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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, 1988; 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 position of a motor neuron in the spinal cord and that of its target in the periphery, therefore, emerges during embryonic development and contributes to the topography 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 Hollyday, 1983a; Tosney and Landmesser, 1984; Phelan and Hollyday, 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 asymmetric divisions of precursor cells and control the fates of many cell types, including neurons (Way and Chalfie, 1988; Bourgouin 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 (Bourgouin 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-

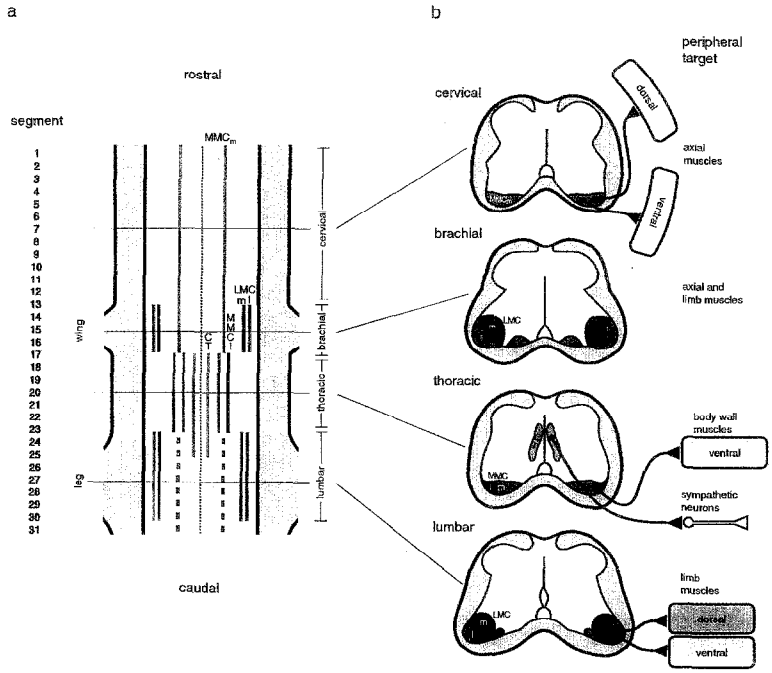

Figure 1. Schematic Representation of the Organization of Motor Columns and the Peripheral Targets of Motor Neurons

(a) Rostrocaudal position. Diagram of the position of individual motor columns along the rostrocaudal axis of the spinal cord of a stage 35 chick embryo. Numbers to the left of the spinal cord indicate spinal cord segments. Broken blue line signifies the decrease in number of motor neurons in the MMC_m at lumbar levels.

(b) Intrasegmental position. Diagram of representative transverse sections through the spinal cord at different segmental levels, showing the location of motor columns. The peripheral targets of motor neurons within each column are also depicted. For simplicity, axonal projections at brachial levels have not been depicted but are consistent with those shown at lumbar levels.

The MMC_m is shown in blue (MMC_m); the MMC_l in red (MMC_l); the LMC_m in red (LMC_m); the LMC_l in green (LMC_l); and the CT in brown (CT). Scheme for motor pool organization adapted from Levi-Montalcini (1950), Landmesser (1978b), Hollyday (1980), and Gutman et al. (1993).

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. *Islet-2* expression in the embryonic spinal cord appears to be restricted to motor neurons (Figure 2), whereas *Islet-1* is also expressed by a subset of cells in the dorsal spinal cord (Ericson et al., 1992).

