

# Motoneuron fate specification revealed by patterned LIM homeobox gene expression in embryonic zebrafish

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## SUMMARY

In zebrafish, individual primary motoneurons can be uniquely identified by their characteristic cell body positions and axonal projection patterns. The fate of individual primary motoneurons remains plastic until just prior to axogenesis when they become committed to particular identities. We find that distinct primary motoneurons express particular combinations of LIM homeobox genes. Expression precedes axogenesis as well as commitment, suggesting that LIM homeobox genes may contribute to the specification of motoneuronal fates. By transplanting them to new spinal cord positions, we demonstrate that

primary motoneurons can initiate a new program of LIM homeobox gene expression, as well as the morphological features appropriate for the new position. We conclude that the patterned distribution of different primary motoneuronal types within the zebrafish spinal cord follows the patterned expression of LIM homeobox genes, and that this reflects a highly resolved system of positional information controlling gene transcription.

Key words: primary motoneuron, commitment, *islet1*, *islet2*, *lim3*, zebrafish, LIM, homeobox gene, gene expression, motoneuron

## INTRODUCTION

The development of a functional vertebrate nervous system requires elaboration of a large number of diverse cell types in highly specific locations. We have been studying the development of a small set of identified neurons, primary motoneurons, in zebrafish to understand how neuronal cell fates become specified. Zebrafish primary motoneurons can be identified by their cell body positions and axonal trajectories (Eisen et al., 1986, 1990). They are serially distributed in bilateral clusters that correspond to the adjacent, segmentally arranged somites. Each cluster is composed of three distinct primary motoneurons, RoP, MiP and CaP with a fourth primary motoneuron, VaP, variably present. Every cell body assumes a stereotyped position within the ventral spinal cord and the axons of a segmental group exit the spinal cord along a common pathway, forming a ventral root. The axons diverge a short distance from the spinal cord to innervate distinct motoneuron-specific regions of the corresponding axial musculature.

Transplantation experiments showed that primary motoneurons become committed to a particular fate after their final division but before axogenesis (Eisen, 1991). Primary motoneurons are born beginning at about 10 hours (postfertilization at 28.5°C) (Myers et al., 1986; Kimmel et al., 1994). Axogenesis begins at about 17 hours depending on the axial level, but the cells can be recognized by their cell body

positions several hours before axonal outgrowth. When transplanted to heterotopic locations within the ventral spinal cord just before axogenesis, primary motoneurons exhibited axonal trajectories and, thus, fates appropriate for their original positions. If, however, a primary motoneuron was transplanted to a new location at an earlier time relative to axogenesis, it adopted a primary motoneuronal fate appropriate for its new position as indicated by its axonal trajectory (Eisen, 1991). These observations suggest that, after a period of plasticity, primary motoneurons become committed to a particular fate that is determined by the local environment.

What are the molecular mechanisms that underlie specification to particular neuronal cell fates? To address this question we have begun to identify genes expressed in primary motoneurons prior to their morphological differentiation. Among these are several members of the homeobox subclass of the LIM gene family from zebrafish, including homologs of the genes described by Tsuchida et al. (1994), which are expressed in chick motoneurons. Previous work has shown that one of these genes, *islet1*, is among the earliest known genes expressed in motoneurons of rat and chick and primary motoneurons of zebrafish (Thor et al., 1991; Ericson et al., 1992; Korzh et al., 1993; Inoue et al., 1994). Genetic studies have shown that LIM homeobox genes are important for development, including the specification of neuronal fates. For example, in *Caenorhabditis elegans*, *mec-3* is necessary for

differentiation of mechanosensory neurons (Way and Chalfie, 1988), in *Drosophila melanogaster*, *apterous* is required for axonal pathway selection by a subset of interneurons (Lundgren et al., 1995) and, in mice, *Lim1* is required for head development (Shawlot and Behringer, 1995).

To begin a detailed analysis of the role of the LIM homeobox gene family in primary motoneuron fate specification, we have isolated several corresponding cDNAs from zebrafish. We show that zebrafish LIM homeobox genes are combinatorially expressed in primary motoneurons and that distinct primary motoneurons express different combinations of these genes. We show that expression of these genes in zebrafish primary motoneurons begins well before overt differentiation and that the patterns are highly dynamic. The resolution of the RNA expression patterns precedes the commitment of a cell to a particular motoneuronal fate. Finally, using cell transplantation experiments, we demonstrate that the expression of a LIM homeobox gene is determined by the position of a primary motoneuron within the spinal cord and, furthermore, that commitment of a primary motoneuron to a particular fate is correlated with a distinct pattern of LIM homeobox gene expression.

## MATERIALS AND METHODS

### Isolation of zebrafish LIM homeobox genes

Approximately  $1 \times 10^6$  plaques of a 20–28 hour lambda ZAP-II cDNA library (gift of Robert Riggleman, Kathryn Helde and David Grunwald) were screened at low stringency using a fragment encompassing the rat *Islet-1* homeobox (Karlsson et al., 1990) to isolate both zebrafish *islet1* and *islet2* clones. cDNA inserts were subcloned by helper-phage cotransfection (Stratagene) and mapped with restriction enzymes. Both strands were sequenced by the dideoxy chain termination method using a T7 Sequencing Kit (Pharmacia). Compressions were resolved by the use of Deaza G/A T7 Sequencing Mixes (Pharmacia). DNA sequences and derived amino acid sequences were analysed on a VAX computer using Genius software package (EMBL, Heidelberg).

Zebrafish *lim3* genomic sequences, isolated by low-stringency screening of a genomic library (gift of Barbara Jones and Martin Petkovich), were the basis for designing the following primers: L3R2, 5'-TATCGAATTCAAATGTAGTACCTACTC; L1F1, 5'-ATATCC-CGGTATGTGCGG; cR1, 5'-CGAAGAGCTGAAAGCTTTCC; L1F2, 5'-TTCTGGATCGCCACTGGC; cR2, 5'-ATCGCT-GCAGTCTATGG. Primer L3R2 was used to initiate cDNA from total RNA (Westerfield, 1994) from 1-day-old zebrafish embryos. The cDNA was amplified with L1F1 and cR1 primers, and the product reamplified with the nested primers L1F2 and cR2, yielding the predicted *lim3* band of 1150 basepairs. This DNA was cloned into the pCR-Script SK (+) vector (Stratagene) to produce cDNA clone pIS8 representing the zebrafish *lim3* coding sequence from 127 nucleotides downstream of the predicted AUG start site to 31 nucleotides past the stop codon. This cDNA was used as probe in the experiments reported here. The remaining N-terminal coding sequence was generated by the RACE procedure using the materials and instructions provided by Clontech.

