

The control of rostrocaudal pattern in the developing spinal cord: specification of motor neuron subtype identity is initiated by signals from paraxial mesoderm

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SUMMARY

The generation of distinct classes of motor neurons is an early step in the control of vertebrate motor behavior. To study the interactions that control the generation of motor neuron subclasses in the developing avian spinal cord we performed *in vivo* grafting studies in which either the neural tube or flanking mesoderm were **displaced between thoracic and brachial levels**. The positional identity of neural tube cells and motor neuron subtype identity was assessed by Hox and LIM homeodomain protein expression. Our results show that the rostrocaudal identity of neural cells is plastic at the time of neural tube closure and is sensitive to positionally restricted signals from the

paraxial mesoderm. Such paraxial mesodermal signals appear to control the rostrocaudal identity of neural tube cells and the columnar subtype identity of motor neurons. These results suggest that the generation of motor neuron subtypes in the developing spinal cord involves the integration of distinct rostrocaudal and dorsoventral patterning signals that derive, respectively, from paraxial and axial mesodermal cell groups.

Key words: Motor neurons, Neural tube, Mesoderm, Positional identity, Cell signalling, Chick, Quail

INTRODUCTION

Vertebrate motor behavior is coordinated by the activity of motor neurons (MNs) located in the spinal cord and brain stem. The refinement of motor function depends, in part, on the existence of subclasses of MNs which participate in distinct circuits in the central nervous system and innervate different target cells in the periphery. Some insight into the molecular events involved in establishing the generic identity of MNs has been obtained (Tanabe and Jessell, 1996). In particular, the differentiation of MNs has been shown to depend on a Sonic Hedgehog-mediated inductive signal transmitted from axial mesodermal cells of the notochord to overlying neural plate cells (Roelink et al., 1995; Tanabe et al., 1995; Marti et al., 1995; Chiang et al., 1996; Ericson et al., 1996). How developing MNs acquire their distinct subtype identities remains poorly understood.

The diversification of MN subtypes first becomes apparent through differences in the settling pattern of MNs in the spinal cord and by the distinct pathways selected by motor axons in the periphery (Landmesser, 1980). One major subdivision of

spinal MNs is based upon their segregation into discrete columns. In the chick embryo, **four major columnar subtypes** of somatic MNs are evident (Landmesser, 1978; 1980; Hollyday, 1980; Gutman et al., 1993). MNs in the medial subdivision of the **median motor column (MMC) project axons to axial muscles**; MNs in the lateral MMC project axons to **body wall muscles**, and MNs in the medial and lateral subdivisions of the lateral motor column (LMC) project axons **respectively to ventrally and dorsally derived limb muscles**. In addition, a class of visceral MNs located in the **Column of Terni (CT) innervates sympathetic neurons** (Levi-Montalcini, 1950; Prasad and Hollyday, 1991). With the exception of the medial MMC, each MN columnar subtype is generated in a discontinuous manner along the rostrocaudal axis of the spinal cord (Fig. 1A). Somatic MNs within individual columns can be further subdivided into pools on the basis of their specific muscle targets (Landmesser, 1978; Hollyday, 1980).

The analysis of motor axon projection patterns after manipulation of the chick embryo has suggested that the columnar and pool identities of somatic MNs are acquired prior to target innervation and enable motor axons to respond

appropriately to guidance cues encountered in the periphery (Tosney, 1991; Landmesser, 1992). Molecular evidence for the early acquisition of MN subtype identity has been provided by the demonstration that the expression of LIM homeodomain (HD) proteins defines each of the columnar subclasses of chick MNs, prior to target innervation (Tsuchida et al., 1994). Moreover, genetic analyses have shown that LIM-HD proteins control neuronal differentiation and axonal pathfinding in both vertebrates and invertebrates (Lundgren et al., 1995; Pfaff, et al., 1996; Thor and Thomas, 1997; Hobert et al., 1997) suggesting that they have a role in specifying the columnar subtype identity of spinal MNs.

The source and nature of inductive signals that control the generation of spinal MN subtypes have not been defined. Early rostrocaudal inversion of chick lumbosacral neural tube results in a respecification of the pool identity of MNs, as assessed by the emergent pattern of muscle innervation (Matisse and Lance-Jones, 1996). Furthermore, the intrasegmental displacement of primary MNs along the rostrocaudal axis of zebrafish embryos changes the initial pattern of motor axon projections (Eisen, 1991) and LIM homeobox gene expression (Appel et al., 1995). Thus, the differentiation of certain MN subtypes appears to be dependent on the rostrocaudal position of neural tube cells. Since notochord inductive activity and Shh signaling appear to be constant along the rostrocaudal axis (Yamada et al., 1991; Fukushima et al., 1996; Ericson et al., 1997), axial mesodermal signals are unlikely to control the formation of distinct MN columnar subtypes at different segmental levels of the spinal cord.

In the embryonic brain there is evidence that signals provided by the paraxial mesoderm can impose rostrocaudal identity on neural cells. An activity expressed transiently by paraxial mesoderm can confer an early caudal character on cells derived from prosencephalic levels of the chick neural plate (Muhr et al., 1997) and similar activities have been detected in zebrafish (Woo and Fraser, 1997) and *Xenopus* (Bang et al., 1997). A later-acting signal from the somitic mesoderm can posteriorize the pattern of *Hox* gene expression within the developing chick hindbrain (Itasaki et al., 1996; Grapin-Botton et al., 1997). At spinal cord levels, signals from the somites have been shown to restrict the rostrocaudal spread of clonally related cells (Stern et al., 1991). Moreover, different rostrocaudal domains of the embryonic spinal cord, as in the hindbrain, can be defined by the overlapping expression of *Hox* genes (Kessel and Gruss, 1991; Krumlauf, 1994). Signals from the paraxial mesoderm may, therefore, also control the rostrocaudal identity of cells at more caudal levels of the neural tube and initiate the generation of distinct MN subtypes at different segmental levels of the spinal cord.

We have examined this possibility through an analysis of the expression of *Hox* and LIM-HD proteins that define the rostrocaudal identity of neural tube cells and the columnar subtype of spinal MNs. Neural tube and mesodermal grafting studies in vivo show that the rostrocaudal identity of neural cells remains plastic at the time of neural tube closure. Positionally restricted signals from the paraxial mesoderm appear to have a critical role in establishing the rostrocaudal identity of cells in the caudal neural tube and later in determining MN subtype identity. Our results suggest that the generation of MN subtypes in the spinal cord involves the integration of rostrocaudal and dorsoventral patterning signals that derive from distinct paraxial and axial mesodermal cells groups.

MATERIALS AND METHODS

This study is based on >200 quail to chick embryonic grafts, and an analysis of the 74 embryos which survived and contained quail tissue in appropriate locations.

Neural tube grafts

To determine the location of prospective brachial (B) and thoracic (T) levels of the neural tube for use in grafting experiments we constructed a fate map of the quail neural tube at Hamburger-Hamilton (HH) (1951) stages 10-13. DiI was injected focally at rostrocaudal levels of the neural tube estimated to correspond to B and T levels. Embryos were incubated until HH stage 28-29, the position of labeled cells determined by fluorescence microscopy and mapped onto the segmental level of that defined by vertebral segmental identity. Based on this analysis, we found that the B level of the neural tube in 10-11 somite stage (s) embryos was located 1-2 somites more rostral than the position predicted by extrapolation of the rostrocaudal length occupied by segmented somites. In 14s embryos, this fate map showed that the T neural tube was located adjacent to the caudal half of the unsegmented mesoderm.

In grafting experiments, T level neural tissue at the 22-25 somite level was isolated from quail donors at the 13-18s and grafted to the 16-21 somite level of 11-15s chick hosts. B level neural tissue was isolated from 11-13s stage quail embryos and grafted to the T level of 11-15s chick hosts. Neural tube tissue for grafting was separated from surrounding mesodermal tissue by digestion with Dispase at 20°C for 7 minutes. Enzymatic treatment was stopped by incubation of neural tissue in L-15 medium containing 2% heat-inactivated normal goat serum (HINGs) at 4°C and tissue was kept for less than 40 minutes prior to transplantation into chick hosts. Grafting and DiI injection methods were generally as outlined by Stern and Holland (1993).

Mesoderm grafts

Prospective B and T regions of quail and chick mesoderm were located on the basis of the fate maps of Chaube (1959) and Hornbruch and Wolpert (1991). Quail embryos were placed in L-15 medium and mesodermal tissue isolated, incubated in Dispase at 20°C for approx. 7 minutes and transferred to L-15 medium containing 4% HINGs before grafting. Grafting methods were essentially as described by Stern and Holland (1993).

Immunohistochemistry

Embryos were fixed in 4% paraformaldehyde and serial 10-15 µm cryostat sections through donor quail and host chick tissue were processed for immunohistochemistry. Rabbit (Rb) (K5) and mAb (4D5) antibodies were used to detect Isl1 and Isl2. Rb antibodies (A7, A8) and mAb 1D5 were used to detect chick Isl1 (Tsuchida et al., 1994; Ericson et al., 1996). Rb antiserum (T4) or mAb 4F2 (Tsuchida et al., 1994) were used to detect Lim1/Lim2. A mAb was used to detect Hoxc8 (Belting et al., 1998). Fluorophore- and peroxidase-conjugated antibodies (Jackson Inc.) were used at 1:100 to 1:500. Quail tissue was detected with mAb QCPN (Developmental Studies Hybridoma Bank). Confocal images were collected on Bio-Rad MRC 600 or Leica TCS4D confocal microscopes.