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

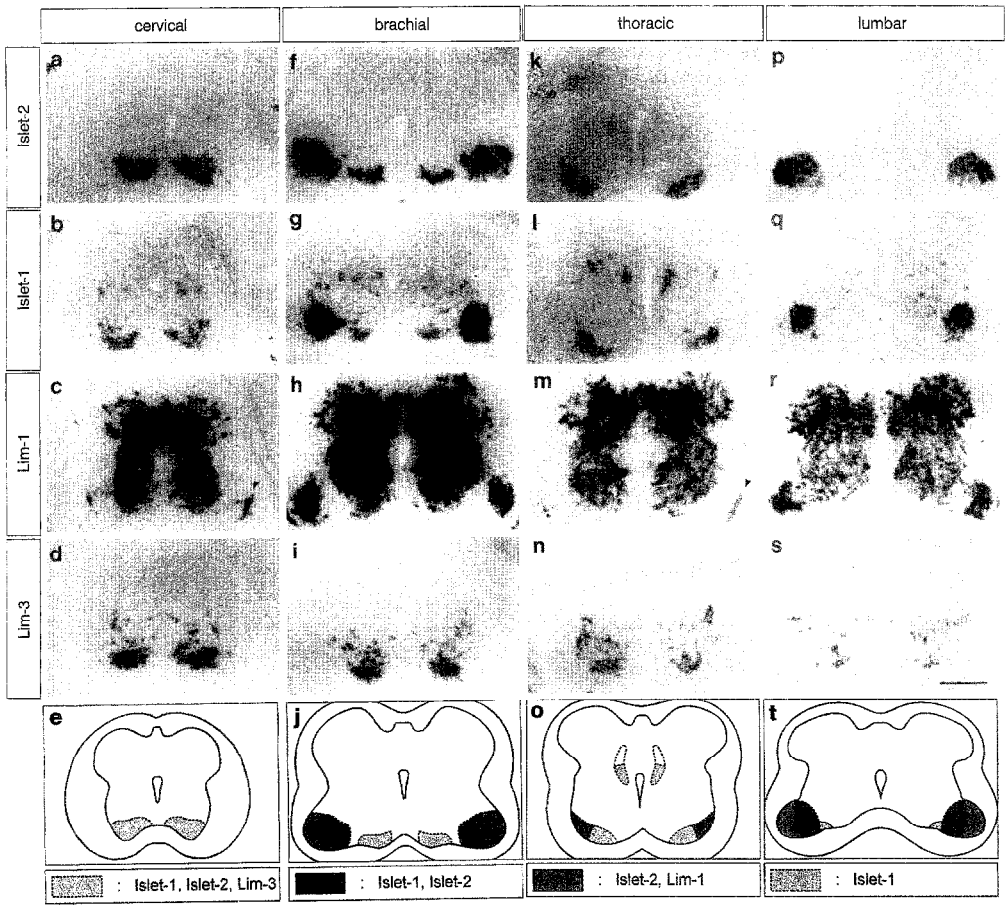

Figure 2. Expression of LIM Homeobox Genes in Chick Spinal Cord

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., 1968). 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), 285 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_m) project to axial muscles that differentiate near the vertebral column; second, motor neurons in the lateral subdivision of the median motor column (MMC_l) project to body wall muscles that differentiate within the ventral lateral plate mesenchyme; third, motor neurons in the medial subdivision of the lateral motor column (LMC_m) 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_l) 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

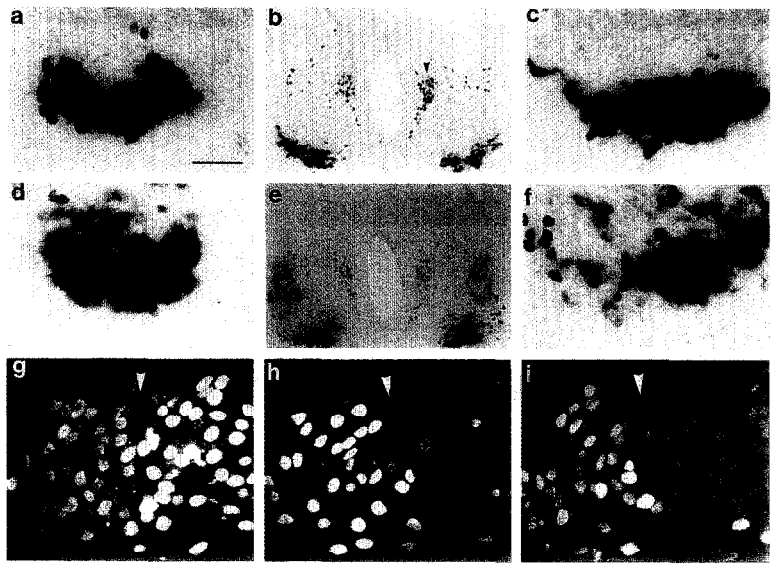

Figure 3. Coexpression of LIM Homeobox Genes by Neurons in the MMC and the LMC

Coexpression of LIM homeobox genes determined by combined immunocytochemistry and in situ hybridization or by double-label immunocytochemistry and confocal microscopy on sections of stage 34–35 chick spinal cord.

(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 *Islet-2* in the MMC_m at cervical levels. Virtually all Islet-1 cells in the MMC_m coexpress *Islet-2*. The Islet-1-labeled nuclei observed dorsal to the MMC_m do not fall 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_m and the MMC_l, but expression of Islet-1 alone in the CT (arrowhead).

(c) High power view of a similar thoracic section showing congruence of expression of Islet-1 and *Islet-2* in the MMC.

(d–f) Colocalization of Islet-1/Islet-2 using a pan-Islet antibody (brown) and *Lim-3* (blue/gray).

(d) High power view showing coexpression of Islet-1/Islet-2 immunoreactivity and *Lim-3* in the MMC at cervical levels. Virtually all Islet-1/Islet-2 cells in the MMC_m coexpress *Lim-3*. *Lim-3* cells that do not show Islet-1/Islet-2 immunoreactivity are found dorsal to the MMC_m.

(e) Thoracic section showing that *Lim-3* is coexpressed with Islet-1/Islet-2 in the MMC_m but not in the MMC_l. The section shows the segregation of Islet-1/Islet-2/*Lim-3* cells medially and Islet-1/Islet-2 cells laterally (arrowhead) within the MMC. CT cells also show Islet-1/Islet-2 immunoreactivity.

(f) High power view of a thoracic section showing the segregation of medial Islet-1/Islet-2/*Lim-3* and lateral Islet-1/Islet-2 cells. The section is representative in showing some intermixing of the two populations.

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

(g) Combined use of Islet-1-specific (red) and pan-Islet (green) antibodies shows that cells in the LMC_m label with both antibodies (yellow), whereas cells in the LMC_l react only with pan-Islet antibodies (green), indicating expression of Islet-2 alone.

