and Haden, 1970). To determine whether the expression of the LIM homeobox genes Islet-1, Islet-2, Lim-1, and Lim-3 is restricted to subclasses of motor neurons that occupy discrete columns, we first examined their
patterns of expression at cervical, brachial, thoracic, and lumbar spinal levels in stage 34–35 chick embryos, defining motor neurons independently by expression of ChAT. Results described below show that subclasses of motor neurons that occupy different columns (see Figure 1a) can be distinguished by the expression of a distinct combination of LIM homeobox genes (Table 1 summarizes results documented in Figures 2–4).

**Differential Expression of Islet-1, Islet-2, and Lim-3 Subdivides the MMC**

The MMC, extends along the entire rostrocaudal length of the spinal cord, although at lumbar levels the number of motor neurons in the MMC decreases markedly. The MMC is restricted to thoracic levels (see Figure 1a). Islet-2, Islet-1, and Lim-3 were expressed uniformly within the MMC (Figures 2a, 2b, 2d, 2f, 2g, 2l, 2k, 2i, and 2n). By contrast, the MMC expressed Islet-1 and Islet-2 but not Lim-3 (Figures 2k–2o). Thus, motor neurons in the MMC, and the MMC, are distinguished by expression of Lim-3. Lim-1 was excluded from the MMC over the entire rostrocaudal length of the spinal cord (Figures 2c, 2h, 2m, and 2r).

**Differential Expression of Islet-1, Islet-2, and Lim-1 Subdivides the LMC**

The LMC is confined to brachial and lumbar levels of the spinal cord (see Figure 1a) and is subdivided into medial (LMC,) and lateral (LMC) columns. Islet-2 was expressed throughout the entire LMC (Figures 2f and 2p). By contrast, Islet-1 (with one exception that is discussed below) was restricted to the LMC, (Figures 2g and 2q). Conversely, Lim-1 expression by motor neurons was restricted to the LMC (Figures 2h and 2r). Lim-3 was not expressed in the LMC (Figures 2i and 2s). Thus, the differential expression of Islet-1 and Lim-1 distinguishes the LMC, from the LMC.

**Islet-1 Expression Subdivides the CT**

The CT extends over thoracic and rostral lumbar segments (Levi-Montalcini, 1950). Neurons in the ventral region of the CT expressed Islet-1 but not Islet-2, Lim-1, or Lim-3 (Figures 2k–2o). However, neurons in the dorsal region of the CT, defined by expression of ChAT, did not express any of the seven LIM homeobox genes examined (Figure 2o; data not shown). These observations indicate that expression of Islet-1 in the absence of Islet-2 distinguishes visceral from somatic motor neurons.

Figure 3. Coexpression of LIM Homeobox Genes by Neurons in the MMC and the LMC. Coexpression of LIM homeobox genes determined by combined immunocytochemistry and in situ hybridization or by double-label immunocytochemistry on sections of stage 34–35 chick spinal cord.

(a–c) Coexpression of Islet-1, identified with an islet-1-specific antibody (brown) and islet-2 mRNA (blue/gray). (a) High power view showing coexpression of Islet-1 and Lim-1 in the MMC, associated with expression of Islet-1 in the cervical spinal cord. (b) Thoracic section showing coexpression of Islet-1, Islet-2, and Lim-1 in cells within the MMC. Virtually all Islet-1/Islet-2 cells in the MMC coexpress Lim-3. Islet-3 cells that do not show Islet-1/Islet-2 immunoreactivity are found dorsal to the MMC, not within the domain of ChAT mRNA expression (data not shown) and therefore are not considered to be motor neurons. (b) Thoracic section showing coexpression of Islet-1 and Islet-2 in cells within the MMC, and the MMC, but expression of Islet-1 alone in the CT (arrowhead). (c) High power view of a thoracic section showing congruence of expression of Islet-1 and Islet-2 in the MMC.

(j–l) Confocal images of double-label immunocytochemical localization of Islet-1, Islet-2, and Lim-1 in neurons within the lumbar LMC.