RESULTS

Columnar identity of motor neurons defined by LIM homeodomain protein expression

To distinguish the columnar subtypes of MNs generated at different rostrocaudal positions in the spinal cord we analyzed the combinatorial expression of LIM-HD proteins (Tsuchida et al., 1994), focusing on MN subtypes generated at brachial (B)

and thoracic (T) levels of the spinal cord (Fig. 1A). At B levels, MNs are organized into three columns: neurons of the medial MMC (MMC_M) which co-express *Isl1*, *Isl2* and *Lim3*; neurons of the medial LMC (LMC_M) which coexpress *Isl1* and *Isl2*; and neurons of the lateral LMC (LMC_L) which co-express *Isl2* and *Lim1* (Fig. 1B-E; Tsuchida et al., 1994). At T levels, MNs are also organized into three columns: MMC_M neurons; lateral MMC neurons which coexpress *Isl1* and *Isl2* but not *Lim3* and dorsomedially positioned CT neurons which express *Isl1* alone (Fig. 1F-I; Tsuchida et al., 1994). This profile of LIM-HD protein expression is identical in chick and quail embryos, becomes apparent by HH stages 25-29, prior to the onset of MN cell death, and accompanies the segregation of MNs into discrete columns (Tsuchida et al., 1994, data not shown).

Changes in motor neuron columnar identity after rostrocaudal displacement of the neural tube

Since neural cells remain sensitive to dorsoventral patterning signals at the time of neural tube closure (Tanabe and Jessell, 1996) we examined whether the columnar identity of MNs can be respecified at a similar developmental stage. Segments of quail B or T neural tube were grafted into chick embryos, exchanging prospective T and B levels of the neuraxis. Grafts were placed in a rostrocaudally inverted orientation to control for a possible influence of the normal rostral-to-caudal gradient of spinal cord maturation on changes in neural pattern. Embryos were permitted to develop until HH stages 28-29 and spinal cord histology, the settling pattern of MNs and the profile of LIM-HD protein expression were examined in both donor and host spinal cord. Graft tissue was identified by expression of a quail-specific antigen, QCPN.

T to B grafts

Grafts of 13-15s quail T neural tube were placed rostrally at the B level of 12-15s chick hosts (Fig. 2A; for summary of grafts in this and subsequent experiments see Table 1). Spinal cord segments that developed from grafted T neural tube exhibited a change in histology and in the settling pattern of MNs, compared to normal T

segments. The ventral horn at T levels is normally compact (Fig. 1G) but in grafted tissue was greatly enlarged and resembled the ventral horn observed normally at B levels (Fig. 1B,C). Dorsomedial *Isl1*⁺/*Isl2*⁻ neurons characteristic of the CT were not detected (Fig. 2C). Ventrally, MNs were organized into an ovoid cluster (Fig. 2C) characteristic of the LMC at B levels (Fig. 1C) rather than the crescent-shaped MMC MN cluster normally present at T levels (Fig. 1G). Consistent with this, MNs in a ventrolateral position coexpressed *Isl2* and *Lim1* but not *Isl1*, a LIM-HD code characteristic of the LMC_L (Fig. 2D-F). These findings suggest that grafted neural cells have been diverted from their normal T fates and that their neuronal progeny acquire the molecular properties of B MNs.

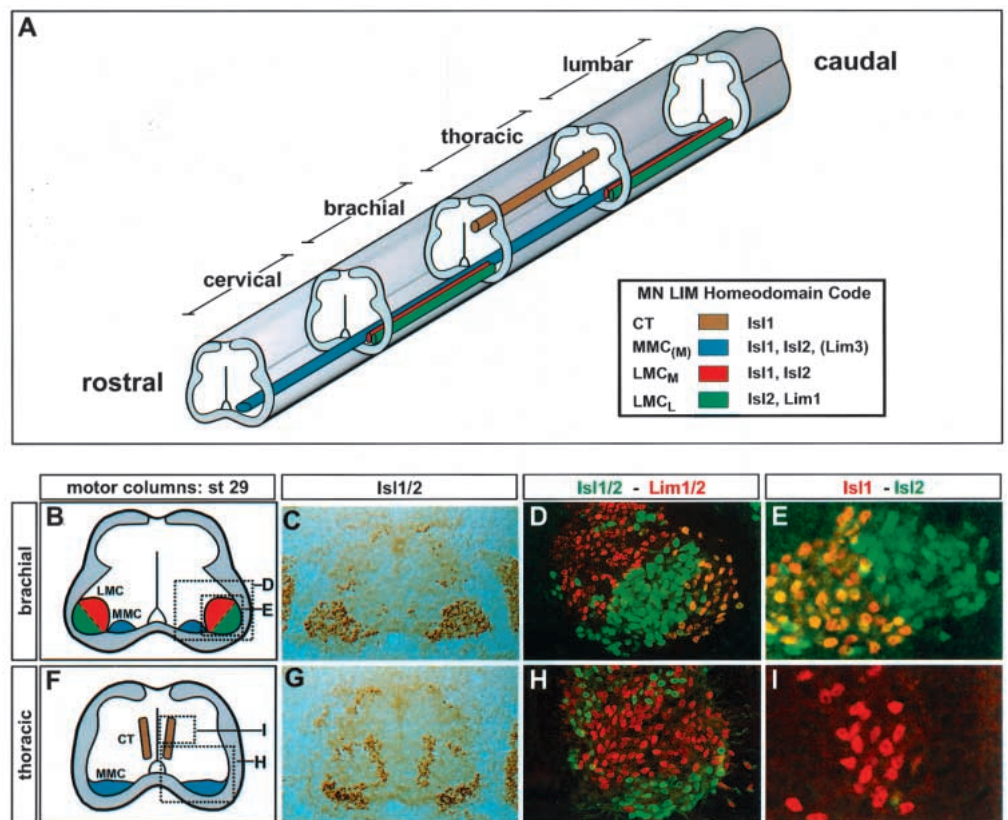


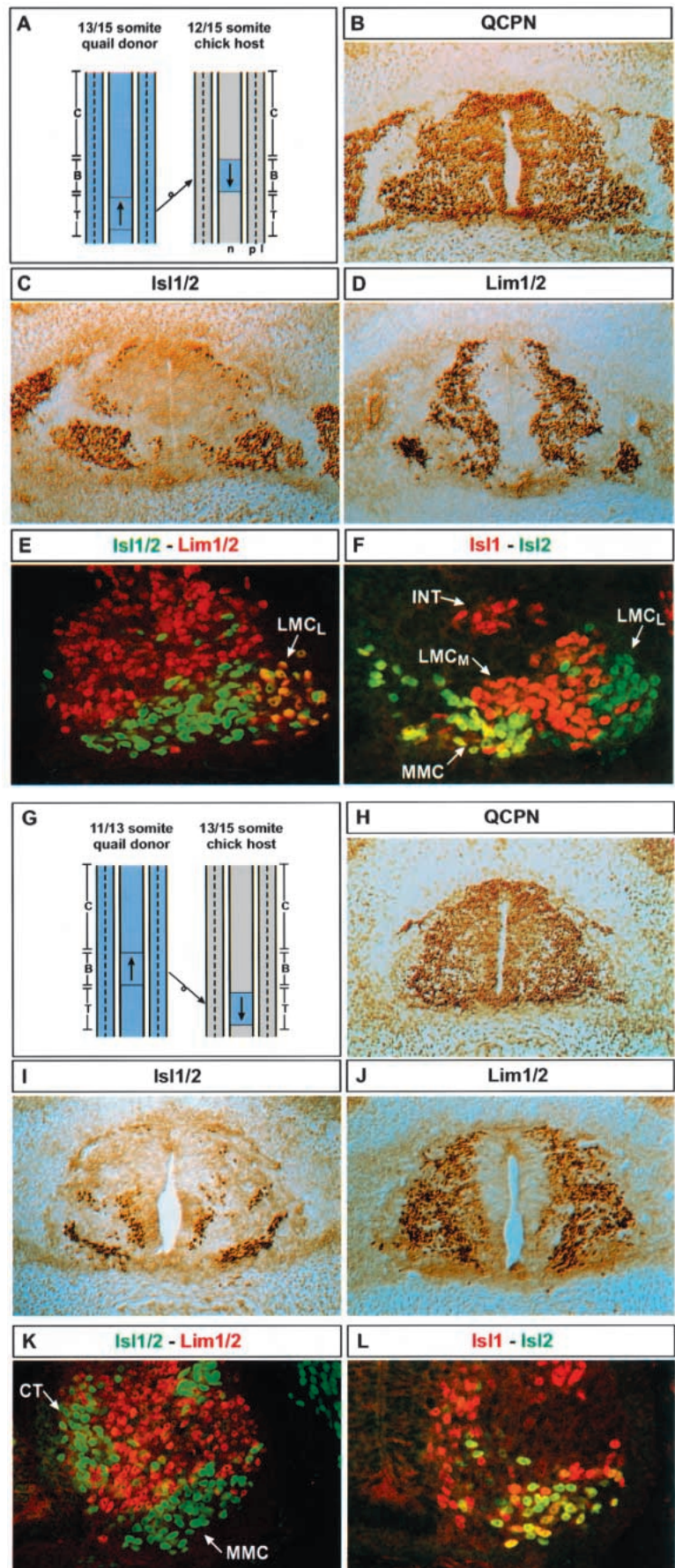
Fig. 1. Rostrocaudal organization and LIM homeodomain protein expression in motor columns. (A) Schematic representation of the organization of motor columns along the rostrocaudal axis of HH stage 28-29 quail (or chick) embryo spinal cord. Color code represents motor columns and LIM-HD protein expression. For simplicity, the lateral MMC is not represented at T levels. MMC_M , medial subdivision of the median motor column; LMC_M , medial subdivision of the lateral motor column; LMC_L , lateral subdivision of the lateral motor column; CT, Column of Terni. (B,F) Diagrams showing the LIM-HD protein code and motor column organization in transverse sections at B (B) and T (F) levels of stage 29 chick and quail embryos. Diagrams use the code and abbreviations in (A). Boxes in B indicate regions shown in D and E. Boxes in F indicate regions shown in H and I. (C,G) *Isl1* and/or *Isl2* are expressed by all quail MNs at both B (C) and T (G) levels. The characteristic enlargement of the LMC at B levels contrasts with the crescent-shaped MMC at T levels. The spinal cord at T levels also contains dorsomedial CT MNs. Some dorsal interneurons express *Isl1* but not *Isl2*. (D,H) Confocal images of the right ventral quadrant of the B and T spinal cord. B levels (D) can be distinguished from T levels (H) by the enlarged B motor column and by coexpression of *Isl2* and *Lim1* by laterally positioned LMC_L cells (yellow cells in D). *Lim1/2* cells (red) located dorsomedial to MNs are ventral interneurons. (E,I) Confocal images of the B level LMC (E) and T level CT (I). At B levels (E), LMC_L neurons express *Isl2* but not *Isl1*. At T levels (I), CT neurons express *Isl1* but not *Isl2*. Lateral is to the right in panels D,E,H and I. For details see Tsuchida et al. (1994).