(h) Combined use of anti-Lim-1/Lim-2 (green) and pan-Islet (red) antibodies shows that cells in the LMC_l coexpress Lim-1 and Islet-2 (yellow), whereas cells in the LMC_m express only the Islet-1/Islet-2 proteins (red). In situ hybridization studies show that the patterns of expression of *Lim-1* and *Lim-2* in nonmotor neurons are similar but that only *Lim-1* is expressed in the region of the LMC (unpublished data). Thus, although the pan-Lim antibodies recognize both Lim-1 and Lim-2, the immunoreacivity in the LMC represents Lim-1.

(i) Combined use of anti-Lim-1/Lim-2 and Islet-1-specific antibodies shows the segregation of neurons in the LMC that express Lim-1 (green) and Islet-1 (red). A few Lim-1 cells that do not express Islet-1 were detected in the LMC. These cells probably correspond to interneurons that are interspersed with motor neurons in the LMC by this stage.

White arrowheads indicate location of the border between the medial (m) and lateral (l) subdivisions of the LMC. Medial is to the right in all high power figures. Micrographs are representative of studies on two to ten embryos. Scale bar reprsents 40 μm in (a), (c), (d), and (f)–(i), and 130 μm in (b) and (e).

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_m extends along the entire rostrocaudal length of the spinal cord, although at lumbar levels the number of motor neurons in the MMC_m decreases markedly. The MMC_l is restricted to thoracic levels (see Figure 1a). *Islet-2*, *Islet-1*, and *Lim-3* were expressed uniformly within the MMC_m (Figures 2a, 2b, 2d, 2f, 2g, 2i, 2k, 2l, and 2n). By contrast, the MMC_l expressed *Islet-1* and *Islet-2* but not *Lim-3* (Figures 2k–2o). Thus, motor neurons in the MMC_m and the MMC_l 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_m) and lateral (LMC_l) 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_m (Figures 2g and 2q). Conversely, *Lim-1* expression by motor neurons was restricted to the LMC_l (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_m from the LMC_l.

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.

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

Motor Neuron Subclass	LIM Homeobox Genes	Color Code
MMC_m	*Islet-1*, *Islet-2*, *Lim-3*	blue
MMC_l	*Islet-1*, *Islet-2*	red
LMC_m	*Islet-1*, *Islet-2*	red
LMC_l	*Islet-2*, *Lim-1*	green
CT_v	*Islet-1*	brown

Motor Neurons within Individual Columns Coexpress LIM Homeobox Genes

The in situ hybridization studies described above do not resolve whether motor neurons coexpress LIM homeobox genes or whether motor neurons that express distinct genes are intermixed within an individual column. To define the expression of LIM homeobox genes by single cells, 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_m express Islet-1 and Islet-2, whereas neurons in the LMC_l express Islet-2 and Lim-1 (Figure 3; data not shown).

We next examined whether individual neurons within somatic motor columns coexpress LIM homeobox genes, by use of double-label immunocytochemistry and a combination of immunocytochemistry and in situ hybridization on single sections. Motor neurons in the MMC_m coexpressed Islet-1, *Islet-2*, and *Lim-3* (Figures 3a and 3d). Cells in the MMC_l coexpressed Islet-1 and *Islet-2* but did not express *Lim-3* (Figures 3b, 3c, 3e, and 3f). Motor neurons in the LMC_m coexpressed Islet-1 and Islet-2 (Figures 3g–3i), whereas cells in the LMC_l coexpressed Islet-2 and Lim-1 (Figure 3h).

These studies show that, at stage 35, motor neurons within the MMC_m coexpress *Islet-1*, *Islet-2*, and *Lim-3* and that motor neurons within both the MMC_l and the LMC_m coexpress *Islet-1* and *Islet-2*, whereas motor neurons in the LMC_l coexpress *Islet-2* and *Lim-1* (Table 1).

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 to HRP and to LIM homeodomain proteins. We focused this analysis on motor neurons in the LMC, since 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_m 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_l 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_l 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_m and coexpressed Islet-1 and Islet-2 but not Lim-1 (Figures 4g–4i).

These results show directly that motor neurons in the LMC_m that coexpress Islet-1 and Islet-2 project to ventrally derived muscles, whereas motor neurons in the LMC_l 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, 1962), is innervated by motor neurons located in the LMC_l (Straznicky and Tay, 1983; Hollyday and Jacobson, 1990) and not, as expected, in the MMC_m. 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_l expressed *Islet-1*, *Islet-2*, and *Lim-3*, a combination of genes characteristic of the MMC_m, 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_l 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_l, 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_m and not LMC_l 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.