(g–i) Confocal images of double-label immunocytochemical localization of Islet-1, Islet-2, and Lim-3 in the MMC at cervical levels. Virtually all Islet-1/Islet-2 cells in the MMC, coexpress Lim-3. Lim-3 cells that do not show Islet-1/Islet-2 immunoreactivity are found dorsal to the MMC, not within the domain of ChAT mRNA expression (data not shown) and therefore are not considered to be motor neurons. (a–c) Colocalization of Islet-1, identified with an islet-1-specific antibody (brown) and islet-2 mRNA (blue/gray). (a) High power view showing coexpression of Islet-1 and Lim-1 in the MMC, associated with expression of Islet-1 in the cervical spinal cord. (b) Thoracic section showing coexpression of Islet-1, Islet-2, and Lim-1 in cells within the MMC. Virtually all Islet-1/Islet-2 cells in the MMC coexpress Lim-3. Islet-3 cells that do not show Islet-1/Islet-2 immunoreactivity are found dorsal to the MMC, not within the domain of ChAT mRNA expression (data not shown) and therefore are not considered to be motor neurons. (b) Thoracic section showing coexpression of Islet-1 and Islet-2 in cells within the MMC, and the MMC, but expression of Islet-1 alone in the CT (arrowhead). (c) High power view of a thoracic section showing congruence of expression of Islet-1 and Islet-2 in the MMC.
Coexpress LIM Homeobox Genes

we first localized Islet-1, Islet-2, and Lim-1 proteins by immunocytochemistry in stage 34–35 chick embryos. The patterns of LIM homeodomain protein and mRNA expression were in close agreement and established that neurons in the MMC and the LMC express Islet-1 and Islet-2, whereas neurons in the LMC express Islet-2 and Lim-1 (Figure 3; data not shown).

Motor Neurons within Individual Columns Coexpress LIM Homeobox Genes

The results described above suggest, but do not establish directly, that the expression of LIM homeobox genes conforms to the columnar subdivision of motor neurons within the embryonic spinal cord. Since neurons within each motor column project to distinct targets, the assignment of motor neurons to particular columns can be defined most clearly by their accumulation of retrogradely transported markers after injection into specific targets. To determine whether the expression of LIM homeobox genes segregates precisely with the position of their muscle target even though the columnar location of these motor neurons in the spinal cord is unusual. At segmental levels C13 to C15, but not at other levels of the brachial or lumbar LMC, a subgroup of motor neurons in the LMC, coexpressed Islet-1 and Islet-2, and Lim-3 cells corresponded to the reported location of motor neurons that innervate the rhomboideus muscle (Straznicky and Tay, 1983; Hollyday and Jacobson, 1990) and not, as expected, in the MMC. This peculiarity permitted us to test whether the expression of LIM homeobox genes by motor neurons segregates with the position of their muscle target even though the columnar location of these motor neurons in the spinal cord is unusual. At segmental levels C13 to C15, but not at other levels of the brachial or lumbar LMC, a subgroup of motor neurons in the LMC, coexpressed Islet-1 and Islet-2, and Lim-3, a combination of genes characteristic of the MMC, whereas surrounding neurons expressed Islet-2 and Lim-1 (Figures 5a–5d). The position of the ectopic Islet-1, Islet-2, and Lim-3 cells corresponded to the reported location of motor neurons that innervate the rhomboideus muscle (Straznicky and Tay, 1983; Hollyday and Jacobson, 1990). To determine the identity of this distinct population of LMC neurons, we injected HRP into the rhomboideus muscle in stage 35 embryos. HRP-labeled motor neurons were found in the lateral region of the LMC, and these neurons expressed Islet-1 but not Lim-1 (Figures 5e–5f), indicating that rhomboideus motor neurons express LIM homeobox genes characteristic of MMC and not LMC neurons. Thus, LIM homeobox gene expression by motor neurons predicts their muscle targets even in instances in which the columnar location of motor neurons in the spinal cord is atypical.

Table 1. Combinatorial Expression of LIM Homeobox Genes by Motor Neuron Subclasses at Stage 35

<table>
<thead>
<tr>
<th>Motor Neuron Subclass</th>
<th>LIM Homeobox Genes</th>
<th>Color Code</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMC, Islet-1, Islet-2, Lim-3</td>
<td>blue</td>
<td></td>
</tr>
<tr>
<td>MMC, Islet-1, Islet-2</td>
<td>red</td>
<td></td>
</tr>
<tr>
<td>LMC, Islet-1, Islet-2</td>
<td>red</td>
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</tr>
<tr>
<td>LMC, Islet-2, Lim-1</td>
<td>green</td>
<td></td>
</tr>
<tr>
<td>CT, Islet-1</td>
<td>brown</td>
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</table>

LIM Homeobox Gene Expression by Motor Neurons Predicts Their Muscle Targets

The results described above suggest, but do not establish directly, that the expression of LIM homeobox genes conforms to the columnar subdivision of motor neurons within the embryonic spinal cord. Since neurons within each motor column project to distinct targets, the assignment of motor neurons to particular columns can be defined most clearly by their accumulation of retrogradely transported markers after injection into specific targets. To determine whether the expression of LIM homeobox genes segregates precisely with motor columns, we therefore injected horseradish peroxidase (HRP) into specific muscle groups and analyzed retrogradely labeled motor neurons with antibodies that detect the three LIM homeodomain proteins expressed by LMC neurons were available.