### Embryos

Embryos were obtained from the University of Oregon laboratory colony and raised at 28.5°C in embryo medium (13.7 mM NaCl, 0.54 mM KCl, 1.3 mM CaCl<sub>2</sub>, 1.0 mM Mg SO<sub>4</sub>, 0.044 mM KH<sub>2</sub>PO<sub>4</sub>, 0.025 mM NaH<sub>2</sub>PO<sub>4</sub>, 0.42 mM NaHCO<sub>3</sub> [pH 7.2]). Staging was according to somite formation (Kimmel et al., 1995).

### Intracellular dye labeling

Individual primary motoneurons were injected with a combination of lysinated rhodamine dextran (3,000 *M<sub>r</sub>*, Molecular Probes) and lysinated FITC dextran (3,000 *M<sub>r</sub>*, Molecular Probes) as described previously (Eisen et al., 1990). Donor embryos for primary motoneuron transplants were labeled by injecting a mixture of lysinated rhodamine dextran and lysinated biotin dextran (10,000 *M<sub>r</sub>*, Molecular Probes) into the yolk of early cleavage stage embryos as described previously (Ho and Kane, 1990). All blastomeres take up dye through cytoplasmic connections with the yolk.

### RNA in situ hybridization and photography

RNA probes were prepared by transcribing linearized cDNA clones with T3 or T7 polymerase in the presence of digoxigenin or fluorescein labeling mix (Boehringer Mannheim). In situ hybridization was carried out essentially as described previously (Thisse et al., 1993) except that the probes were not hydrolyzed. Double RNA in situ hybridization and detection was described previously (Hauptmann and Gerster, 1994). For injection and transplantation experiments, injected labels were histochemically detected, following completion of the hybridization procedure, using anti-fluorescein (Boehringer Mannheim) and anti-biotin (Sigma) antibodies, respectively, conjugated to alkaline phosphatase, which were used with Fast Red substrate (Boehringer Mannheim) to form a red precipitate. Embryos were mounted in 75% glycerol in PBS and photographed with Nomarski optics using a Zeiss Axioplan and a Kodak DCS 400 digital camera. The Fast Red reaction product is fluorescent when illuminated and viewed with a Texas Red filter set, which we used to visualize axon projections and low levels of RNA in double labeling procedures. Digital images were processed on a Power Macintosh using Adobe Photoshop 3.0, assembled with Microsoft Powerpoint and printed with a Tektronix Phaser 440 dye sublimation printer. Image processing consisted of cropping and resizing, contrast enhancement, color correction, and brightness, hue and saturation adjustment. Fluorescence and bright-field images of transplanted cells were recorded prior to fixation with an image intensifier plate connected to a video camera and stored separately on an optical disc using Axovideo software (Myers and Bastiani, 1991; Axon Instruments, Inc.). Processing consisted of addition of images from different focal planes, contrast and image enhancement, pseudocolor addition, and addition of fluorescence and bright-field images.

### Primary motoneuron transplants

Transplants were carried out as described previously (Eisen, 1991). Donor and host embryos ranged between the 12.5-somite stage and the 18-somite stage. MiPs were removed from segment levels 6–14 of donor embryos and transplanted to segment levels 5–11 of hosts. Native primary motoneurons were removed from recipient spinal segments prior to the transplantation.

## RESULTS

### Isolation of LIM homeobox genes from zebrafish

We cloned zebrafish LIM homeobox genes by screening libraries with probes generated from rat and *Xenopus laevis* cDNA clones (Materials and Methods). Isolation of zebrafish *islet1* has been reported (Inoue et al., 1994). Relationships to LIM homeobox genes identified in other vertebrates were determined by nucleotide sequence comparison (Dawid et al., 1995). Fig. 1 shows an alignment of the amino acid sequences spanning the LIM domains and homeodomains encoded by the *islet1*, *islet2* and *lim3* genes, which we focus on for the present study. The *islet1* and *islet2* proteins are very similar having a single amino acid difference within the homeodomain, and

94% and 70% amino acid sequence identity in the first and second LIM domains, respectively. The *lim3* protein is quite distinct from the *islet1* and *islet2* proteins, sharing 47% sequence identity within the homeodomain, and 56% and 51% identity within the first and second LIM domains. Zebrafish *lim3* is very similar to *lim3* of other vertebrates. For example, it has 98% sequence identity in the homeodomain and 95% identity in the LIM domains compared to its *X. laevis* ortholog (Taira et al., 1993).

### LIM homeobox genes are differentially expressed in primary motoneurons

We examined the embryonic expression of six LIM homeobox genes by in situ RNA hybridization. We did not detect RNA of *lim1* (Toyama et al., 1995a), *lim5* (Toyama et al., 1995b) or *lim6* (R. Toyama and I. B. D., unpublished results) in the ventral spinal cord of 18 hour embryos nor did double labeling experiments of the type described below identify *lim6* expression in primary motoneurons (B. A. and J. S. E., unpublished results); therefore, these genes do not appear to be expressed in primary motoneurons prior to axogenesis. Probes for *islet1*, *islet2* and *lim3*, however, detected expression patterns consistent with the positions of primary motoneurons.

We identified the primary motoneurons that express *islet1*, *islet2* and *lim3* by injecting fluorescent vital dye into individual cells and, subsequently, probing for gene expression by in situ RNA hybridization. Dye was injected into cells of live 18-22 hour embryos, which were viewed periodically with epifluorescence. Embryos were fixed and hybridized with digoxigenin-labeled RNA probes once motoneuron axons developed their characteristic projections. At the time primary motoneurons develop their axonal projections, additional cells within the spinal cord express *islet1*, *islet2* and *lim3* (see below); however, the double-labeling technique allows us to distinguish clearly primary motoneurons from other cell types. The midsegmentally located CaP motoneuron expresses *islet2* (Fig. 2A,B) and *lim3* RNAs (Fig. 2C,D). VaP, which is closely apposed to CaP and has equivalent developmental potential (Eisen, 1992), also expresses *islet2* and *lim3* (data not shown). MiP, located near the overlying somite furrow, expresses *islet1* (Fig. 2E,F) as well as *lim3* (Fig. 2G,H). RoP, which lies just rostral to MiP near the somite border, also expresses *islet1* (Fig. 2I,J) and *lim3* (Fig. 2K,L).