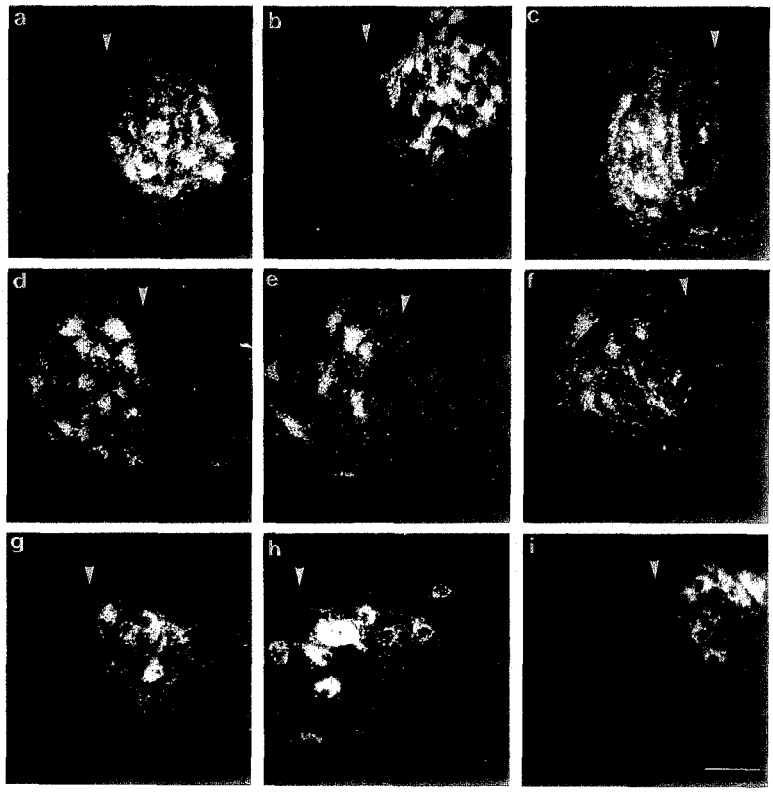

Figure 4. Motor Neurons in the LMC that Express Distinct LIM Homeobox Genes Project to Different Muscle Targets

LIM homeodomain proteins are restricted to nuclei, whereas retrogradely transported HRP is localized in the cytoplasm of motor neurons. All sections are taken at lumbar levels. In all micrographs, LIM homeodomain proteins are detected with Texas red–labeled antibodies (red) and HRP with BODIPY–FL-labeled antibodies (yellow/green).

(a) Expression of Islet-1 in HRP-labeled motor neurons in the LMC_m after injection of HRP into the ventral limb muscle mass in a stage 30 embryo.

(b) Segregation of Lim-1 to the LMC_l and HRP-labeled motor neurons to the LMC_m after injection of HRP into the ventral limb muscle mass in a stage 30 embryo.

(c) Lim-1 expression in HRP-labeled motor neurons in the LMC_l after injection of HRP into the dorsal limb muscle mass in a stage 30 embryo. To avoid leakage of HRP, injections were confined to the region of the dorsal muscle mass that gives rise to the femorotibialis muscle. This explains why neurons in the lateral region of the LMC_l do not contain HRP. The extreme lateral Lim-1 cells are in Hofmann's nucleus major. Micrographs in (a)–(c) are representative of studies on ten embryos.

(d) Motor neurons labeled with HRP after injection into the sartorius muscle at stage 37 are restricted to the LMC_l. The section has been colabeled with pan-Islet antibodies to delineate the entire LMC.

(e) Segregation of Islet-1 to the LMC_m and HRP-labeled motor neurons to the LMC_l after injection of HRP into the sartorius muscle at stage 37, as determined by labeling with an Islet-1-specific antibody.

(f) Expression of Lim-1 in LMC_l motor neurons labeled with HRP after injection into the sartorius muscle at stage 37. Lim-1 cells near the LMC_m are likely to be interneurons that are interspersed with motor neurons in the LMC at this stage. Consistent with this, they are never retrogradely labeled after HRP injection into muscle targets.

(g) Motor neurons labeled with HRP after injection into the adductor muscle at stage 37 are restricted to the LMC_m, as determined by pan-Islet immunoreactivity.

(h) Expression of Islet-1, detected with an Islet-1-specific antibody, in LMC_m motor neurons labeled with HRP after injection into the adductor muscle at stage 37.

(i) Segregation of Lim-1- and HRP-labeled motor neurons after injection of HRP into the adductor muscle at stage 37. The small number of Lim-1 cells in the LMC_l reflects the fact that at this stage Lim-1 expression has already been lost from femorotibialis motor neurons although not from sartorius motor neurons or other lateral motor nuclei in the LMC_l (data not shown). Micrographs are representative of studies on eleven embryos. White arrowheads indicate approximate position of the boundary between the lateral (left) and medial (right) subdivisions of the LMC. Scale bar, 38 μm.

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_m 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 15 (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_m 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 stages 17–18 showed the presence of Islet-1 cells that did not express *Islet-2* (Figure 6f). Moreover, in an analysis of serial sec-

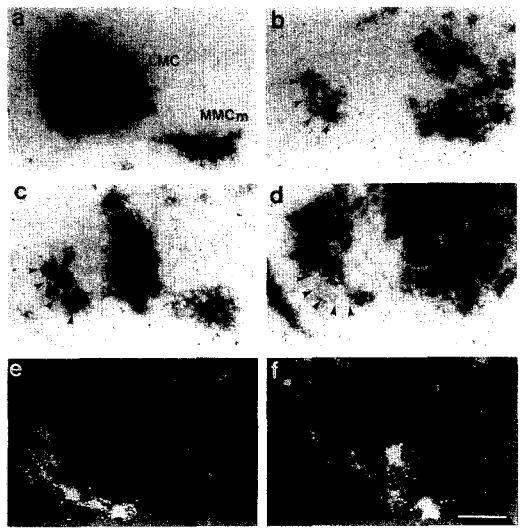

Figure 5. Motor Neurons in the Brachial LMC that Project to the Rhomboideus Muscle Express LIM Homeobox Genes Characteristic of MMC_m Neurons

Expression of LIM homeobox genes was determined by in situ hybridization and immunocytochemistry. The identity of rhomboideus motor neurons was defined by retrograde labeling after injection of HRP into the rhomboideus muscle. Sections are shown at level C14.