We first injected HRP into the ventral or dorsal limb muscle masses at stage 30. Motor neurons that were retrogradely labeled by injection of HRP into the ventral muscle mass were located in the LMC, and expressed Islet-1 but not Lim-1 (Figures 4a and 4b). Motor neurons labeled after injection of HRP into the dorsal muscle mass were located in the LMC and expressed Lim-1 and Islet-2 but not Islet-1 (Figure 4c; data not shown). To determine whether the expression of LIM homeodomain proteins segregates with motor neurons that project to individual limb muscles of dorsal or ventral origin, we injected HRP into either the sartorius, a dorsally derived muscle, or the adductor, a ventrally derived muscle, at stage 37. After injection into the sartorius muscle, HRP-labeled motor neurons were located in the LMC and coexpressed Islet-2 and Lim-1 but not Islet-1 (Figures 4d–4f). Conversely, after injection into the adductor muscle, HRP-labeled motor neurons were located in the LMC, and coexpressed Islet-1 and Islet-2 but not Lim-1 (Figures 4g–4i).

These results show directly that motor neurons in the LMC, that coexpress Islet-1 and Islet-2 project to ventrally derived muscles, whereas motor neurons in the LMC that coexpress Islet-2 and Lim-1 project to dorsally derived muscles. The expression of different LIM homeodomain proteins by motor neurons in the LMC, therefore, conforms precisely to their columnar organization and to their muscle targets.

The only exception to the general relationship between the columnar organization of motor neurons and the position of the muscle target occurs at brachial levels C13 to C15, where an axial muscle, the rhomboideus (Sullivan, 1952), is innervated by motor neurons located in the LMC (Straznicky and Tay, 1983; Hollyday and Jacobson, 1990) and not, as expected, in the MMC. This peculiarity permitted us to test whether the expression of LIM homeobox genes by motor neurons segregates with the position of their muscle target even though the columnar location of these motor neurons in the spinal cord is unusual. At segmental levels C13 to C15, but not at other levels of the brachial or lumbar LMC, a subgroup of motor neurons in the LMC, coexpressed Islet-1, Islet-2, and Lim-3, a combination of genes characteristic of the MMC, whereas surrounding neurons expressed Islet-2 and Lim-1 (Figures 5a–5d). The position of the ectopic Islet-1, Islet-2, and Lim-3 cells corresponded to the reported location of motor neurons that innervate the rhomboideus muscle (Straznicky and Tay, 1983; Hollyday and Jacobson, 1990). To determine the identity of this distinct population of LMC neurons, we injected HRP into the rhomboideus muscle in stage 35 embryos. HRP-labeled motor neurons were found in the lateral region of the LMC, and these neurons expressed Islet-1 but not Lim-1 (Figures 5e–5f), indicating that rhomboideus motor neurons express LIM homeobox genes characteristic of MMC and not LMC neurons. Thus, LIM homeobox gene expression by motor neurons predicts their muscle targets even in instances in which the columnar location of motor neurons in the spinal cord is atypical.
Expression of LIM Homeobox Genes by Motor Neurons Precedes the Selection of Distinct Axon Pathways and Segregation into Columns

By stage 35, when the segregation of motor neurons into columns is complete, subclasses of motor neurons that project to distinct peripheral targets express LIM homeobox genes in different combinations. The selection by motor axons of peripheral pathways and the innervation of muscle targets, however, occurs well before this stage. We therefore examined the time at which motor neurons first express LIM homeobox genes. Results described below provide evidence that the expression of LIM homeobox genes occurs before the segregation of motor neurons into columns and before distinct motor axon pathways are established (for a summary of results described in Figures 6 and 7, see Figure 8).

MMC Neurons

The onset of expression of LIM homeobox genes by MMC neurons can be determined most clearly at cervical levels (see Figure 1a). Islet-1, Islet-2, and Lim-3 cells were first detected at stages 14 to 1b (data not shown), and the number of cells had increased markedly by stages 17 to 18 (Figures 6a–6f; data not shown). These results provide evidence that the onset of expression of LIM homeobox genes by MMC neurons occurs soon after they are born and before their axons project to axial muscle targets (Hollyday and Hamburger, 1977; Tosney and Landmesser, 1985a, 1985b).