B to T grafts

Grafts of 11-13s quail B neural tube were placed caudally into the T level of 13-15s chick hosts (Fig. 2G). In a host T environment, the grafted B neural tube developed into spinal cord tissue that exhibited histological features of T spinal cord (Fig. 2H). The grafted tissue contained dorsomedial $Isl1^+/Isl2^-$ MNs characteristic of the CT (Fig. 2I,L). Moreover, ventral $Isl1^+/Isl2^+$ MNs were organized into a crescent-shaped cluster characteristic of the MMC (Fig. 2I,L) and no $Isl2^+/Lim1^+$ $LMCL$ -like MNs were detected within the graft (Fig. 2J-L). Thus, cells in such grafts appear to have been diverted from their normal B fates and give rise to MN subtypes characteristic of the T spinal cord.

Segments of 12-15s quail T or B level neural tube were also grafted into chick hosts at their appropriate axial level but still in an inverted rostrocaudal orientation. These grafts developed into spinal cord segments with the MN columnar settling pattern characteristic of their position of origin (Table 1A; data

Fig. 2. Transplantation of neural tube between thoracic and brachial levels changes the columnar identity of MNs. (A) Diagram of thoracic to brachial neural tube grafts. Blue shading indicates quail tissue and gray shading chick tissue. Arrows indicate rostrocaudal orientation of grafted neural tube. C, cervical; B, brachial; T, thoracic; n, neural tube; p, paraxial mesoderm; l, lateral plate mesoderm. The same convention is used in subsequent figures. (B-F) Transverse sections of grafted quail T spinal cord at B level of HH stage 29 chick hosts. (B) QCPN expression defines quail tissue. The spinal cord and neural crest-derived dorsal root ganglion neurons and Schwann cells which ensheath the ventral root are of quail origin. (C) Ventral $Isl1^+/Isl2^+$ MNs are detected in an ovoid-LMC-like cluster and no dorsomedial $Isl1^+$ CT-like MNs are detected. (D) A ventrolateral group of $LMCL$ -like MNs expresses $Lim1$. There are also many $Lim1^+/Lim2^+$ interneurons in medial and dorsal positions. (E,F) Confocal images of the lower right quadrant of the grafted quail spinal cord. E shows cells that coexpress $Isl2$ and $Lim1$ in a lateral position appropriate for the $LMCL$. F shows that lateral $LMCL$ MNs express $Isl2$ but not $Isl1$. Coexpression of $Isl2$ and $Isl1$ is detected in more medially located $LMCM$ and MMC_M MNs, albeit at different levels in each motor column. (G) Diagram of brachial to thoracic neural tube grafts. (H-L) Transverse sections of spinal cord at T levels of stage 29 embryos. (H) QCPN expression defines quail cells in the spinal cord and in dorsal and ventral roots. The adjacent mesoderm is of chick origin. (I) Ventral $Isl1/Isl2$ expression reveals a crescent-shaped cluster of MMC -like MNs and a separate dorsally positioned CT-like MN column. In these B to T grafts the most complete transformation in MN identity was usually observed at caudal levels of the graft. At more rostral (formerly caudal) levels, both LMC and CT-like MNs were often detected (not shown). (J) $Lim1/Lim2$ expression is restricted to medial and dorsal interneurons and no ventrolateral $LMCL$ -like $Lim1^+$ neurons are detected. (K,L) Confocal images of the lower right quadrant of the grafted quail spinal cord. K shows that no ventral MNs coexpress $Isl1/2$ and $Lim1$. Dorsomedial $Isl1^+$ CT-like MNs are present. L shows that most MNs coexpress $Isl1$ and $Isl2$. Dorsomedially positioned CT-like MNs express $Isl1$ but not $Isl2$.



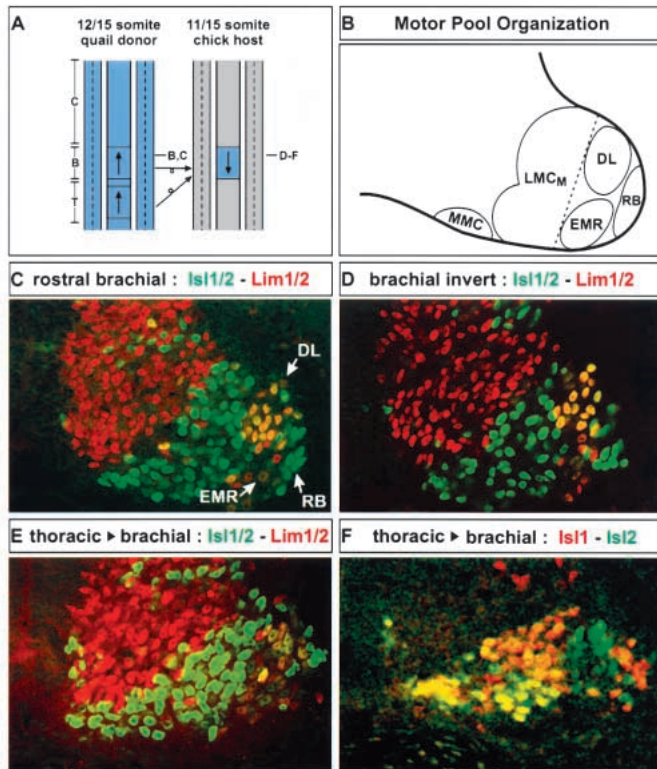


Fig. 3. Rostrocaudal transplantation of the neural tube changes motor pool identity within the brachial spinal cord. (A) Diagram of grafts used to assess changes in motor pool identity. (B) Simplified diagram of motor pool organization at rostral B levels of quail (and chick) spinal cord. Modified from Hollyday and Jacobson (1990). MNs marked DL may also include the Scapulohumeralis pool and MNs marked EMR may also include the Triceps pool. (C) Motor pool-dependent variation in expression of LIM-HD proteins at rostral B levels. Dorsomedial DL-like LMC_L neurons co-express higher levels of Isl2 and Lim1 than ventromedial EMR-like LMC_L neurons. Lateral RB MNs in the LMC_L do not express Lim1 but co-express Isl1 and Isl2 (not shown). (D) Rostral (formerly caudal) level section through quail B level neural tube grafted in an inverted rostrocaudal orientation into chick B level. The pattern of LIM-HD protein expression in the LMC_L resembles that found normally at rostral B levels and is markedly different from that normally detected at caudal B levels (see Fig. 1 D). (E, F) Sections through stage 29 spinal cord after grafts of 13-15s quail T neural tube after grafting to 12-15s B levels of chick hosts. At rostral B levels the pattern and level of LIM-HD protein expression by MNs in the LMC_L resembles that normally detected at rostral B levels. (F) After quail T to chick B grafts the lateral-most MNs in the LMC_L coexpress Isl1 and Isl2 but not Lim1, typical of RB MNs, whereas more medial LMC_L neurons express Isl2 but not Isl1. LMC_M and MMC_M MNs also coexpress Isl1 and Isl2.

not shown), although as discussed below, under this condition the pool identity of MNs in the B LMC is altered.