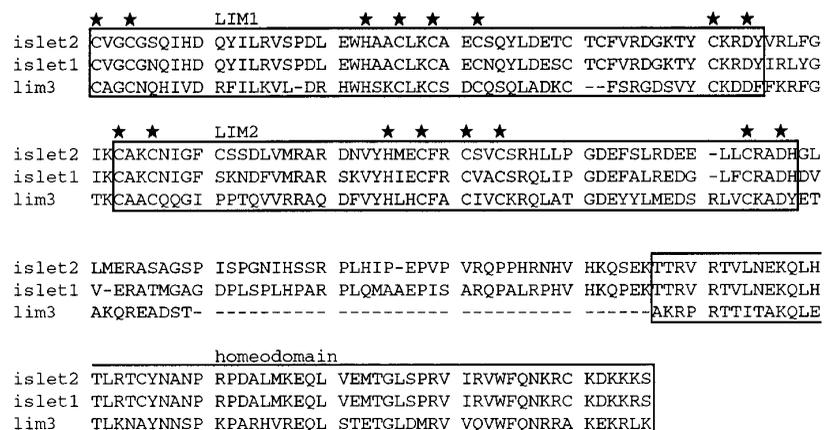
Thus, RoP and MiP can be differentiated from CaP and VaP on the basis of LIM homeobox gene expression. All four cells express *lim3* RNA. RoP and MiP express *islet1* RNA, but not *islet2*, while CaP and VaP express *islet2* RNA, but not *islet1*.

### Expression of LIM homeobox genes precedes primary motoneuron axogenesis

Expression of LIM homeobox genes is initiated at early stages of spinal cord development. The zebrafish spinal cord arises from convergent movements of cells within the neuroepithelium toward the dorsal midline of the embryo (Schmitz et al., 1993; Kimmel et al., 1994; Papan and Campos-Ortega, 1994). These movements initially

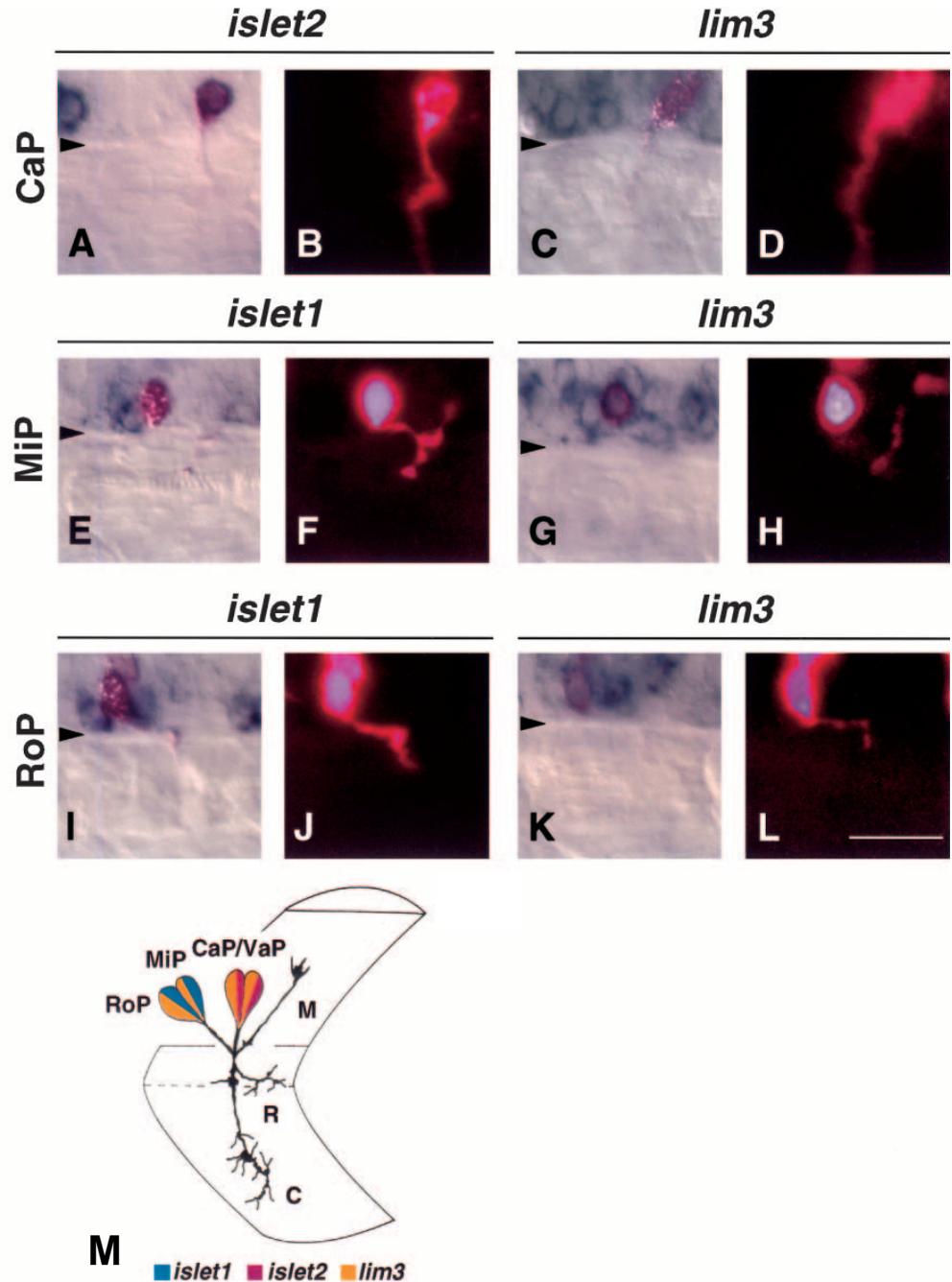
form a neural keel, which continues to thicken with further condensation of the neural plate where the ventral spinal cord will arise (Papan and Campos-Ortega, 1994). We first detect *islet1* RNA near the midline in the early stages of keel formation, when one or two somites are evident (10.3-10.7 hours). *islet1* RNA is initially confined to several irregularly spaced cells on both sides of the differentiating floorplate at the level of the first somites to form (Fig. 3A). Expression rapidly expands posteriorly into the neural plate adjacent to unsegmented mesoderm, forming two longitudinal columns of cells (data not shown). *islet1* expression proceeds posteriorly at approximately the same rate as somite furrow formation, but maintains a posterior limit of expression about four somite equivalent lengths caudal to the most recently formed somite furrow (data not shown). *lim3* expression is first evident at the 3-somite stage (11 hours) in bilateral, discontinuous, longitudinal columns bordering the floorplate. Like *islet1*, *lim3* expression shows no clear segmental pattern at early stages (Fig. 3B). *lim3* expression also extends into the unsegmented region of the embryo, but about one half as far as *islet1*. *islet2* expression is first detected at the 7-somite stage (12.5 hours) in bilateral columns bordering the floorplate (data not shown). The posterior extent of *islet2* expression roughly corresponds with the most posterior somite.