In addition, analysis of Islet-1 and Islet-2 expression in single sections of cervical spinal cord at stage 17 showed the presence of Islet-1 cells that did not express Islet-2 (Figure 6f). Moreover, in an analysis of serial sec-
the ventricular zone (Figures 6a and 6b), whereas Lim-3 cells were detected only at the extreme lateral edges of the ventricular cord (compare Figures 6a, 6b, and 6c; data not shown). Newborn motor neurons emerge from the ventricular zone and migrate laterally (Langman and Haden, 1983b). Thus, those motor neurons generated at early times populate the LMC, and those at later times, the LMG.

To determine the onset of expression of LIM homeobox genes by LMC neurons, we examined segments L4 and L5, a level where there are few MMC, neurons and the MMC, and CT are not present (see Figure 1a). At lumbar levels, most LMC, neurons are born at stages 10–19 and most LMC neurons at stages 20–21 (Whitelaw and Holliday, 1983b). Thus, those motor neurons generated at early times populate the LMC, and those at later times, the LMC.

To examine the onset of LIM homeobox gene expression by LMC neurons, we monitored the appearance of Islet-1 and Islet-2. At stage 17, serial sections through the lumbar spinal cord of individual embryos revealed cells that expressed Islet-1 but not Islet-2 (data not shown), providing evidence that in the LMC, as in the MMC, Islet-1 expression precedes that of Islet-2. By stage 18, both genes were detected (Figures 6g and 6h). At these stages, Lim-1 was not expressed in the ventral spinal cord (Figure 6i).

To examine the onset of LIM homeobox gene expression by LMC neurons, we analyzed, from stages 20 to 27, cells in the ventral spinal cord that expressed Lim-1. At stage 21, soon after the birth of LMC neurons, the ventral spinal cord contained only a few Lim-1 cells, and, somewhat surprisingly, these cells coexpressed Islet-1 (Figures 7a–7c). By stage 23, the number of Lim-1 cells had increased markedly, and by now these cells expressed Islet-2 but not Islet-1 (Figures 7d–7f). To provide more detailed information on the expression of Islet-1, Islet-2, and Lim-1 at this stage, we performed an in situ hybridization analysis on serial sections. Islet-1 was expressed in a medial zone that does not express Islet-2 or Lim-1 (see Figure 6j) but was expressed only at very low levels in an intermediate zone that contained Islet-2 and Lim-1 cells (compare Figures 6k and 6l). A lateral zone that appears to correspond to the LMC, expressed Islet-1 and Islet-2 but not Lim-1 (see Figures 6j–6l). These results indicate that LMC neurons express Islet-1 before Lim-1 or Islet-2 and also that Islet-1 expression is transient, decaying as LMC neurons migrate laterally and begin to express Islet-2 and Lim-1.

This analysis has also provided information on the timing of LIM homeobox gene expression in relation to the segregation of motor neurons into columns. At stage 23, cells that coexpress Lim-1 and islet-2 were located medial to LMC, neurons (Figures 7d–7f), but by stage 25, cells with this phenotype were observed in a more lateral position (Figures 7g–7i), and by stage 27, they occupied a domain that was almost exclusively lateral to LMC, neurons (Figures 7j–7l). These observations suggest that neurons that coexpress Lim-1 and Islet-2 migrate through LMC, neurons that have been generated at earlier times to reach their final position in the LMC. Thus, the LIM homeobox gene phenotype of LMC neurons appears to be established prior to their lateral migration and before the segregation of motor neurons into columns.
Figure 6. Onset of Expression of LIM Homeobox Genes by Motor Neurons

(a) Expression of Islet-1. Note that some medial cells close to the ventricular zone (arrowhead) as well as lateral cells express Islet-1.

(b) Expression of Islet-2. Note the absence of expression in medial cells.

(c) Expression of Lim-3 is restricted to cells located at an extreme lateral position in the ventral spinal cord. The number of Lim-3 cells is lower than the number of Islet-1 or Islet-2 cells.

(d) Islet-1 expression in motor neurons in the ventral spinal cord, detected with an Islet-1-specific antibody.

(e) Islet-1 and Islet-2 expression in motor neurons detected with pan-Islet antibodies.