(a) *Islet-2* is expressed throughout the LMC and the MMC_m.

(b) *Lim-3* is expressed in the MMC_m and in a few cells in the far lateral (left) region of the LMC_l (arrowheads). Nonmotor neurons dorsal to the MMC_m also express *Lim-3*.

(c) *Islet-1* is expressed by neurons in the MMC_m, the LMC_m, and cells in the far lateral region of the LMC_l (arrowheads). A few nonmotor neurons dorsal to the MMC_m are also detected.

(d) *Lim-1* is expressed by neurons in the LMC_l with the exception of cells in its most ventrolateral region (arrowheads), which corresponds to the domain that expresses *Lim-3* and *Islet-1*. Motor neurons in the LMC_m and the MMC_m are not labeled, but many interneurons express *Lim-1*. *Lim-1* labeling lateral to the LMC_l corresponds to neurons in Hofmann's nucleus major.

(e) Motor neurons in the lateral region of the LMC_l are retrogradely labeled after injection of HRP (green) into the rhomboideus muscle. These neurons but not other LMC_l neurons express Islet-1 (red).

(f) Motor neurons in the far lateral region of the LMC_l, labeled retrogradely after injection of HRP into the rhomboideus muscle (green), do not express Lim-1 (red), whereas neighboring LMC_l neurons do.

Micrographs (a)–(d) are representative of studies on three embryos, and (e) and (f) of studies on five embryos. In all micrographs, medial is to the right. Scale bar represents 40 μm in (a)–(d), 30 μm in (e), and 22 μm in (f).

tions, *Islet-1* but not *Islet-2* was detected in cells close to the ventricular zone (Figures 6a and 6b), whereas *Lim-3* cells were detected only at the extreme lateral edges of the spinal cord (compare Figures 6a, 6b, and 6c; data not shown). Newborn motor neurons emerge from the ventricular zone and migrate laterally (Langman and Haden, 1970); thus, the mediolateral position of a motor neuron provides an indication of the time that has elapsed since its birth. These observations, taken together with the double-label analysis, provide evidence that *Islet-1* is the first LIM homeobox gene to be expressed in the MMC_m, followed by *Islet-2* and then by *Lim-3*.

LMC Neurons

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_m neurons and the MMC_l and CT are not present (see Figure 1a). At lumbar levels, most LMC_m neurons are born at stages 18–19 and most LMC_l neurons at stages 20–21 (Whitelaw and Hollyday, 1983b). Thus, those motor neurons generated at early times populate the LMC_m, and those at later times, the LMC_l.

To examine the onset of LIM homeobox gene expression by LMC_m neurons, we monitored the appearance of *Islet-1* and *Islet-2*. At late 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_m as in the MMC_m, *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_l 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_l 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_m expressed *Islet-1* and *Islet-2* but not *Lim-1* (see Figures 6j–6l). These results indicate that LMC_l neurons express *Islet-1* before *Lim-1* or *Islet-2* and also that *Islet-1* expression is transient, decaying as LMC_l neurons migrate laterally and begin to express *Islet-2* and *Lim-1*. Since motor axons begin to establish dorsal or ventral pathways in the hindlimb mesenchyme only at stages 24–25 (Lance-Jones and Landmesser, 1981a; Tosney and Landmesser, 1985a, 1985b), these results indicate that the combinatorial expression of LIM homeobox genes by LMC neurons precedes the formation of their distinct axon trajectories.

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_m 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_m neurons (Figures 7j–7l). These observations suggest that neurons that coexpress Lim-1 and Islet-2 migrate through LMC_m neurons that have been generated at earlier times to reach their final position in the LMC_l. Thus, the LIM homeobox gene phenotype of LMC_l neurons appears to be established prior to their lateral migration and before the segregation of motor neurons into columns.