A critical period of neural tube plasticity

We next determined the period over which cells at T and B levels of the neural tube remain competent to respond to signals from the host environment with a change in MN columnar subtype. To assess this, quail T neural tube was isolated from later stage donors and grafted into the B level of chick hosts (Table 1A). Spinal cord tissue derived from 15-16s T grafts

contained only a few Isl2⁺/Lim1⁺ LMC_L-like MNs and dorsal Isl1⁺/Isl2⁻ CT-like MNs were still detected (Table 1 A; data not shown). Spinal cord tissue derived from 18s quail T neural tube contained no Isl2⁺/Lim1⁺ LMC_L-like MNs and all MNs were characteristic of the T spinal cord, (Table 1A, data not shown). Conversely, 14-16s quail B neural tube grafted into the T level of 14-15s chick hosts contained both dorsal CT-like MNs and LMC_L-like MNs (Table 1A, data not shown). 17s quail B neural tube grafts to T levels generated LMC but no CT MNs (Table 1A). These results suggest that by these stages cells from B and T levels of the neural tube have been exposed to signals that specify their rostrocaudal identity and thus are no longer able to respond to host signals with a change in the columnar identity of MNs.

Changes in motor pool identity within the LMC after rostrocaudal displacement of the neural tube

Early rostrocaudal inversion of lumbosacral level neural tube has been shown to change the pool identity of MNs, as assessed by the pattern of muscle innervation (Matise and Lance-Jones, 1996). We examined whether the rostrocaudal inversion of B neural tube results in a change in the molecular properties of MNs within individual motor pools. To address this issue we made use of an observation that certain motor pools within the rostral two segments of the B LMC can be distinguished at HH stage 29 by the combination and/or level of LIM-HD protein expression. At rostral B levels, a dorsomedial group of MNs within the LMC_L expresses high levels of Isl2 and Lim1 (Fig. 3C) and appears to correspond to the Deltoid (DL) motor pool (Fig. 3B; Hollyday and Jacobson, 1990). A ventromedial group of LMC_L MNs expresses low levels of Isl2 and Lim1 and appears to correspond to the Extensor Metacarpi Radialis/Ulnaris (EMR) motor pools (Fig. 3B,C; Hollyday and Jacobson, 1990). A third MMC_M-like group of rhomboideus (RB) MNs is embedded in the lateral region of the LMC_L (Hollyday and Jacobson, 1990; Straznicki and Tay, 1983) and expresses Isl1, Isl2 and Lim3 but not Lim1 (Fig. 3B,C; Tsuchida et al., 1994). This pool-dependent variation in LIM-HD protein expression was not observed within the two caudal segments of the B spinal cord where all LMC_L MNs co-expressed high levels of Isl2 and Lim1 (Fig. 1D; data not shown).

We first analyzed the pattern of LIM-HD protein expression in segments of a 12s quail B neural tube grafted in a rostrocaudally inverted orientation into the B level of a 10s host chick embryo (Fig. 3A). The profile of LIM-HD expression by MNs within the LMC_L was altered in the rostral two segments of the graft, formerly the caudal level of the donor B neural tube. A dorsomedial high level Isl2⁺/Lim1⁺ (DL-like) pool, a ventromedial low level Isl2⁺/Lim1⁺ (EMR-like) pool and a lateral Isl1⁺/Isl2⁺/Lim1⁻ (RB-like) pool were detected (Fig. 3D). The LIM-HD protein profile of these MN pools resembles closely that normally generated in the LMC_L in rostral B spinal cord (Fig. 3C). In the caudal (formerly rostral) two segments of the inverted B spinal cord, all LMC_L MNs co-expressed high levels of Isl2 and Lim1 (data not shown), similar to the normal caudal B spinal cord.

We next examined whether an equivalent change in the pool-specific pattern of LIM-HD protein expression was elicited in the LMC that develops in a 15s quail T neural tube segment grafted into the rostral B level of a 14s host chick embryo (Fig.

Table 1. Summary of neural tube and mesodermal grafting studies

A	Neural tube grafts	Rostro caudal axis shift	Quail donor age	Chick host age	Motor column identity		Incidence of columnar transformation
					LMC	CT	
	Thoracic	T↔T	13–15s	14–15s	–	+	0 (2)
		T→B	13–15s	12–15s	+	–	5 (5)
		T→B	15–16s	14s	+/-	+	2 (3)
		T→B	18s	14–15s	–	+	0 (3)
	Brachial	B↔B	12s	11s	+	–	0 (2)
		B→T	11–13s	13–15s	–	+	6 (6)
		B→T	14–16s	14–15s	+/-	+	5 (5)
		B→T	17s	14–15s	+	–	0 (2)
B	Mesoderm grafts	Rostro caudal axis shift	Quail donor age	Chick host age	Motor column identity		Incidence of columnar transformation
					LMC	CT	
	Non-axial	B→T	11–12s	12–15s	+	–	9 (15)
	Non-axial	T→B	17–20s	9–2s	–	–	3* (4)
	Paraxial	B→T	13–15s	12–16s	+	–	3 (8)
	Paraxial	B→T	18–20s	12–16s	–	+	0 (3)
	Paraxial	T→B	15–18s	10–11s	–	–	2* (3)
	Paraxial	C→B	5s	10–11s	–	–	3* (5)
	Lateral plate	B→T	11–15s	14–16s	–	+	0 (8)

Age of donor and host embryos is given as somite number (s). 'Incidence of columnar transformation' is the number of embryos in which a transformation in anticipated MN columnar identity was detected and numbers in brackets indicate the total number of embryos examined under each condition. In many cases of mesodermal grafts, the failure to detect a change in MN identity was found to correlate with the presence of only a small number of donor mesodermal cells. Embryos in which no donor mesodermal cells remained adjacent to the spinal cord are not included in this analysis. In 'Motor column identity' the identification of the LMC is based on LIM homeodomain expression and in particular on the coexpression of Isl2 and Lim1 by MNs. This LIM homeodomain code permits the unambiguous identification of LMC_L neurons. The presence of LMC_L MNs is an indication of brachial (B) character. The presence of CT MNs was determined both by the LIM homeodomain code, the expression of Isl1 in the absence of other LIM homeodomain proteins, and by the dorsomedial position of these MNs. The presence of CT neurons is an indication of thoracic (T) character. Asterisks indicate that some embryos (1 for T→B non-axial; 2 for T→B paraxial; 3 for cervical→B paraxial) were analyzed at stage 25/26.

3A). In the graft, dorsal LMC_L neurons expressed higher levels of Isl2/Lim1 than ventromedial LMC_L neurons and laterally located MNs coexpressed Isl1 and Isl2 but not Lim1 (Fig. 3E,F). Thus a similar, albeit less ordered, change in the organization of MN pools was detected. These results provide evidence that the emergence of a pool-specific pattern of LIM-HD protein expression by MNs within the LMC is dependent on the rostrocaudal position of neural tube cells.

Altered Hoxc8 expression after rostrocaudal displacement of the neural tube

The alteration in histological features of the spinal cord after rostrocaudal displacement of the neural tube suggested that the positional identity of cells other than MNs might be changed. To assess this, we analyzed the pattern of expression of Hoxc8, a Hox protein with a domain of expression known to encompass B and T levels of the spinal cord (Le Mouellie al., 1988; Awgulewitsch and Jacobs, 1990; Belting et al., 1998).

We first analyzed in detail the normal pattern of expression of Hoxc8 in chick and quail embryos at HH stage 28/29. Hoxc8 is expressed over distinct rostrocaudal domains of the spinal cord and paraxial mesoderm. Neural expression of Hoxc8 extends throughout both T and B levels (Fig. 4A), but the pattern varied markedly at different levels. In rostral B

segments (Fig. 4A), expression is largely restricted to cells in the dorsal spinal cord whereas at more caudal B levels, expression is detected in ventral interneurons and at the level of the most caudal B segments, also in ventral progenitor cells and MNs (Fig. 4A). In the paraxial mesoderm, the rostral boundary of Hoxc8 expression is located at the junction of T and B levels and expression extends caudally throughout the T level, decaying at rostral lumbar levels (Fig. 4A; Burke et al. 1995; Belting et al., 1998). The pattern of Hoxc8 expression, therefore, provides a sensitive indicator of the rostrocaudal positional identity of cells at B and T levels of the spinal cord and in addition, distinguishes T and B paraxial mesoderm.

We therefore examined whether the pattern of neural Hoxc8 expression is changed after rostrocaudal displacement of T and B segments of the neural tube. In 15s quail T neural tube grafted rostrally into the B level of a 14s chick embryo (Fig. 4B), Hoxc8 expression was restricted to dorsal cells at rostral levels of the graft (Fig. 4C). However, expression expanded ventrally at more caudal levels (Fig. 4D,E), mimicking closely the normal B pattern (Fig. 4A). In 11-12s quail B neural tube grafted in a rostrocaudally inverted orientation into the B level of an 11s chick host (Fig. 4F), Hoxc8 expression was dorsally restricted at rostral, formerly caudal, levels of the graft, (Fig. 4G). Again expression expanded ventrally at more caudal levels (Fig. 4H), generating a pattern similar to that normally

detected at B levels. Finally, in 11-13s rostral quail B neural tube grafted caudally into the T level of 13-15s chick hosts (Fig. 4F), a uniform pattern of *Hoxc8* expression was detected (Fig. 4I), similar to that found normally at T levels (Fig. 4A).

This analysis of *Hoxc8* expression patterns provides evidence that the change in cell identity after neural tube displacement is not restricted to MNs, occurs in a graded manner along the rostrocaudal axis of the spinal cord and is associated with both a rostral and a caudal respecification in cell fate. In contrast, neural tube grafts between B and T levels did not change the pattern of *Hoxc8* expression in the flanking paraxial mesoderm (Fig. 4C-E,G-I).