Patterns of *islet1*, *islet2* and *lim3* expression in the spinal cord are very dynamic through early neurogenesis. Therefore, we focus on the patterns at a single axial level, somites 6 and 7, at different developmental stages. The patterns that we describe, although typical, are not invariant as minor differences in the timing of expression are evident in individual embryos. *islet2* exhibits the simplest pattern. In 8-somite-stage (13 hour) embryos, *islet2* RNA is found in one or two cells per hemisegment, but without a clear spatial arrangement (Fig. 4A). By the 10-somite stage (14 hours), *islet2* is still expressed in one or two cells per hemisegment, but they are located in a regular position in the ventral spinal cord, midway between somite borders (hereafter referred to as midsegment cells) (Fig. 4B). This pattern is maintained through the 12-somite (15 hour), 16-somite (17 hour) and 19-somite (18.5 hour) stages



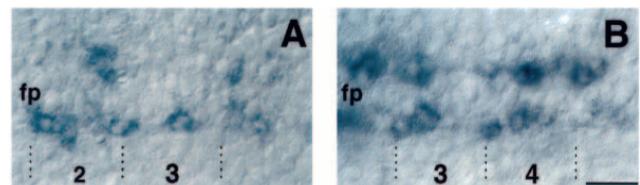
**Fig. 1.** Alignment of amino acids comprising the LIM domains and homeodomains derived from conceptual translation of *islet1*, *islet2* and *lim3* cDNAs. The LIM domains and homeodomains are boxed and the metal coordinating residues defining the LIM domains (Dawid et al., 1995) are indicated by stars. The GenBank accession number for the *islet2* nucleotide sequence is X88805; for *lim3*, U34590. The *islet1* sequence has been previously published (Inoue et al., 1994).

**Fig. 2.** LIM homeobox gene expression in identified primary motoneurons. Primary motoneurons were injected with fluorescent dyes, fixed at 22-24 hours, and hybridized with LIM homeobox RNA probes. The fluorescent signal was enhanced using an alkaline phosphatase-catalyzed colorimetric reaction (Materials and Methods). A, C, E, G, I, K show Nomarski optics images of doubly labeled neurons. B, D, F, H, J, L are fluorescent images of the same motoneurons showing axonal trajectories. Triangles indicate the ventral boundary of the spinal cord. Scale bar equals 25  $\mu\text{m}$ . (A,B) Injected CaP showing hybridization with *islet2* probe. (C,D) Injected CaP hybridized with *lim3* probe. At this stage, *lim3* probe labels more cells than the primary motoneurons in the ventral spinal cord. (E,F) Injected MiP hybridized with *islet1* probe. (G,H) Injected MiP hybridized with *lim3* probe. (I,J) Injected RoP showing hybridization with *islet1* probe. A second cell, dorsal to RoP, was injected but does not show *islet1* labeling. Also, RoP is bordered by *islet1*-labeled cells. At this stage, *islet1* begins to label cells in addition to the primary motoneurons (see Fig. 5). (K,L) Injected RoP hybridized with *lim3* probe. (M) Schematic representation of primary motoneuron positions within the spinal cord, their axonal projections into the adjacent somite and the combination of LIM homeobox genes each expresses at axogenesis. Yellow represents *lim3* expression, blue represents *islet1*, and red represents *islet2*. M indicates the MiP axon, R the RoP axon and C the CaP axon. The primary motoneurons were drawn by tracing and superimposing images of cells from different 24 hour embryos from a video display.



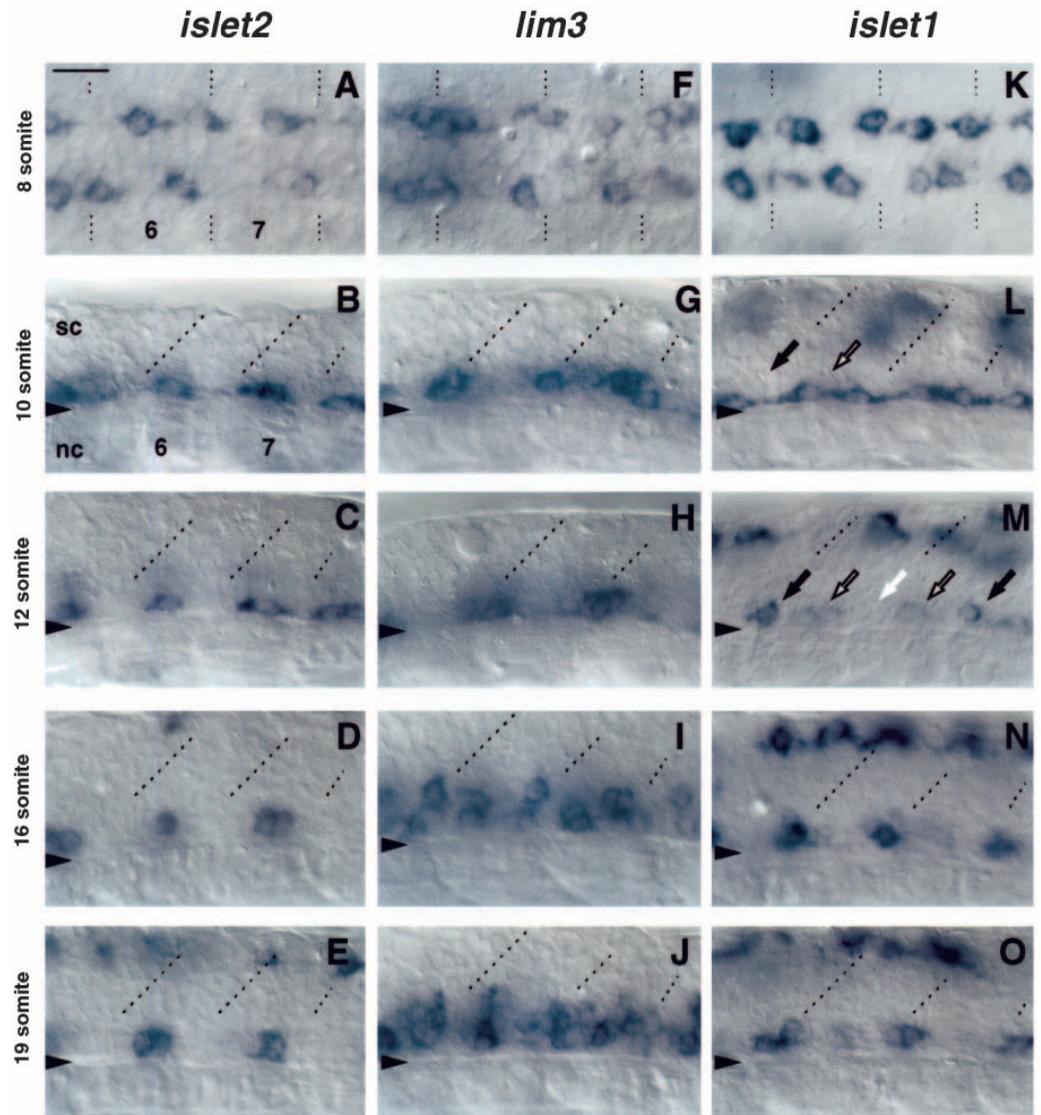
(Fig. 4C,D,E). From our cell labeling analysis described above, we conclude that these cells are CaP and VaP. Additionally, *islet2* expression appears in dorsally located cells at the 16-somite and the 19-somite stages (Fig. 4D,E). The positions of these cells are consistent with the distribution of Rohon-Beard (RB) sensory neurons (Bernhardt et al., 1990).