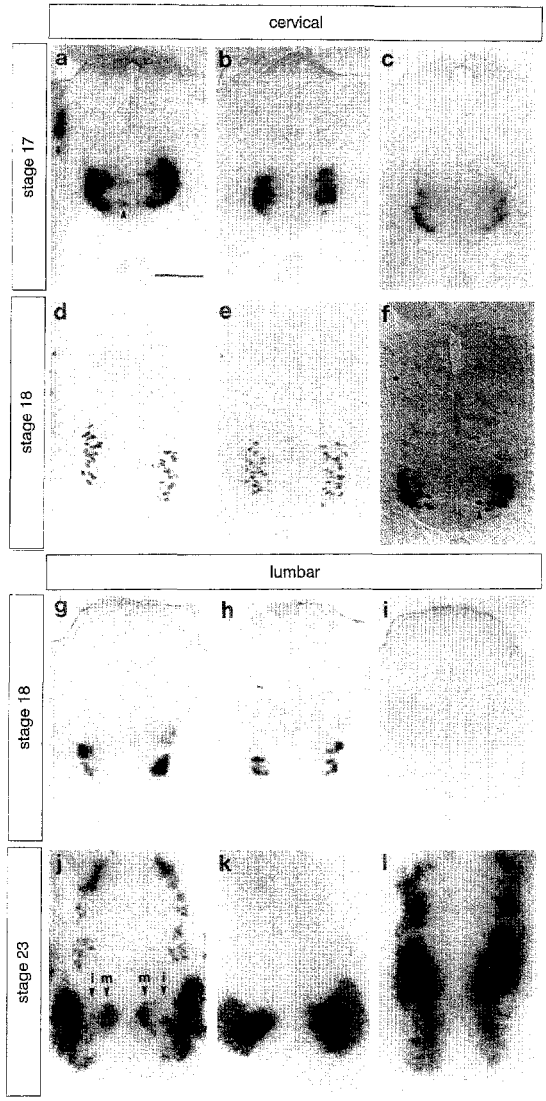

Figure 6. Onset of Expression of LIM Homeobox Genes by Motor Neurons

(a)–(f) show the expression of LIM homeobox genes in MMC_m neurons at cervical levels in late stage 17 (a–c) and stage 18 (d–f) embryos.
(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 medially located 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-1* 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 (i 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_m 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 (j)–(l).

MMC_l and CT Neurons

The generation of MMC_l and CT neurons overlaps temporally and spatially with that of MMC_m neurons (Prasad and Hollyday, 1991). Because of this, it was not possible to define the onset of LIM homeobox gene expression by MMC_l 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

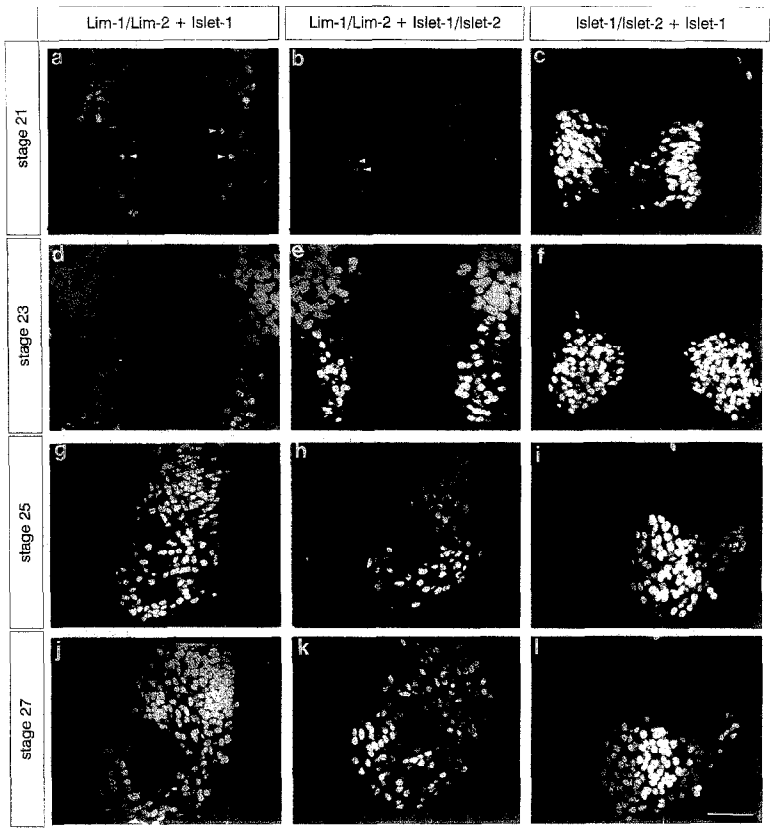

Figure 7. Early Expression of LIM Homeobox Genes by LMC Neurons

Dual immunofluorescence confocal images showing the onset of LIM homeodomain protein expression by neurons in the lumbar LMC.

(a) At stage 21, a few Lim-1 cells can be detected in the ventral spinal cord, and these coexpress Islet-1 (orange cells marked by white arrowheads). The more ventral Lim-1/Lim-2 cells (green) adjacent to the floor plate are interneurons found in region X (Yamada et al., 1991).

(b) A few Lim-1/Islet double-labeled cells (orange) are also detected with pan-Islet antibodies (white arrowheads). It remains unclear whether these cells express Islet-2 as well as Islet-1 at this stage.

(c) Cells detected with pan-Islet antibodies are also detected with Islet-1-specific antibodies (yellow).

(d) At stage 23, ventral Lim-1 cells (green) do not express Islet-1 (red) and are found medial to the LMC_m cells that occupy the lateral region of the spinal cord at this stage.

(e) The ventral Lim-1 cells colabel with pan-Islet antibodies (yellow), indicating that Lim-1 cells coexpress Islet-2.

(f) LMC_m cells that coexpress Islet-1 and Islet-2 (yellow) are found lateral to cells in the intermediate region of the LMC that express only Islet-2 (green). Compare this figure with the in situ hybridization patterns shown in Figures 6j–6l.

(g) At stage 25, ventrolateral Lim-1 cells (green) do not express Islet-1 (red) and are now interspersed with and lateral to the Islet-1 LMC_m cells.

(h) Ventral Lim-1 cells do, however, coexpress Islet-2 (yellow cells).