Alteration in the columnar identity of motor neurons by signals from non-axial mesoderm

The change in identity of MNs detected after displacement of the neural tube led us to examine the source of the patterning signals. Studies of the neuraxis, at hindbrain levels, have raised the possibility that signals transmitted along the neural tube contribute to the early establishment of rhombomere identity (Grapin-Botton et al., 1997). We therefore examined whether there is any change in MN settling pattern or in the profile of LIM-HD protein expression in host chick spinal cord tissue adjacent to the grafted quail neural tube. We did not detect any change in the cellular or molecular features of MN differentiation in adjacent host chick T or B spinal cord (data not shown). We conclude, therefore, that signals transmitted rostrocaudally within neural tube are unlikely to underlie the marked change in positional identity and pattern of cells detected within grafted neural tissue.

We next examined whether signals derived from non-axial mesoderm cell groups might control the subtype identity of MNs. The neural tube at prospective spinal cord levels is flanked by paraxial, intermediate and lateral plate mesoderm (collectively termed non-axial mesoderm) and signals transmitted between these tissues appear to control many aspects of mesodermal differentiation (Stephens et al., 1991; Pourquie et al., 1996; Michaud, et al., 1997). In view of this, our initial analysis of mesodermal influences on rostrocaudal neural tube patterning attempted to preserve any ongoing intra-mesodermal signaling by transplanting all non-axial mesodermal tissues coordinately.

B to T non-axial mesoderm grafts

11-12s quail B non-axial mesoderm was grafted ipsilaterally to the T level of 12-15s chick hosts (Fig. 5A) and embryos were analyzed at stage HH 28-29. QCPN expression showed that quail cells contributed to mesodermal tissues only on the side of the graft (Fig. 5B). These embryos developed an additional limb bud at the T level (data not shown), indicating that the signaling activities of non-axial mesoderm required for limb generation (Stephens et al., 1991; Cohn et al., 1995; Crossley et al., 1996; Fernandez-Teran et al., 1997) are preserved. Moreover, there was no expression of *Hoxc8* in donor quail mesodermal cells grafted to T levels, whereas *Hoxc8* was expressed by contralateral host paraxial mesoderm (Fig. 5F). Thus, the positional identity of the grafted mesodermal tissue appears unchanged by its rostrocaudal displacement.

A marked change in MN settling pattern and LIM-HD protein expression was detected within the host spinal cord on the side ipsilateral to the B mesoderm graft. Ipsilateral T spinal

cord did not contain dorsally positioned *Isl1*⁺/*Isl2*⁻ CT-like MNs (Fig. 5C,D). Ventrally, MNs settled in a pattern characteristic of the LMC (Fig. 5C-E) and lateral LMC MNs co-expressed *Isl2* and *Lim1* (Fig. 5D,E,H, data not shown). In contrast, the MN settling pattern and profile of LIM-HD protein expression on the contralateral side remained characteristic of T levels (Fig. 5C-E,G). The widespread neural expression of *Hoxc8* on the side of the graft (Fig. 5F) suggested that the T neural tube possessed a caudal B character and consistent with this, there was not a clear distinction in the level of *Isl2*/*Lim1* co-expression by LMC_L-like MNs (Fig. 5H; data not shown).

T to B non-axial mesoderm grafts

17-20s quail T non-axial mesoderm was grafted rostrally in place of B mesoderm (Fig. 5I). QCPN⁺ mesodermal cells remained on the side of the graft (Fig. 5J). The positional identity of the grafted mesoderm appeared unchanged, as assessed by the maintenance of *Hoxc8* expression in quail mesodermal cells (Fig. 5N) and by the generation of ribs and the absence of a limb bud ipsilaterally (data not shown). These non-axial T mesoderm grafts induced a marked reorganization of the ipsilateral side of the host spinal cord. *Isl1*⁺/*Isl2*⁺ MNs were characteristic of the MMC_M at T levels, not of B level LMC (Fig. 5K-M,O) and no MNs coexpressed *Isl2* and *Lim1* (Fig. 5K-M,P). Although there was a change in somatic MN identity, we detected few if any dorsally positioned *Isl1*⁺/*Isl2*⁻ CT-like MNs on the side of the spinal cord ipsilateral to the graft (Fig. 5K,L). In these experiments, successful mesodermal grafts were always positioned at caudal B levels and consistent with this, widespread neural expression of *Hoxc8* was detected (Fig. 5N). However, because of this the analysis of these grafts did not reveal whether changes in MN columnar identity are accompanied by a rostralization in the identity of other cell types in the spinal cord.

This series of grafts therefore provides evidence that signals from non-axial mesoderm can change the columnar subtype identity of somatic MNs and can suppress but apparently not induce the generation of visceral MNs.

Signals from paraxial mesoderm are sufficient to respecify motor neuron columnar identity

To determine more precisely the origin of non-axial mesodermal signals, quail paraxial mesoderm was separated from intermediate and lateral plate mesoderm and grafted alone in place of chick paraxial mesoderm.

B to T paraxial mesoderm grafts

13-15s quail B level paraxial mesoderm was placed unilaterally at the T level of 12-16s chick host embryos (Fig. 6A). QCPN⁺ mesodermal cells remained on the side of the graft (Fig. 6B) and retained their B identity, as assessed by the absence of *Hoxc8* expression (Fig. 6E). The host T spinal cord on the side adjacent to grafted B paraxial mesoderm assumed a B identity: it did not contain dorsally positioned *Isl1*⁺/*Isl2*⁻ CT-like MNs (Fig. 6C) and ventral MNs settled in a pattern characteristic of LMC neurons with the most lateral of these co-expressing *Isl2* and *Lim1* (Fig. 6C,D,H).

In contrast, grafts of later (18-20s) segmented B paraxial mesoderm into the T level of chick hosts did not change the pattern of MN differentiation in the adjacent T level spinal cord (Table 1B; data not shown), suggesting that the patterning activity of the paraxial mesoderm is expressed transiently.

Moreover, grafts of 11-15s quail B lateral plate mesoderm adjacent to the T neural tube of chick hosts produced no detectable change in MN settling pattern or in LIM-HD protein expression (Table 1B; data not shown). Thus, the patterning activities of the non-axial mesoderm appear to be mediated by signals from the paraxial mesoderm.

T to B paraxial mesoderm grafts

15-18s T paraxial mesoderm was grafted rostrally in place of B level paraxial mesoderm (Fig. 6I). QCPN⁺ donor cells remained on the side of the graft (Fig. 6J) and retained their positional identity as assessed by the maintenance of Hoxc8 expression (Fig. 6M; data not shown). In these experiments embryos were analyzed at HH stage 25 since we encountered problems with survival at later stages. This precluded an analysis of the generation of CT neurons which have not yet begun to migrate dorsally (Prasad and Hollyday, 1991). However, the ipsilateral side of the host level B spinal cord did not contain Isl2⁺/Lim1⁺ LMC_L-like MNs (Fig. 6K,L,O,P). Furthermore, there was an increase in the number of ventral Hoxc8⁺ cells on the side of the graft (Fig. 6M), suggesting that the B neural tube had acquired a more caudal identity.

Cervical to B paraxial mesoderm grafts

We also examined whether B neural tube could be induced to acquire a more rostral identity in response to signals from paraxial mesoderm. To test this, we grafted 5s cervical level paraxial mesoderm caudally, in place of B paraxial mesoderm (Fig. 7A). QCPN⁺ quail mesodermal cells remained on the side of the graft (Fig. 7B) and did not express Hoxc8 (Fig. 7F), as expected for cervical or B mesoderm (Fig. 4A). The ipsilateral B spinal cord contained MNs with a settling pattern characteristic of cervical MNs (Fig. 7C; Ericson et al., 1997; data not shown) and few if any MNs exhibited the B property of co-expression of Isl2 and Lim1 (Fig. 7G,H). The LIM-HD protein code does not clearly distinguish somatic MN subtypes generated at the cervical and T levels of the spinal cord (Tsuchida et al., 1994; Ericson et al.,

1997). This precluded a molecular assessment of whether the depletion of LMC_L MNs resulted from a rostral (to cervical) or a caudal (to T) shift in MN identity. However, Hoxc8 expression was almost completely extinguished on the side of the spinal cord ipsilateral to the graft (Fig. 7F). Since Hoxc8 is not expressed by cells at cervical levels of the spinal cord (Fig. 4A), this result suggests that the loss of LMC_L MNs elicited by cervical paraxial mesoderm grafts is associated with a rostral, rather than a caudal transformation in the identity of B neural tube cells. This result also provides additional evidence that the pattern of Hoxc8 expression by neural cells can be altered by signals from the paraxial mesoderm.