*lim3* is expressed in a continuously expanding number of cells. At the 8-somite stage, *lim3* RNA is detected in one to three cells near somite borders (border cells) (Fig. 4F). From the 10-somite through 12-somite stages, *lim3* is typically expressed in three border cells (Fig. 4G,H). In 16-somite-stage embryos, *lim3* expression is detected in several border cells as well as midsegment cells (Fig. 4I). By the 19-somite stage, *lim3*-expressing cells are broadly distributed in the ventral spinal cord (Fig. 4J).



**Fig. 3.** Early pattern of *islet1* and *lim3* expression in the neural keel. Dorsal views of neural-keel-stage embryos. At this stage, nascent primary motoneurons form bilateral, discontinuous columns separated by the floorplate (fp). Note that expression patterns are not bilaterally symmetric. (A) Dorsal view of 3-somite-stage embryo probed for *islet1* RNA. (B) Dorsal view of 5-somite-stage embryo probed for *lim3* RNA. Dotted lines mark approximate somite boundaries. Numbers indicate somite level. Scale bar equals 20  $\mu\text{m}$ .

**Fig. 4.** Developmental profile of LIM homeobox gene expression in the trunk ventral spinal cord. Expression of *islet2*, *lim3* and *islet1* RNA at similar axial positions at different times of development. All photos were taken at somite levels 6 and 7. Anterior is to the left for each panel. Dotted lines mark approximate positions of somite boundaries. Triangles indicate the ventral boundary of the spinal cord. A, F and K are dorsal views of the neural keel at the 8-somite stage. Remaining panels are lateral views with dorsal at the top. Scale bar equals 20  $\mu\text{m}$ . sc, spinal cord; nc, notochord. (A) *islet2* RNA is expressed in 1-2 irregularly spaced cells per hemisegment at the 8-somite stage. (B) *islet2* RNA in 1-2 midsegment cells at the 10-somite stage. (C) *islet2* expression in 1-2 midsegment cells at the 12-somite stage. (D) *islet2* RNA in 1-2 midsegment cells at the 16-somite stage. (E) *islet2* expression maintained in 1-2 midsegment cell pattern at the 19-somite stage. Dorsal labeling, here and in D (one cell), indicates expression in cells that are probably Rohon-Beard sensory neurons, based on their locations. (F) *lim3* is expressed in a variable number of cells clustered near somite boundaries in 8-somite-stage embryos. (G) Clusters of cells expressing *lim3* RNA near somite borders at the 10-somite stage. (H) Approximately three cells near somite borders express *lim3* at the 12-somite stage. (I) *lim3* RNA expressed in border and midsegment cells at the 16-somite stage. (J) Expression of *lim3* in numerous ventral spinal cord cells at the 19-somite stage. (K) *islet1* RNA is expressed in 2-3 variably spaced cells per hemisegment in the anterior neural keel of an 8-somite-stage embryo. (L) *islet1* RNA detected in one cell near somite boundaries (black arrow) and one midsegment cell (outlined arrow) at the 10-somite stage. (M) Down regulation of *islet1* RNA at the 12-somite stage. Single cells expressing high levels of *islet1* RNA are seen at the anterior borders of the 6th and 8th somites (black arrows) but not of the 7th (white arrow). Low levels of RNA are seen in midsegment cells (outlined arrows). Labeling in the dorsal spinal cord represents expression in putative Rohon-Beard sensory neurons. (N) High levels of *islet1* RNA in single cells at each somite border at the 16-somite stage. (O) *islet1* RNA in two cells at each somite border at the 19-somite stage.



We have shown that *lim3* is expressed in all four primary motoneurons. Thus, two of the three *lim3*-expressing border cells are likely MiP and RoP. The third cell is probably an interneuron known as VeLD (Bernhardt et al., 1990) (B. A. and J. S. E., unpublished results). The *lim3*-expressing midsegment cells likely include CaP and VaP. The additional *lim3*-expressing cells are probably secondary motoneurons, which develop after primary motoneurons (Myers, 1985; Myers et al., 1986).

The pattern of *islet1* RNA in the ventral spinal cord undergoes dramatic changes. In 8-somite-stage embryos, *islet1* is expressed in two or three unevenly spaced cells per hemisegment (Fig. 4K). By the 10-somite stage, the distribution of *islet1*-expressing cells is more regular. *islet1* RNA is usually found in one border cell

and one or two midsegment cells (Fig. 4L). A transition of the *islet1* pattern can be seen in 12-somite-stage embryos (Fig. 4M). First, expression in midsegment cells is low or undetectable. Second, expression in border cells at somite levels 7 or 8 is also low or undetectable. In contrast, border cells at both anterior and posterior axial levels exhibit high levels of *islet1*. Because the *islet1* pattern develops in an anterior-to-posterior sequence (see below), this means high levels of *islet1* expression are lost and then regained in single border cells within approximately one hour. We are, however, unable to determine if reinitiation of *islet1* expression occurs in the same border cell as the original expression, or in a nearby cell. At the 16-somite stage, single border cells located directly below somite furrows express *islet1*

(Fig. 4N) and in 19-somite-stage embryos, *islet1* is expressed in a second border cell adjacent to the first (Fig. 4O). The two border cells that stably express *islet1* are MiP and RoP. The position of the first of these two cells to express *islet1* RNA relative to the somite furrow indicates that it is MiP. Double *islet1* and *islet2* RNA labeling experiments show that the transient expression of *islet1* in midsegment cells occurs in CaP and VaP (see below). Thus, *islet1* RNA is expressed in each of the primary motoneurons during some part of the cell's history. Like *islet2*, *islet1* RNA is also detected in dorsally located cells that are likely RB neurons.