(f) Double-label detection of Islet-1 protein (brown) and Islet-2 mRNA (blue/grey) shows directly that some neurons express Islet-1 but not Islet-2 (arrowheads).

(g)–(l) show the expression of LIM homeobox genes in LMC neurons at lumbar levels at late stage 18 and stage 23.

(g)–(i), late stage 18; (j)–(l), stage 23. (g), expression of Islet-7 in ventral cells. (h), expression of Islet-2 in ventral cells. (i), absence of expression of Lim-1 in ventral cells at this stage.

(j)–(l) Expression of Islet-1 (j), Islet-2 (k), and Lim-1 (l) divides the ventral spinal cord into three zones: a medial zone (m arrowhead) close to the ventricular zone that expresses Islet-1 (j) but not Islet-2 (k) or Lim-1 (l), an intermediate zone (l arrowhead) in which Islet-1 expression is low or absent but which expresses Islet-2 and Lim-1, and a lateral zone (corresponding to LMC neurons) that expresses Islet-1 and Islet-2 but not Lim-1. Cells dorsal to the LMC also express Islet-1 or Lim-1.

Micrographs are representative of studies on at least three embryos at each stage. Scale bar represents 96 μm in (a)–(f), 82 μm in (j)–(i), and 180 μm in (g)–(l).

MMC, and CT Neurons

The generation of MMC, and CT neurons overlaps temporally and spatially with that of MMC neurons (Prasad and Hollyday, 1991). Because of this, it was not possible to define the onset of LIM homeobox gene expression by MMC, or CT neurons. However, by stage 26, after the axons of CT neurons have reached their targets (Yip, 1990), the cell bodies of CT neurons begin to translocate dorsally, away from the MMC (Prasad and Hollyday, 1991) and thus can be distinguished by their position (Yip, 1990). Between stages 26 and 30, migrating CT neurons expressed both Islet-1 and Islet-2 (data not shown), although by stage 35, these neurons expressed only Islet-1 (see Figure 2). These results indicate that Islet-2 is expressed transiently by CT neurons.

Extinction of LIM Homeobox Gene Expression Occurs after Innervation of Muscle

We next asked whether the pattern of LIM homeobox gene expression that is established by stage 35 is maintained throughout embryonic development (see Figure 8 for a summary of these data). Islet-2 expression by somatic motor neurons persisted at least until stage 45. By contrast, Islet-1, Lim-3, and Lim-1 were not expressed by motor neurons at this stage. The expression of LIM homeobox genes by somatic motor neurons, therefore, changes markedly at late stages of embryogenesis, possibly in response to signals from their targets.

Discussion

Motor neurons located at different positions in the spinal cord project their axons in a stereotyped manner to innervate distinct peripheral targets. The topography of motor connections has three primary levels of organization. First, motor neurons within different columns project to targets at discrete locations in the periphery (Landmesser, 1978a, 1978b; Hollyday, 1980). Second, motor neurons that project to specific muscles, termed motor pools, are clustered within a column (Landmesser, 1978b; Hollyday, 1980; Lance-Jones and Landmesser, 1980b, 1981a, 1981b). Third, motor neurons that occupy different rostrocaudal positions within the spinal cord project to target cells at corresponding rostrocaudal levels (Wigston and Sanes, 1982, 1985; Lichtman et al., 1980; Laskowski and Sanes, 1987). These observations have led to the suggestion that motor neurons located at different positions within the spinal cord possess intrinsic differences that permit them to establish specific connections in the periphery. We therefore sought to identify genes that distinguish subclasses...
of motor neurons on the basis of their position in the spinal cord, their axonal projection patterns, and their targets.

We have cloned a family of chick LIM homeobox genes, four of which, *Islet-1*, *Islet-2*, *Lim-1*, and *Lim-3*, are expressed by motor neurons in the embryonic chick spinal cord. LIM homeobox genes do not obviously delineate individual motor pools, nor do they reveal rostrocaudal differences between motor neurons within a single motor column. However, the combinatorial expression of these genes defines subclasses of motor neurons that segregate into different columns, select specific axonal pathways and innervate distinct targets. Moreover, the expression of LIM homeobox genes by motor neurons occurs prior to the segregation of motor neurons into columns and before the selection of distinct axonal pathways.