DISCUSSION

These studies provide evidence that the paraxial mesoderm has a critical role in imposing a rostrocaudal identity on cells at

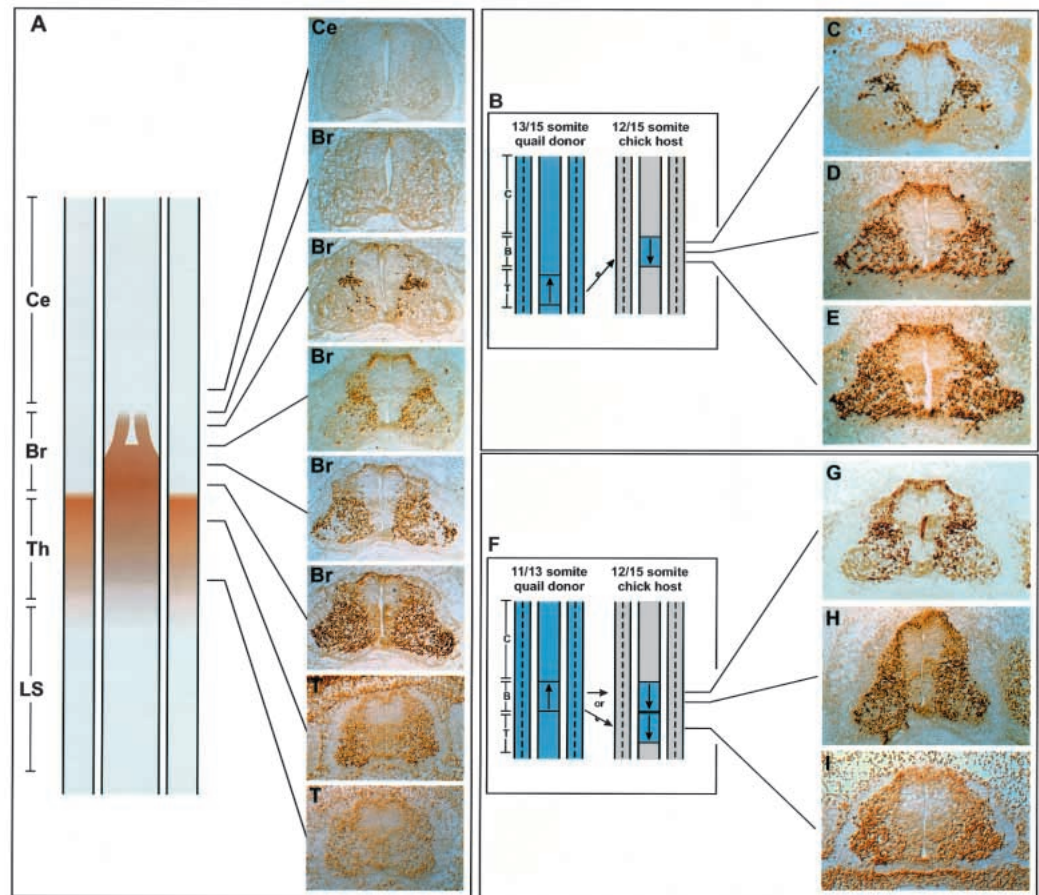


Fig. 4. Rostrocaudal neural tube displacement changes the pattern of Hoxc8 expression. (A) Pattern of Hoxc8 expression in HH stage 28-29 quail spinal cord and paraxial mesoderm. The intensity of brown shading is an approximate indication of the number of labeled cells and level of Hoxc8 expression. (B) Diagram of transplantation of quail T neural tube to chick B level. (C-E). The rostrocaudal pattern of Hoxc8 expression in grafted T neural tube resembles closely the pattern detected normally at B levels. (F) Diagram of quail B neural tube inversion at B levels and of B to T grafts. (G,H) Quail B neural tube grafted in reversed rostrocaudal orientation exhibits an inverted pattern of Hoxc8 expression, characteristic of normal B neural tube. (I) Grafts of B level neural tube to T levels generates a uniform pattern of neuronal Hoxc8 expression, typical of the normal T spinal cord. Note also the expression of Hoxc8 in ventral ventricular zone cells which is characteristic of T levels, but is absent from the majority of the B spinal cord. Ce, cervical; Br, brachial; Th, thoracic; LS, lumbosacral.

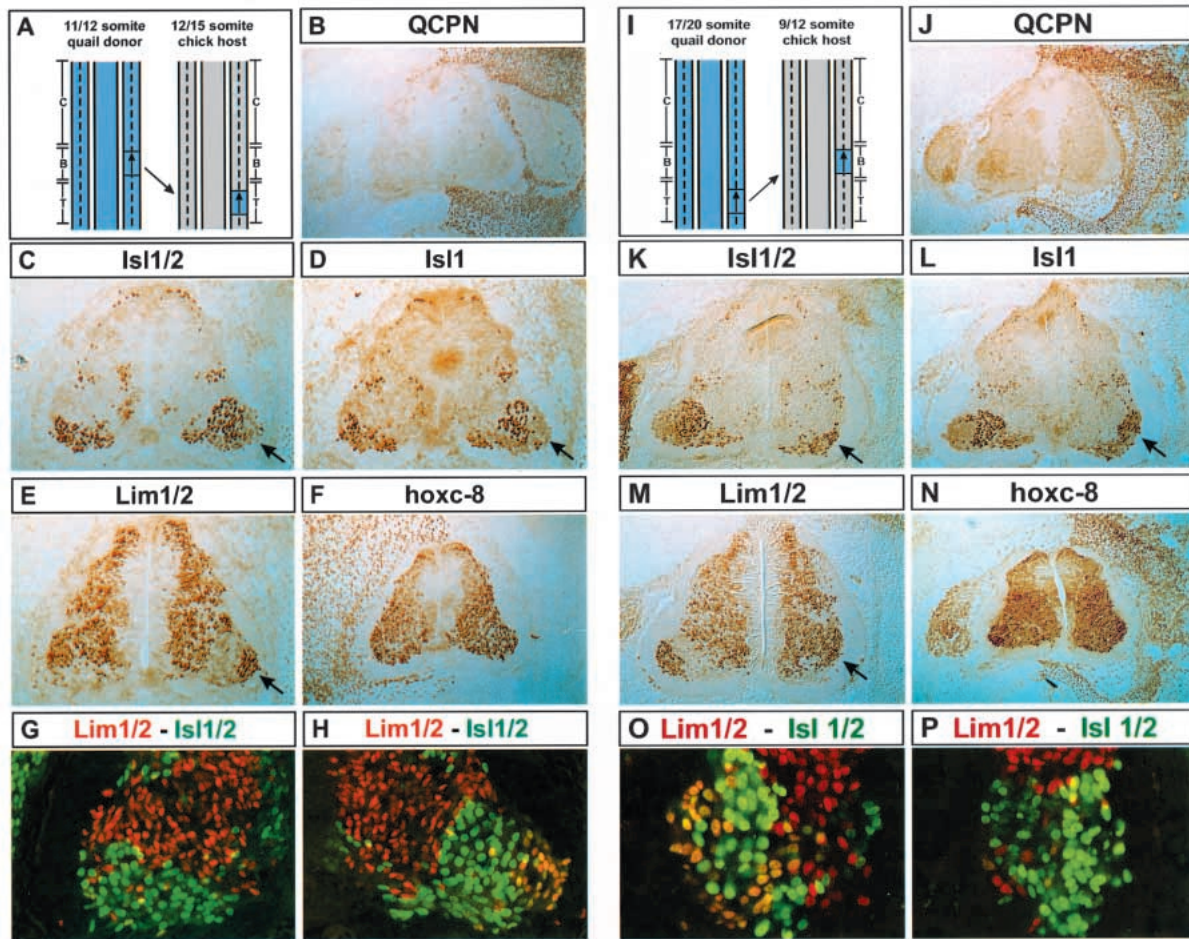


Fig. 5. Signals from non-axial mesoderm change the columnar identity of motor neurons. (A) Diagram of ipsilateral B to T non-axial mesoderm grafts. (B) Grafted QCPN⁺ quail mesoderm cells are confined to the side of the graft. (C) Isl1/Isl2 expression shows that ventral MNs on the side of the graft (arrow) settle in an ovoid LMC-like cluster but on the contralateral side settle in a crescent-shaped MMC-like cluster. No dorsomedial Isl1⁺ CT-like MNs are detected on the side of the graft. (D) Isl1 expression shows that on the side of the graft, ventrolateral LMC_L-like MNs express Isl2 but not Isl1 (arrow) whereas contralateral ventral MMC MNs coexpress Isl2 and Isl1. No dorsal Isl1 MNs are detected on the side of the graft. (E) Lim 1/Lim2 expression shows that lateral LMC_L-like MNs express Lim1 on the side of the graft (arrow). (F) Mesodermal cells on the grafted side do not express Hoxc8, consistent with their B origin whereas contralateral host mesodermal cells do express Hoxc8. The widespread pattern of neural Hoxc8 expression, including the ventral ventricular zone, suggests that cells possess a caudal B identity. (G) Confocal image of the lower left (control) quadrant of spinal cord showing a MMC-like T motor column morphology and the absence of Isl2 and Lim1 coexpression by MNs. (H) Confocal image of the lower right (graft) quadrant of spinal cord showing an enlarged ovoid LMC-like motor column and laterally positioned LMC_L-like MNs that coexpress Isl2 and Lim1. (I) Diagram of T to B non-axial mesoderm grafts. (J) QCPN⁺ quail mesodermal cells are confined to the side of the graft. (K) Isl1/Isl2 expression shows the generation of a crescent-shape cluster of ventral MMC-like MNs on the side of the graft (arrow). Few if any dorsally located CT-like MNs are detected. (L) Isl1 expression shows that all MMC-like MNs on the side of the graft express Isl1 (arrow) whereas most lateral LMC_L-like MNs on the contralateral side do not. (M) Lim1/Lim2 expression shows that there is no lateral LMC_L-like Lim1 MN cluster on the side of the graft (arrow). (N) Mesodermal expression of Hoxc8 is detected on the side of the graft but not on the contralateral side. (O) Confocal image of the lower left (control) quadrant of spinal cord showing the presence of LMC_L-like MNs that coexpress Isl2 and Lim1. (P) Confocal image of the lower right (graft) quadrant of spinal cord showing the absence of LMC_L-like MNs that coexpress Isl2 and Lim1. No dorsomedially positioned CT-like MNs cells are detected. Images in O and P are derived from a different embryo analyzed at stage 25.

caudal levels of the neural tube that give rise to the spinal cord. The acquisition of such positional identity appears to underlie the generation of distinct columnar subclasses of MNs at different rostrocaudal positions. Our results add to the emerging evidence (Itasaki et al., 1996; Grapin-Botton et al., 1997; Muhr et al., 1997) that signals from the paraxial mesoderm influence rostrocaudal pattern within the avian neural tube. These prior studies on the patterning of the hindbrain and forebrain have indicated that the paraxial mesoderm exerts a caudalizing influence on neural cell fates.