### The *islet1* expression pattern reveals an anteroposterior sequence of spinal cord development

In vertebrates, morphogenesis of the trunk occurs in an anterior-to-posterior sequence, as can be seen most easily in the formation of midbody somites. The dynamic nature of *islet1* expression allowed us to determine that the development of molecular pattern in the spinal cord occurs in a similar fashion. Fig. 5 shows *islet1* expression at different anteroposterior positions in a 19-somite-stage embryo; the anteroposterior pattern reflects the patterns observed at a single axial level during development (compare to Fig. 4K-O). *islet1*-expressing cells are closely spaced in the spinal cord coincident with unsegmented mesoderm and newly formed somites (Fig. 5A). At the 15- to 16-somite level, *islet1* RNA can be seen in one border cell and at low levels in one or two midsegment cells (Fig. 5B). At somite levels 13-14, expression at the somite 14 border cell declines, presumably transiently, and midsegment cell expression remains at low levels (Fig. 5C). At somite levels 10-11, single cells at the somite boundaries express *islet1* RNA and midsegment cell expression is absent (Fig. 5D). Two border cells express *islet1* at somite level 8-9 (Fig. 5E) and, at somite levels 5-6, a cluster of about four cells express *islet1* near the somite boundaries (Fig. 5F). As development proceeds, additional cells in the ventral spinal cord express *islet1* (data not shown). In addition to MiP and RoP, this growing cluster of *islet1*-expressing cells likely includes a subset of secondary motoneurons.

Although the progression of the *islet1* pattern is played out along the anteroposterior axis of the trunk, the initial pattern in the posterior trunk and tail differs from the initial *islet1* pattern in the most anterior spinal segments. From the onset of expression in the anterior regions of the spinal cord, *islet1* RNA appears in cells separated by cells in which *islet1* is not detected. In the posterior trunk, *islet1*-positive cells are more closely spaced, often being contiguous (compare Fig. 3A and Fig. 5A).

### *islet1* RNA is transiently expressed in CaP and VaP motoneurons

The position of the *islet1*-positive cells midway between somite borders in young segments is indicative of CaP and VaP motoneurons. As *islet2* expression is a stable marker of CaP and VaP fates, we asked whether CaP and VaP transiently express *islet1* RNA by simultaneously probing 21-somite-stage embryos for both gene products. At the level of somite 14, *islet1* and *islet2* probes label different cells (Fig. 6A,B). In contrast, in more posterior regions, somite 17 for example, *islet1* and *islet2* RNAs are coexpressed in midsegment cells

(Fig. 6C,D). Thus, the initial expression of *islet1* includes CaP and VaP. Downregulation of *islet1* RNA in CaP and VaP, as indicated by decreased staining intensity, occurs within 1-2 hours after *islet2* is first detected in cells at a comparable anteroposterior level.

### Positional determination of gene expression and primary motoneuronal fate

The intricate patterns of gene expression that we have described indicate that transcription of *islet1*, *islet2* and *lim3* is highly regulated. We predict that these patterns arise as a result of positional information that determines in which cell each gene is transcribed, but it is unclear how such information might be distributed within the spinal cord and when it is utilized by a cell to determine a program of LIM homeobox gene expression. To explore these issues, we transplanted individual primary motoneurons to new positions at different developmental stages and asked whether position influenced LIM homeobox gene expression. We chose to transplant cells from the MiP position of dye-labeled donor embryos to various positions of unlabeled hosts and probe for *islet2* since it is expressed in CaP and VaP, and not in MiP, through at least 30 hours (B. A. and J. S. E., unpublished results). Thus, *islet2* expression in the transplanted cell would indicate that its fate had been altered. When cells were transplanted from the MiP position of a donor to the MiP position of a host, they developed a MiP morphology and did not express *islet2* (Table 1). When cells were transplanted from the MiP position to the CaP position within approximately 1 hour before the initiation of axogenesis, they maintained a MiP fate and, also, did not express *islet2* (Fig. 7A; Table 1). In contrast, cells transplanted from the MiP position to the CaP position 2-3.5 hours before MiP axogenesis adopted the CaP fate. All of these cells expressed *islet2* (Fig. 7B; Table 1). These experiments demonstrate that the transcriptional state of *islet2* is highly sensitive to cell position within the spinal cord and does not become

**Table 1. *islet2* expression is correlated with commitment to primary motoneuron fate**

Original position	Transplant position	Stage of transplant*	Fate†	No. of <i>islet2</i> <sup>+</sup>	No. of <i>islet2</i> <sup>-</sup>
MiP	MiP	14-17 som	MiP	0	2
MiP	CaP	12.5-15 som	CaP or VaP‡	8	0
MiP	CaP	17 som	CaP§	1	0
MiP	CaP	17.5 som	MiP	0	5

\*Developmental stage determined by number of somites (som) formed. 0.5 som designations indicate initiation, but not completion, of the most posterior somite furrow.

†Fate was determined by identifying the axonal projection of the fluorescently labeled, transplanted cell prior to fixation.

‡In all cases, the native CaP was removed from the recipient segment of the host. However, previous work (Eisen et al., 1990) showed that, occasionally, there is a cryptic primary motoneuron in this position. Thus, in some cases, a native CaP was still present and the transplanted cell developed as VaP (see Eisen, 1992). Of the transplanted cells, four developed as CaPs and four developed as VaPs.

§This cell was transplanted from somite level 14 of a 17-somite embryo. Previous work that determined MiP is uncommitted until at least 2 hours prior to axogenesis was based on transplants made between somite levels 6-10 (Eisen, 1991). Transplantation of this motoneuron from the posterior spinal cord of a 17-somite-stage embryo likely occurred at least 2 hours before axogenesis.

fixed until shortly before axogenesis. Furthermore, the exact correlation of *islet2* expression with CaP, but not MiP identity, shows that *islet2* expression is tightly coupled to cell fate.

## DISCUSSION

### LIM homeobox genes and motoneuronal identity

Specification of motoneuronal identity should be reflected in patterns of gene expression that uniquely demarcate motoneurons prior to their overt differentiation. We have shown that this is the case for zebrafish primary motoneurons, each of which express a particular temporal sequence of LIM homeobox genes. Initiation of LIM homeobox gene expression precedes overt differentiation of primary motoneurons, marked by axogenesis, by several hours. The patterns of gene expression, although initially dynamic, are resolved before axogenesis so that distinct primary motoneurons express specific combinations of LIM homeobox genes. Thus, the expression patterns of LIM homeobox genes show that primary motoneurons are specified before the initiation of axonal outgrowth.