(i) Cells that express Islet-2 alone (green) are intermingled with or are lateral to LMC_m cells that coexpress Islet-1 and Islet-2 (yellow).

(j) At stage 27, ventrolateral Lim-1 cells (green) do not express Islet-1 (red) and are now found almost exclusively lateral to the Islet-1 LMC_m cells. The Lim-1/Lim-2 cells at an extreme medial position are interneurons. Islet-1 cells at the extreme left of the figure are dorsal root ganglion neurons.

(k) Lim-1 cells coexpress Islet-2 (orange cells).

(l) Most cells that express Islet-2 alone (green) are found lateral to LMC_m cells that coexpress Islet-1 and Islet-2 (yellow), although a few medial Islet-2 cells (green) can still be seen at this stage. In addition, a few cells that coexpress Islet-1 and Islet-2 (green/yellow) can be seen in an extreme medial position. Because these cells do not appear to express Lim-1 (see [j] and [k]), the columnar fate of these neurons is unclear.

In all unilateral images, medial is to the right. Micrographs are representative of studies on at least three embryos at each stage. Scale bar represents 60 μm in (a)–(c), 30 μm in (d)–(f), and 25 μm in (g)–(l).

of motor neurons on the basis of their position in the spinal cord, their axonal projection pattern, 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 (Tosney, 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

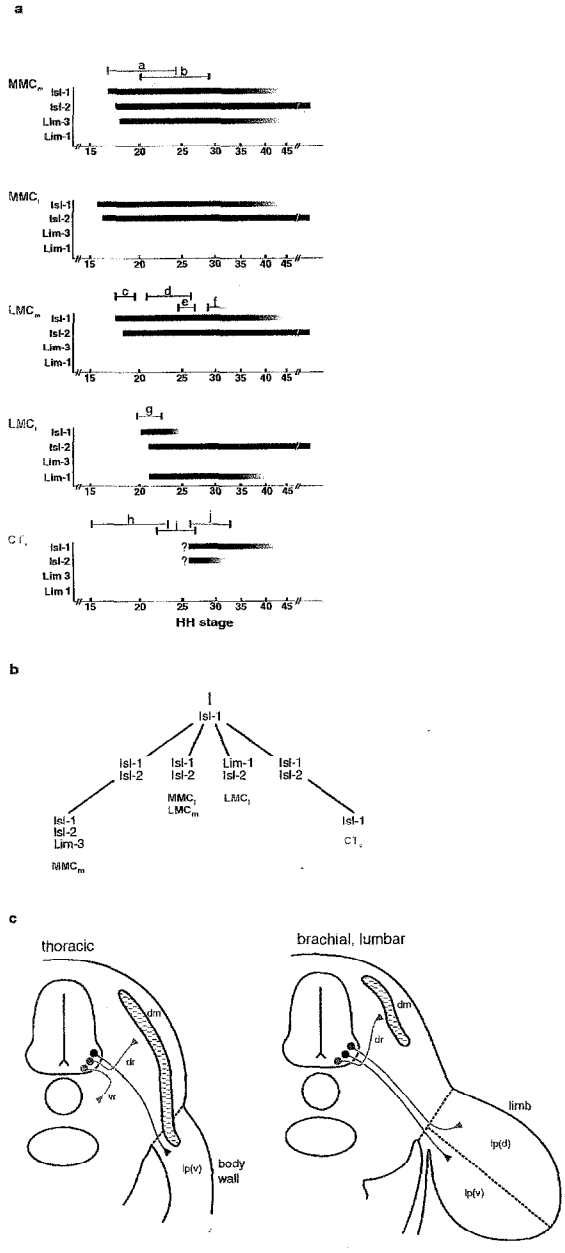

Figure 8. LIM Homeobox Gene Expression and the Early Axonal Trajectories of Motor Neurons

(a) Temporal expression in motor columns. Summary of the time course of expression of LIM homeobox genes by neurons within individual motor columns. Because of limited information on motor neuron birthdays and the timing of motor axon extension within particular columns at specific axial levels, the timing of gene expression in the MMC$_m$ is depicted at lumbar levels, in the MMC$_l$ and the CT at thoracic levels, and in the LMC$_m$ and the LMC$_l$ at lumbar levels. A detailed time course of the extinction of gene expression has not been performed. Colors designate the combination of LIM homeobox genes expressed at the time that motor axons select distinct pathways, except for CT neurons, which are defined at stage 35. Blue, *Islet-1*, *Islet-2*, *Lim-3*; red, *Islet-1*, *Islet-2*; green, *Islet-2*, *Lim-1*; brown, *Islet-1*. Critical events in the early history of motor neurons are indicated by the bars above the plot for each motor column as follows: a, time of birthday of MMC$_m$ neurons (Prasad and Hollyday, 1991); b, detection of axons that deviate dorsally from the ventral root at lumbar levels (Tosney and Landmesser, 1985b); c, birthdate of lumbar LMC$_m$ neurons (Whitelaw and Hollyday, 1983b); d, time at which the growth cones of LMC neurons arrive at the base of the limb (Tosney and Landmesser, 1985a, 1985b); e, time at which LMC motor axons enter the limb mesenchyme (Tosney and Landmesser, 1985a, 1985b); f, onset of muscle innervation (Landmesser, 1978a); g, time of birthdate of LMC$_l$ neurons (Whitelaw and Hollyday, 1983b); h, time of birthdate of CT neurons (Prasad and Hollyday, 1991); i, time at which motor axons are found in the vicinity of the primary sympathetic chain (Yip, 1990; Prasad and Hollyday, 1991); j, time at which the dorsal migration of CT neurons begins (Levi-Montalcini, 1950; Prasad and Hollyday, 1991).