In contrast, the present studies suggest that at spinal cord levels the paraxial mesoderm is able to respecify cell fates in both a rostral and a caudal direction. We discuss these findings in the context of the progression of progenitor cell differentiation into MNs, the rostrocaudal patterning of the neural tube and the signaling properties of paraxial mesoderm.

Timing of motor neuron subtype specification

The pattern of cell differentiation observed after displacement of the neural tube or paraxial mesoderm indicates that the

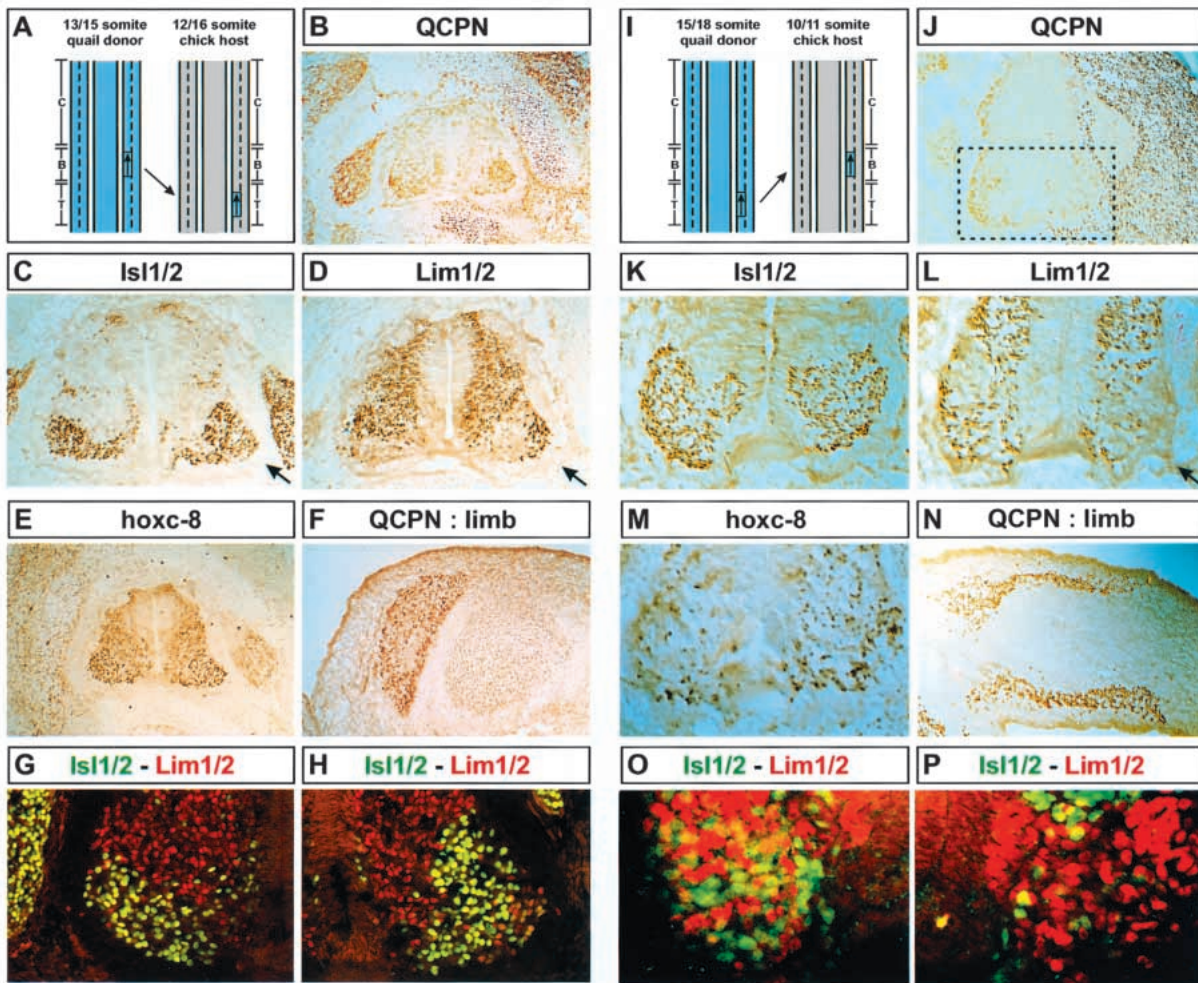


Fig. 6. Signals from paraxial mesoderm are sufficient to change the columnar identity of motor neurons. (A) Diagram of B to T paraxial mesoderm grafts. (B) QCPN expression shows that quail paraxial mesodermal cells remain on the side of the graft. Apparent labeling on control side reflects non-specific peroxidase background. In some embryos, such B grafts induced the formation of a limb-bud like structure at T levels (not shown). (C) Ventral Isl1/2 expression shows the presence of an ovoid cluster of LMCL-like MNs (arrow) and the absence of dorsomedial CT-like MNs on the side of the graft. (D) Lim1/Lim2 expression shows the presence of lateral Lim1⁺ LMCL-like MNs on the side of the graft (arrow). (E) Grafted mesodermal cells do not express Hoxc8, suggesting that they have retained B identity. The widespread neural expression of Hoxc8 suggests that cells possess a caudal B identity. (F) In some embryos, quail mesodermal cells are detected at B levels even though the majority of grafted cells were located at T levels. We used this situation to evaluate the precision of paraxial mesoderm grafts by analyzing the species of origin of different cell types within the ipsilateral limb bud. After such grafts, QCPN expression shows that lateral plate mesoderm-derived limb mesenchymal cells are of host chick origin whereas paraxial mesoderm-derived muscle cells are of quail origin, confirming the transfer of paraxial but not lateral plate mesodermal cells. (G) Confocal image of the lower left (control) quadrant of spinal cord showing that no MMC MNs coexpress Isl2 and Lim1. (H) Confocal image of the lower right (graft) quadrant of spinal cord showing that ventrolateral LMCL-like MNs coexpress Isl2 and Lim1. (I) Diagram of T to B paraxial mesoderm grafts. In this series of grafts, embryos were analyzed at stage 25 because of poor survival at later stages. (J) QCPN expression reveals that grafted mesodermal cells remain on the side of the graft. Such T grafts resulted in a marked perturbation in the development of the ipsilateral limb bud (not shown). Box indicates region of the spinal cord shown in K-M. (K) Isl1/2 expression reveals the position of ventral MNs. Laterally positioned MNs on the side of the graft do not express Isl1 (not shown). (L) Lim1/Lim2 expression shows the absence of lateral Lim1⁺ LMCL-like MNs on the side of the graft. (M) Hoxc8 expression reveals an increase in the number of Hoxc8⁺ cells in the ventral spinal cord on the side of the graft. Grafted mesodermal cells retain Hoxc8 expression but contralateral host mesodermal cells do not express Hoxc8 (not shown at this magnification). (N) QCPN⁺ muscle cells in the ipsilateral limb bud derive from the quail donor whereas limb mesenchymal cells derive from the chick host, confirming the selectivity of paraxial mesoderm grafts. (O) Confocal image of the left (control) ventral quadrant of the spinal cord showing LMCL-like MNs that coexpress Isl1/2 and Lim1 (orange cells). (P) Confocal images of the right (graft) ventral quadrant of the spinal cord, showing MMC-like MNs that express Isl1/2 but not Lim1. Apparent yellow cells result from the superimposition of separate nuclei of cells that express either Isl1/2 or Lim1 but not both proteins.

rostrocaudal identity of neural cells remains plastic at the time of neural tube closure but that there is only a brief period (approx. 12 hours) within which respecification is possible.