LIM homeobox genes may have a role in motoneuronal fate specification that is widely conserved among vertebrates. Tsuchida et al. (1994) isolated a number of chick LIM homeobox genes and found correlations between different combinations of gene products and the columnar organization of motoneurons. Some, but not all, features of LIM homeobox gene expression are shared between chick motoneurons and zebrafish primary motoneurons. First, zebrafish primary motoneurons express three of four LIM homeobox gene homologs expressed in chick motoneurons; only *Lim-1* is expressed in chick motoneurons and not zebrafish primary motoneurons. In chick, *Lim-1* expression is restricted to motoneurons of the lateral subdivision of the lateral motor column (LMC<sub>1</sub>), which innervate limb muscle. Zebrafish primary motoneurons do not innervate limb muscle, suggesting that *Lim-1* may have a distinct role in specifying limb-specific motoneurons. Second, all zebrafish primary motoneurons express *lim3*. In chick, *Lim-3* expression is limited to motoneurons of the medial subdivision of the median motor column (MMC<sub>m</sub>). MMC<sub>m</sub> motoneurons and zebrafish primary motoneurons both innervate axial muscle, suggesting that *lim3* may be particularly involved in specifying motoneurons that innervate this muscle type. Third, all chick motoneurons express *Islet-1* and *Islet-2* at some stage of their differentiation. This is not the case for all zebrafish primary motoneurons as *islet2* is never expressed in MiP and RoP. Finally, the order in which these genes are expressed differs somewhat between zebrafish primary motoneurons and chick motoneurons. In all chick motoneurons, *Islet-1* RNA is detected before RNA of other LIM homeobox genes. The same pattern is observed in zebrafish CaP, VaP and MiP. However, our results raise the possibility that *lim3* expression precedes that of *islet1* in RoP. Thus, although primary motoneurons appear to employ the same set of LIM homeobox genes as chick motoneurons, there are significant differences in combinatorial expression and timing, perhaps identifying fundamental differences between specification of zebrafish primary motoneurons and chick motoneurons. It will be necessary to characterize LIM homeobox gene expression in secondary motoneurons of zebrafish, which innervate axial and pectoral fin muscle, as well

as in motoneurons of other vertebrate species to identify essential features of a putative combinatorial code of LIM homeodomain proteins involved in motoneuronal fate specification

The set of transcription factors encoded by the *islet1*, *islet2* and *lim3* genes cannot account for the full range of motoneuronal identities evident within chick spinal cord nor among primary motoneurons in zebrafish. For example, distinct motor pools that lie at characteristic positions within the chick MMC<sub>m</sub> and innervate either dorsal or ventral axial muscles (Gutman et al., 1993) are not identified by differential expression of LIM homeobox genes (Tsuchida et al., 1994). Similarly, zebrafish RoP cannot be differentiated from MiP, nor CaP from VaP on the basis of LIM homeobox gene expression at the time of axogenesis. What, then, might account for individual fate differences? Ablation and transplantation experiments showed that CaP and VaP have equivalent developmental potential, which is maintained nearly until the time of VaP death (Eisen, 1992). Differentiation of CaP and VaP fates, therefore, may not result from the patterned, differential expression of transcription factors controlling sets of downstream genes. Instead, we suspect that CaP and VaP fate differentiation results from the initiation of a cell death pathway resulting from an interaction between CaP and VaP. RoP maintains its identity after MiP ablation (Pike and Eisen, 1990), as does MiP after RoP ablation (J. S. E., unpublished results) showing, in contrast to CaP and VaP, that MiP and RoP do not constitute an equivalence pair. MiP and RoP could be differentiated by the timing of *islet1* expression relative to morphological differentiation. Approximately 3 hours separates the time at which we can first identify *islet1* expression in a single border cell and expression in two cells in that region, therefore, it is possible that early *islet1* expression determines one cell fate, and late expression, another. Alternatively, an unidentified, differentially expressed gene or set of genes, acting in combination with *lim3* and *islet1*, may specify the individual MiP and RoP fates. Such a function may be provided by additional genes of the LIM homeobox class or by transcription factors of another class. The differential expression of a LIM domain binding partner that affects the activity of a LIM homeodomain protein could, also, determine a distinction between MiP and RoP. For example, DNA-binding specificity is enhanced by a cooperative interaction of the LIM homeodomain protein MEC-3 and the POU homeodomain protein UNC-86 as a heterodimer (Xue et al., 1993). LIM domains act as negative regulatory elements of *Xlim-1* function in experiments assaying induction of neural and muscle tissue in *X. laevis* suggesting a means for the modification of LIM homeodomain protein function (Taira et al., 1994).

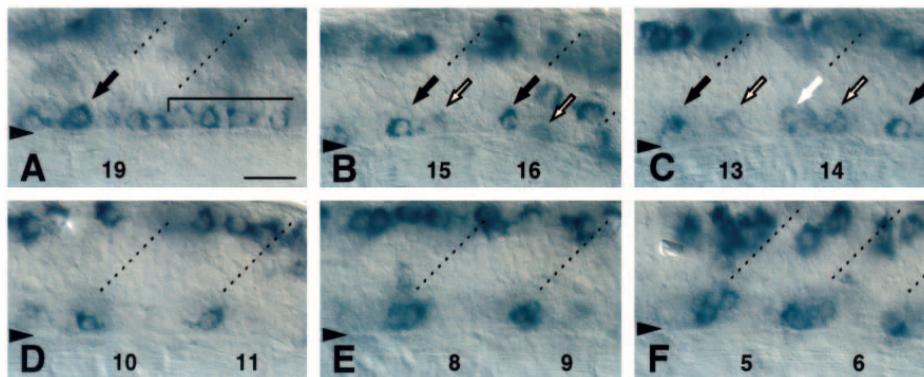
### Patterned LIM homeobox gene expression and commitment of cell fate

Single cell transplantation experiments have shown that zebrafish primary motoneurons become committed to particular fates after their terminal division, but before morphological differentiation (Eisen, 1991). How does commitment correlate with expression of LIM homeobox genes? Expression of each of the LIM homeobox genes described here is initiated in CaP and VaP several hours before commitment. The event that correlates best with the time of commitment to the CaP fate is the loss of *islet1* RNA. In trunk segments, CaP becomes

**Fig. 5.** Anteroposterior progression of the *islet1* RNA expression pattern in the ventral spinal cord. Panels show *islet1* RNA distribution in the ventral spinal cord of a 19-somite-stage embryo at different anteroposterior positions. Anterior is to the left and dorsal is up in all panels.