Importantly, the expression of a single LIM homeobox gene is not sufficient to distinguish individual subclasses of motor neurons. *Islet-2* and, transiently, *Islet-1* are expressed by all somatic motor neurons (Figure 8b). Expression of the two *Islet* genes might, therefore, be required to specify features common to the development of all motor neurons. The differential expression of *Lim-3* and *Lim-1* is more informative in distinguishing motor neuron subclasses. *Lim-3* subdivides the MMC into medial and lateral columns, and *Lim-1* similarly subdivides the LMC. Since the expression of *Lim-1* and *Lim-3* is not restricted to motor neurons, it is the combinatorial expression of *Lim-1* or *Lim-3* together with one or another *Islet* gene that distinguishes subclasses of motor neurons.

Selection of Distinct Motor Axon Pathways

We consider first the relationship between the combinatorial expression of LIM homeobox genes and the pathfinding of motor axons.

The specificity of motor projections to muscle targets at different peripheral locations depends on the pathfinding choices made by motor axons during embryonic development (Tooney, 1991; Landmesser, 1992; Eisen, 1994). The axons of all chick motor neurons project from the spinal cord along a common path in the ventral root, at
which point subclasses of motor axons establish different trajectories (Figure 8c). The axons of MMCmotor neurons break away from the ventral root and form a nerve branch, the dorsal ramus, that projects to the dermomyotome (Tosney and Landmesser, 1985b; Tosney, 1991). Ablation of the dermomyotome prevents the dorsal deviation of MMCmotor axons, suggesting that the growth cones of these axons respond to cues, possibly chemoattractants, that derive from dermomyotomal cells (Tosney, 1987, 1988). By contrast, the axons of MMCmotor neurons appear to ignore the dermomyotome and instead project ventrolaterally to reach the border of the lateral plate mesoderm. At the base of the limb, the axons of MMCmotor neurons select either a dorsal or a ventral pathway in response to cues provided by mesenchymal cells of the lateral plate mesoderm (Lance-Jones and Landmesser, 1991a; Ferguson, 1983; Tosney and Landmesser, 1984; Phelan and Hollyday, 1990). Neurons in the LMCmotor column project into ventral mesenchyme, whereas neurons in the LMCmotor column project into dorsal mesenchyme. At thoracic levels, motor neurons in the MMCmotor column are defined to innervate body wall muscles as well as project into ventral mesenchyme of lateral plate origin (Wachtler and Christ, 1992; Gutman et al., 1993), a pathway similar to that taken by MMCmotor neurons.

Our results show, therefore, that LIM homeobox genes subdivide somatic motor neurons on the basis of their ability to select one of three distinct axonal pathways. Expression of Islet-1, Islet-2, and Lm-3 defines MMCmotor neurons, which point subclasses of motor axons establish different trajectories (Figure 8c). The axons of MMCmotor neurons break away from the ventral root and form a nerve branch, the dorsal ramus, that projects to the dermomyotome (Tosney and Landmesser, 1985b; Tosney, 1991). Ablation of the dermomyotome prevents the dorsal deviation of MMCmotor axons, suggesting that the growth cones of these axons respond to cues, possibly chemoattractants, that derive from dermomyotomal cells (Tosney, 1987, 1988). By contrast, the axons of MMCmotor neurons appear to ignore the dermomyotome and instead project ventrolaterally to reach the border of the lateral plate mesoderm. At the base of the limb, the axons of MMCmotor neurons select either a dorsal or a ventral pathway in response to cues provided by mesenchymal cells of the lateral plate mesoderm (Lance-Jones and Landmesser, 1991a; Ferguson, 1983; Tosney and Landmesser, 1984; Phelan and Hollyday, 1990). Neurons in the LMCmotor column project into ventral mesenchyme, whereas neurons in the LMCmotor column project into dorsal mesenchyme. At thoracic levels, motor neurons in the MMCmotor column are defined to innervate body wall muscles as well as project into ventral mesenchyme of lateral plate origin (Wachtler and Christ, 1992; Gutman et al., 1993), a pathway similar to that taken by MMCmotor neurons.

Our results show, therefore, that LIM homeobox genes subdivide somatic motor neurons on the basis of their ability to select one of three distinct axonal pathways. Expression of Islet-1, Islet-2, and Lm-3 defines MMCmotor neuron,
rons that appear to respond to target-derived cues from the dermomyotome. Coexpression of Islet-1 and Islet-2 defines MMC neurons that extend axons into lateral plate mesenchyme of ventral character. Coexpression of Lim-1 and Islet-2 defines LMC neurons that extend axons into lateral plate mesenchyme of dorsal character. Our results also provide evidence that the onset of expression of LIM homeobox genes by motor neurons precedes the selection of their distinct axonal pathways (Tosney and Landmesser, 1985a, 1985b; see Figure 8a). Strikingly, the ability to predict the peripheral target of a motor neuron on the basis of the LIM homeobox genes it expresses is maintained even when motor neurons are found in apparently ectopic positions in the spinal cord. Thus, rhomboideus neurons that express LIM homeobox genes characteristic of MMC neurons innervate an axial muscle target, even though they are located in the LMC. Taken together, these results suggest that one function of LIM homeobox genes is to convey subcellulars of motor neurons with the ability to recognize selectively the guidance cues that direct axons along distinct pathways to muscle targets at different positions.