(b) Sequential expression by individual neurons. Proposed sequence of LIM homeobox gene expression by individual motor neurons within each embryonic motor column. This scheme indicates that all motor neurons express *Islet-1* initially, prior to the expression of other LIM homeobox genes. Details of the early expression of these genes by CT neurons and MMC$_l$ neurons are uncertain, because at thoracic levels they are intermingled with MMC$_m$ neurons.

(c) Motor axon trajectories. Summary of the early axonal trajectories of subclasses of motor neurons. The diagram depicts sections through a stage 25 embryo. At this stage, motor neurons are defined by combinatorial expression of LIM homeobox genes, and their axons have selected different pathways, but they have not segregated into distinct columns. The axons of MMC$_m$ neurons (blue) break away from the ventral root and project toward the dermomyotome (dm) to form a dorsal ramus (dr) at thoracic, brachial, and lumbar levels. At brachial and lumbar levels, the axons of LMC$_m$ neurons (red) project ventrolaterally and enter lateral plate mesenchyme of ventral character (lp(v)), whereas LMC$_l$ neurons (green) also project ventrolaterally but enter lateral plate mesenchyme of dorsal character (lp(d)). At thoracic levels, MMC$_l$ neurons (red) follow a path similar to that of LMC$_m$ neurons, entering lateral plate mesenchyme of ventral character in the body wall. At cervical levels (data not shown), MMC$_m$ neurons follow a pathway similar to that shown at thoracic, brachial, and lumbar levels. CT neurons (brown) project into the sympathetic chain along a ventral ramus (vr), although the combination of LIM homeobox genes that they express at this early stage has not been resolved. For further details, see Tosney and Landmesser (1985a, 1985b), Tosney (1991), Prasad and Hollyday (1991), and text.

which point subclasses of motor axons establish different trajectories (Figure 8c). The axons of MMC$_m$ 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 MMC$_m$ 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 MMC$_l$ and LMC 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 LMC 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, 1981a; Ferguson, 1983; Tosney and Landmesser, 1984; Phelan and Hollyday, 1990). Neurons in the LMC$_m$ project into ventral mesenchyme, whereas neurons in the LMC$_l$ project into dorsal mesenchyme. At thoracic levels, motor neurons in the MMC$_l$ destined to innervate body wall muscles also project into ventral mesenchyme of lateral plate origin (Wachtler and Christ, 1992; Gutman et al., 1993), a pathway similar to that taken by LMC$_m$ 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. Coexpression of *Islet-1*, *Islet-2*, and *Lim-3* defines MMC$_m$ neu-

rons that appear to respond to target-derived cues from the dermomyotome. Coexpression of *Islet-1* and *Islet-2* defines MMC_l and LMC_m neurons that extend axons into lateral plate mesenchyme of ventral character. Coexpression of *Lim-1* and *Islet-2* defines LMC_l 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_m neurons innervate an axial muscle target, even though they are located in the LMC_l. Taken together, these results suggest that one function of LIM homeobox genes is to confer subclasses 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_m that innervate dorsally and ventrally located axial muscles. Motor neurons that project to ventral axial (hypaxial) muscles are located medially in the MMC_m, 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_m 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_m neurons is established in a manner that does not involve the differential expression of transcription factors. MMC_m 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_m and LMC_l 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_l but express *Islet-1*, *Islet-2*, and *Lim-3*, a profile characteristic of MMC_m neurons. The atypical position of rhomboideus motor neurons raises the possibility that they express genes that subvert the normal pattern of segregation of MMC_m 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_m 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_m, the LMC_l, and the CT, express distinct combinations of LIM homeobox genes. Motor neurons within the MMC_l and the LMC_m, 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_l from those in the LMC_m. 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_l and the LMC_m 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_l and LMC_m 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_l and the LMC_m 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_l 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*, *Lmx-1*, and *Gsh-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; T. T., unpublished data) and control the fate and pathfinding of subsets of neurons in the Drosphila and nematode nervous systems (Bourgouin 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*, *Lim-3*, 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 (4D5) 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 (1D5) 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 (4F2) were generated against residues 1–360 of rat Lim-2 expressed in E. coli. The specificity of antibodies was determined by comparison of the labeling patterns obtained by immunocytochemistry and by in situ hybridization.

Immunohistochemistry

For immunohistochemisty, 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, 4D5 at 1:100, A7 and A8 at 1:5000, T4 at 1:3000, and 1D5 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.

Retrograde Labeling of Motor Neurons

Fertilized chick eggs (Spafas) were incubated at 37°C, and experiments were performed on embryos at stages 30, 35, and 37 (Hamburger and Hamilton, 1951) according to Hollyday (1980). To inject the rhomboideus muscle, 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 Immunochemicals, 1:1000).

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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.