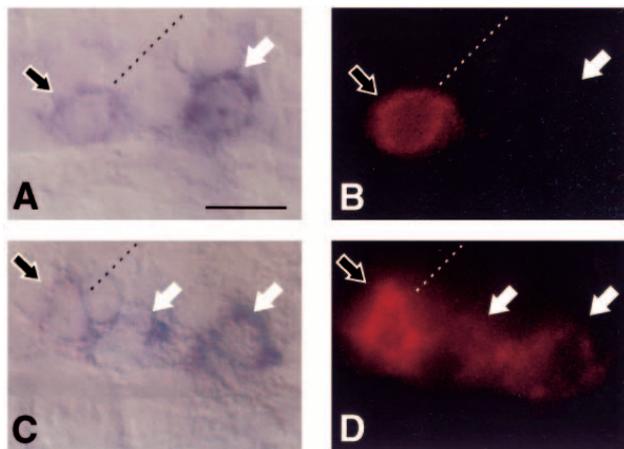
Approximate somite boundaries are indicated by dashed lines. Numbers at the bottom of each panel indicate somite level. Triangles mark boundary between spinal cord and notochord. Scale bar equals 20  $\mu\text{m}$ .

(A) Somite level 19 and unsegmented region. High level expression (arrow) at somite 19 boundary. *islet1* expression in a nearly contiguous line of cells in unsegmented region (bracket). (B) Somite level 15-16. High level expression in single cells (black arrows) near somite boundaries. Low level expression in midsegment cells (outlined arrows). Dorsal labeling in putative Rohon-Beard sensory neurons. Labeling in segment 16 just dorsal of primary motoneurons represents transient expression in unidentified cells. (C) Somite level 13-14. High level of *islet1* expression in single cells at 13 and 15 somite boundaries (black arrows). Low level expression (white arrow) near somite 14 boundary as well as in midsegment cells (outlined arrows). (D) Somite level 10-11. *islet1* is expressed in 1 cell at each somite boundary. (E) Somite level 8-9. *islet1* is expressed in 2 cells near each somite boundary. (F) Somite level 5-6. *islet1* is expressed in approximately 4 cells clustered near the somite boundaries.



committed to its fate by 16 hours (Eisen, 1991). Detectable amounts of *islet1* RNA are nearly absent in CaP and VaP of trunk segments by 15 hours. Thus, *islet1* expression could prevent a cell from adopting the CaP fate. MiP becomes committed by about 17-18 hours, which follows the onset of *islet1* and *lim3* expression in trunk segments. The expression of particular combinations of *islet1*, *islet2* and *lim3* in primary motoneurons before commitment and before axonal development suggests these genes may contribute to primary motoneuron specification.

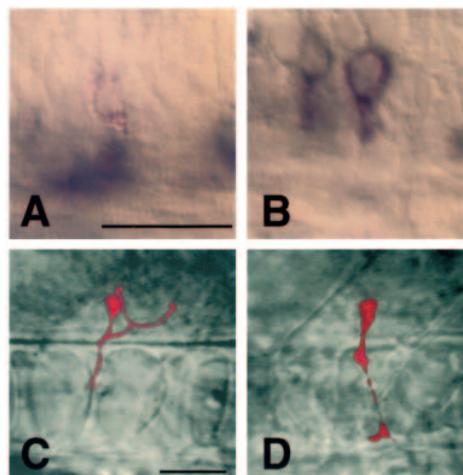
By transplanting primary motoneurons and probing for



**Fig. 6.** Simultaneous detection of *islet1* and *islet2* RNA in primary motoneurons. (A and C) Double labels for *islet1* RNA (magenta) and *islet2* RNA (blue). (B and D) Corresponding fluorescent images showing low levels of *islet1* RNA. Photos are from a 21-somite-stage embryo. Anterior is left and dorsal is up. Dashed lines represent approximate positions of somite borders. Scale bar equals 10  $\mu\text{m}$ .

(A,B) At somite level 14, only *islet1* is expressed in a cell near the somite border (outlined arrow) and *islet2*, alone, is expressed in a midsegment cell (white arrow). (C,D) At somite level 17, *islet1*, and not *islet2*, RNA is detected in a cell near the somite border (outlined arrow) while both *islet1* and *islet2* RNAs are detected in two midsegment cells (white arrows).

gene expression, we have demonstrated a tight correlation between commitment to a particular motoneuronal fate and LIM homeobox gene expression. Cells fated to become MiP, when transplanted to the CaP position at least 2 hours prior to axogenesis, developed as CaP or VaP and initiated *islet2* expression. When the same type of transplant was done within 1 hour of axogenesis, the transplanted cell maintained



**Fig. 7.** *islet2* gene expression in transplanted primary motoneurons determined by in situ RNA hybridization. (A,B) Cells transplanted from the MiP position to the CaP position at different developmental stages and fixed and probed for *islet2* expression after the transplanted cells developed axons. Transplanted cells are marked by red staining and *islet2* localization by blue. Scale bar in A equals 25  $\mu\text{m}$  for A,B. Scale bar in C equals 25  $\mu\text{m}$  for C,D. (A) This cell was transplanted about 1 hour before axogenesis. It maintained the MiP fate and did not express *islet2*. Hybridization signal in the ventral spinal cord represents late *islet2* expression, probably in secondary motoneurons. (B) This cell was transplanted 2-3 hours before axogenesis, adopted a CaP fate, and expressed *islet2*. (C) Image of the cell in A prior to fixation showing its MiP axonal projection. (D) Image of the cell in B prior to fixation showing its CaP axonal projection.

a MiP fate and did not express *islet2*. These results support a possible role for *islet2* in the specification of CaP and VaP fates.

Perhaps most importantly, the transplantation experiments provide insight to the patterning mechanisms that must exist within the spinal cord. Previous studies have shown that notochord and floorplate can induce the expression of motoneuronal markers in spinal cord (Yamada et al., 1991, 1993). In zebrafish, the onset of *islet1* expression occurs in cells close to the differentiating notochord and floorplate. Thus, the time and place of *islet1* expression in zebrafish is consistent with induction by a signal originating in the notochord and/or floorplate. How an axial inductive signal is converted to the patterned distribution of different cell types is considerably less clear. We have shown that transplantation of a cell to a position only a few cell diameters away can change both gene expression and cell fate. Thus, a finely grained system of positional information, to which transcriptional control of LIM homeobox genes is responsive, must exist within the spinal cord. It will be necessary to determine how patterned gene expression is established and maintained to understand how diverse cell types are generated in the vertebrate central nervous system.

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