There is a clear distinction in LIM homeobox gene expression by motor neurons in the LMC that project axons to dorsally and ventrally derived limb muscles (Figure 8c), raising the question of why a similar distinction is not apparent in motor neurons of the MMC that innervate dorsally and ventrally located axial muscles. Motor neurons that project to ventral axial (hypaxial) muscles are located medially in the MMC, whereas neurons that project to dorsal axial (epaxial) muscles are located laterally (Gutman et al., 1993). Yet, our results show that motor neurons within the MMC coexpress the same combination of LIM homeobox genes irrespective of their mediolateral location. One possible explanation for this is that the innervation of dorsally and ventrally located axial muscles by MMC neurons is established in a manner that does not involve the differential expression of transcription factors. MMC neurons are born and extend axons over a protracted period (Hollyday and Hamburger, 1977; Tosney and Landmesser, 1985b). The time at which an axon extends might, therefore, determine whether it contacts a dorsal or ventral region of the myotome before its cleavage, and consequently whether it innervates an epaxial or hypaxial muscle.

By contrast, the pathfinding choices of LMC neurons cannot easily be explained by differences in the time at which their axons grow out, since all LMC axons undergo a prolonged waiting period at the base of the limb (Tosney and Landmesser, 1985a, 1985b). As a consequence, the ability of axons of LMC and LMC neurons to select ventral or dorsal pathways in the limb may depend on the generation of molecular differences between LMC neurons.

**Columnar Organization of Motor Neurons**

The combinatorial expression of LIM homeobox genes by subclasses of motor neurons conforms closely to their columnar organization as well as to their axonal projection pattern (Figures 1 and 8). Since the expression of LIM homeobox genes by motor neurons occurs prior to their segregation into columns, it is possible that these genes contribute to the process of motor neuron segregation.

In one instance, however, the expression of LIM homeobox genes by motor neurons violates the prevailing columnar organization. Rhomboideus motor neurons are located in the LMC, but express Islet-1, Islet-2, and Lim-3, a profile characteristic of MMC neurons. The atypical position of rhomboideus motor neurons raises the possibility that they express genes that subvert the normal pattern of segregation of MMC neurons. Recent studies have identified a murine LIM homeobox gene, Gsh-4, that is closely related to Lim-3 and is expressed by motor neurons in the MMC (Li et al., 1994; S. L. P. et al., unpublished data). Thus, Gsh-4 or indeed other genes might distinguish rhomboideus motor neurons from neurons in the MMC and account for their unusual position.

Each of the five major motor columns (Figure 1) innervates a different peripheral target. The neurons in three of these columns, the MMC, the LMC, and the CT, express distinct combinations of LIM homeobox genes. Motor neurons within the MMC and the LMC, however, coexpress the same LIM homeobox genes, Islet-1 and Islet-2. What might account for the expression of the same combination of LIM homeobox genes by motor neurons in two different motor columns? One possibility is that additional genes distinguish neurons in the MMC from those in the LMC.

A second possibility is that the identical profile of LIM homeobox gene expression is a reflection of properties that are shared by these two subclasses of motor neurons. It is striking that the axons of both these classes of motor neurons project into lateral plate mesenchyme of ventral character. The common environment through which their axons project suggests that motor neurons in the MMC and the LMC might recognize the same guidance cues and, therefore, require the same combination of LIM homeobox genes. Furthermore, the absence of any overlap in the rostrocaudal domains of MMC and LMC neurons (Figure 1a) would permit the use of the same set of cues to guide the axons of these two classes of motor neurons to distinct targets, body wall and ventrally derived limb muscles.

Another feature of motor organization that derives from these observations is that motor neurons of the MMC and the LMC form a continuous column that extends from brachial through lumbar levels of the spinal cord, united by common expression of LIM homeobox genes and by similar axonal trajectories (Figure 1b). By extension, then, the LMC appears to be the only subclass of motor neurons that is restricted to limb levels.

**Homeobox Genes and the Control of Neuronal Identity**

The neural tube can be subdivided into broad domains along its anteroposterior axis by the expression of members of the Hox, Dlx, and Emx homeobox gene families (Graham et al., 1989; Hunt et al., 1991; Puelles and Rubenstein, 1993). These families of homeobox genes, however, do not obviously define distinct neuronal cell types (Graham et al., 1991; Puelles and Rubenstein, 1993). The present studies on spinal motor neurons, together with
analyses of expression of Lh-2, *Lm* -1, and *Os* -4 (Li et al., 1994; T. T. et al., unpublished data; G. Tremml, unpublished data) show that LIM homeobox genes define distinct subclasses of neurons throughout the spinal cord. LIM homeobox genes are also expressed in restricted regions of the developing brain (Taira et al., 1992, 1993; Korzh et al., 1993; Xu et al., 1993; Barnes et al., 1994; Fujii et al., 1994; Inoue et al., 1994; I. I., unpublished data) and control the fate and pathfinding of subsets of neurons in the Drosophila and nematode nervous systems (Bourgoin et al., 1992; Cohen et al., 1992; Way and Chalfie, 1988; J. Thomas, personal communication). Thus, LIM homeobox genes might participate more generally in determining the fates and early axonal projections of subclasses of neurons in both invertebrate and vertebrate embryos.

**Experimental Procedures**

**Isolation of Chick LIM Homeobox Genes**

cDNAs encoding the chick *Islet* -1, *Islet* -2, *Lim* -1, *Lim* -2, *Chat*, *rat Islet-2*, and *Lim* -2 genes were cloned by homology (details available on request).

**RNA In Situ Hybridization**

Linearized cDNA clones were transcribed with T3 or T7 RNA polymerase and digoxigenin labeling mix (Boehringer Mannheim). In situ hybridization was performed on sections essentially as described by Schaeren-Wiemers and Gerfin-Moser (1993). For combined in situ hybridization and immunocytochemistry, sections were then incubated simultaneously with anti-LIM homeodomain protein and anti-digoxigenin antibodies at 22°C overnight. Primary antibodies were detected with Vectastain Elite ABC (Vector Laboratories).

**Generation of Antibodies**

Polyclonal (K5) and monoclonal (4DS) antibodies that recognize both islet-1 and islet-2 were generated against carboxy-terminal residues 178-349 of rat islet-1 (Thor et al., 1991). Islet-1-specific rabbit antibodies (A7, A8) and mouse monoclonal antibody (1D6) were generated against residues 86-175 of chick islet-1 expressed in Escherichia coli. The anti-Lim-1/Lim-2 rabbit antibody (T4) and monoclonal antibody against residues 86-175 of chick *Islet* -1 expressed in Escherichia coli. The specificity of antibodies was determined by comparison of the labeling patterns obtained by immunocytochemistry and by in situ hybridization.

**Immunohistochemistry**

For immunohistochemistry, 10-15 μm cryostat sections were processed as described (Yamada et al., 1991; Ericson et al., 1992). Primary antibody dilutions were as follows: K5 at 1:2000, 4DS at 1:100, A7 and A8 at 1:5000, T4 at 1:3000, and 1D6 and 4F2 at 1:1. Fluorophore-conjugated antibodies were used at 1:100 to 1:500, and confocal images were collected on a Bio-Rad MRC 600 confocal laser scanning microscope.

**Retрограде Labeling of Motor Neurons**

Fertilized chick eggs (Spafas) were incubated at 37°C, and experiments were performed on embryos at stages 33, 35, and 37 (Hamburger and Hamilton, 1951) according to Hollyday (1980). To inject the rhomboid muscles, stage 35 embryos were withdrawn from the amniotic sac and held with forceps under the neck to expose the dorsal scapular area.

After injections, the eggs were sealed and incubated for 2-4 hr at 37°C. The embryos were fixed by intracardiac perfusion with PF at 4°C and processed for immunohistochemistry (Ericson et al., 1992). HRP was detected with an affinity-purified rabbit anti-HRP antibody (Jackson Immunoresearch Laboratories, 1:1000) or with a mouse monoclonal anti-HRP antibody (Sigma Immunocchemicals, 1:1000).

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**References**


GenBank Accession Numbers

The accession numbers for the genes reported in this paper are as follows: chick islet-1, L35567; chick islet-2, L35568; rat islet-2, L35571; chick lim-1, L35569; rat lim-2, L35572; and chick lim-3, L35570.