These observations probably explain the failure of later-stage grafts to alter the pattern of MN differentiation (Wenger, 1951; Narayanan and Hamburger, 1971; O'Brien et al., 1990). An

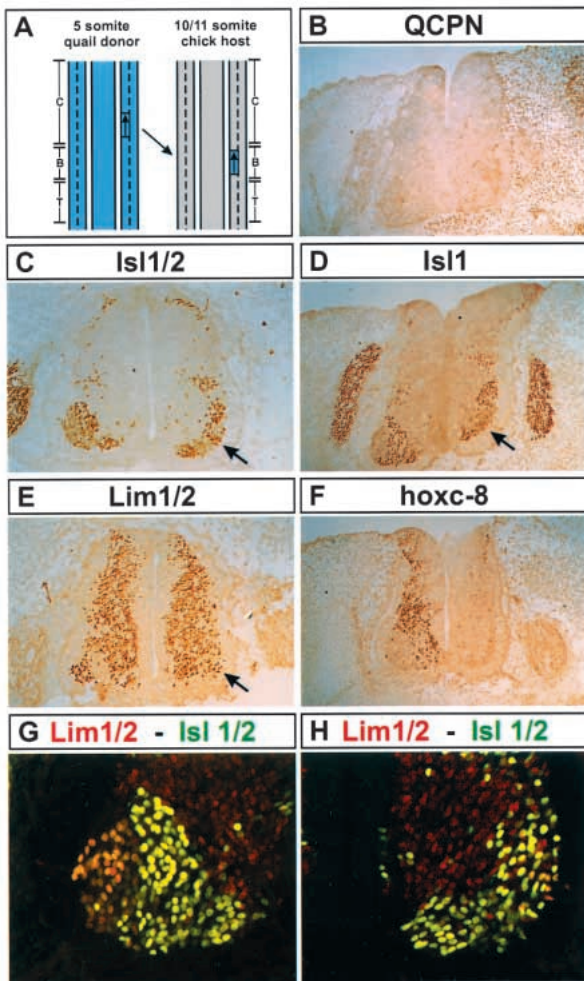


Fig. 7. Cervical paraxial mesoderm changes motor neuron columnar identity and brachial *hoxc8* expression. (A) Diagram of cervical to B paraxial mesoderm graft. Embryos were analyzed at HH stage 25/26. (B) QCPN expression shows that grafted quail mesodermal cells remain on the side of the graft. (C) Ventral *Isl1/2* expression reveals the loss of an ovoid LMC-like MN cluster on the side of the graft (arrow). (D) Ventral *Isl1* expression shows that all MNs on the side of the graft express *Isl1* (arrow), in contrast to the contralateral side. The dorsolateral MN group is characteristic of spinal accessory and phrenic MNs found at cervical levels of the spinal cord. (E) *Lim1/Lim2* expression shows the absence of a discrete lateral group of *Lim1*⁺ LMC_L-like MNs on the side of the graft (arrow). (F) *Hoxc8* is not expressed in the graft or host mesoderm. No neural expression of *Hoxc8* is detected on the side of the graft. (G) Confocal image of the lower left (control) quadrant of spinal cord showing that lateral LMC_L-like MNs coexpress *Isl2* and *Lim1*. (H) Confocal image of the lower right (graft) quadrant of spinal cord showing that few if any MNs coexpress *Isl2* and *Lim1*. *Isl1* MNs dorsal to the few *Isl2*⁺/*Lim1*⁺ neurons are likely to be spinal accessory or phrenic motor neurons.

apparent transformation of MN fate, assessed histologically, has been described after grafting cervical neural tube to T levels (Shieh, 1951). However, the age at which most of these grafts were performed is beyond the critical period defined here and more recently, similarly late grafts have not resulted in a change in cervical MN fate (Yaginuma et al., 1996).

Although the critical period for specification of the

rostrocaudal identity of neural cells appears to end soon after stage 11, post-mitotic MNs are not generated at B and T levels until stage 15, some 18–24 hours later (Hollyday and Hamburger, 1977; Prasad and Hollyday, 1991; Ericson et al., 1992). The loss of competence for transformation could reflect the time at which progenitor cells in the neural tube commit to MN columnar fates. However, the acquisition of a generic MN fate remains sensitive to local *Shh* signaling until late in the final cell cycle of MN progenitors (Ericson et al., 1996) which would require that MNs commit to their columnar subtype identity before their generic identity. An alternative possibility is that the loss of competence reflects the time at which progenitor cells within the neural tube acquire secondary inductive signaling activities that are independent of the paraxial mesoderm. In this case the columnar subtype identity of MNs may be determined only at later stages. In support of this idea, studies of MN differentiation in zebrafish have provided evidence that primary MN subtype identity is determined in post-mitotic cells after the specification of generic MN fate (Eisen et al., 1991; Appel et al., 1995).

The extent of transformation of motor neuron subtype identity

Our analysis of LIM-HD protein expression suggests that both the columnar and pool subtype identities of MNs are influenced by signals from the paraxial mesoderm. The molecular evidence for a change in MN columnar identity is strongest in the case of the LMC_L, a subtype for which an unambiguous LIM-HD protein code exists. Analysis of the pattern of LIM-HD expression by MNs, however, suggests that signals from the paraxial mesoderm coordinately regulate the generation of all LMC neurons. The sufficiency of paraxial mesodermal signals for the differentiation of CT neurons is less clear. Displacement of B neural tube adjacent to T level paraxial mesoderm leads to the generation of CT neurons but placement of T paraxial (or non-axial) mesoderm adjacent to B level neural tube does not. This result could reflect the requirement for a higher level of paraxial mesodermal signaling in CT neuron generation. Since all mesodermal grafts were performed unilaterally, a competing influence of signals from the contralateral host B mesoderm might therefore be sufficient to prevent the generation of CT but not somatic MNs. We cannot exclude, however, that an additional T-level restricted signal provided by cells other than the paraxial mesoderm, normally contributes to the generation and/or dorsal migration of CT neurons.

An alteration in motor pool identity, determined on the basis of the emergent pattern of muscle innervation, has been described after inversion of lumbosacral neural tube segments at early stages (Matise and Lance-Jones, 1996). Our results show that at B levels, distinct LMC motor pools express different levels and/or combinations of LIM-HD proteins and it is likely that this pool-specific pattern is also established in response to signals from the paraxial mesoderm. Since the organization of motor pools varies between individual segmental levels of the B spinal cord, signals from the paraxial mesoderm may therefore influence MN subtype at a spatial resolution finer than that revealed by the columnar organization of MNs. Consistent with this idea, the mesoderm induces a graded reorganization of the pattern of *Hoxc8* expression within the B spinal cord. Thus, a graded control of the

positional identity of neural tube cells may result later in the establishment of the distinct segmental (pool) and plurisegmental (column) organizational features of MN subtypes.

Although our studies show that the paraxial mesoderm changes the LIM-HD coding of MN columnar identity they have not addressed whether this code predicts the pattern of motor axon projections. An anatomical analysis of axonal projection patterns after early interchange of B and T levels of the neural tube, however, is unlikely to be informative in assessing the degree to which the molecular and anatomical changes in MN columnar identity are coordinated. Large displacements of the neural tube at stages beyond the critical period for respecification of MN identity lead to axonal projection patterns that are inappropriate for both the normal columnar (O'Brien and Oppenheim, 1990; O'Brien et al., 1990) and pool (Lance-Jones and Landmesser, 1980) identities of MNs. The intrasegmental displacement of primary MNs in zebrafish, has, however, been shown to result in a coordinate change in the expression of LIM homeobox genes and motor axon projections (Eisen et al., 1991; Appel et al., 1995). It is likely, therefore, that LIM-HD protein expression by avian MNs is a pertinent indicator of their columnar subtype identity.

Signaling by paraxial mesoderm

The present studies provide evidence that rostrocaudal differences in the signaling properties of the paraxial mesoderm specify MN subtype identity. Studies of primary MN differentiation in *spadetail* mutant zebrafish embryos have revealed changes in MN identity and in the profile of LIM homeobox gene expression at levels in which mesodermal development is perturbed (Eisen and Pike 1991; Tokumoto et al., 1995). These changes, however, may reflect a general permissive requirement for mesodermal signaling rather than a more specific positional influence.

What is the basis of the positional signaling properties of the paraxial mesoderm? After its segmentation, the paraxial mesoderm gives rise to sclerotomal and myogenic cells. Sclerotomal cells possess a positional identity which is retained when cells are transplanted to different rostrocaudal levels of the embryonic axis (Kieny, 1971; Kieny et al., 1972; Chevallier et al., 1977; Chevallier, 1979). Myogenic progenitor cells may also express stable and heritable markers of their rostrocaudal position of origin (Donoghue et al., 1992; Grieshammer et al., 1992). The mechanisms that establish intrinsic rostrocaudal distinctions in sclerotomal and/or myogenic cells might also confer upon the paraxial mesoderm its positional signaling properties, reflected later in the pattern of cell types generated in the neural tube. The positional identity of the sclerotomal derivatives of the paraxial mesoderm is dependent on *Hox* gene activity (Krumlauf, 1994). In turn, there is evidence that the mesodermal expression of *Hox* genes is regulated by the *Cdx* class of homeobox genes and by FGF signaling (Subramanian et al., 1995; Shashikant et al., 1995; Pownall et al., 1996; Chawengsaksophak et al., 1997). Defining how the positional identity of the paraxial mesoderm is established may help to determine whether its neural patterning activity results from the spatially restricted expression of different signals or from variations in the level or timing of expression of a single inductive signal.

It remains unclear when signaling from the paraxial

mesoderm is initiated and whether cells of the caudal neural plate exhibit relevant anteroposterior positional differences before the action of paraxial mesoderm signals. Analysis of the pattern of *Hox* gene expression has revealed early and dynamic domains of *Hox* gene expression at caudal levels of the neural plate that give rise to the spinal cord (Wilkinson et al., 1989; Sundin and Eichele, 1992; Deschamps and Wijgerde, 1993; Gaunt and Strachan, 1994; Krumlauf, 1994). Consistent with this, *Hoxc8* is initially expressed over a wider rostrocaudal domain of the neural tube than it is at HH stage 28 (LeMouellic et al., 1988; Awgulewitsch and Jacobs, 1990; Belting et al., 1998). Paraxial mesoderm-derived signals that operate at the time of neural tube closure may therefore refine pre-existing but coarse positional values that are established at neural plate stages. Such early positional differences in *Hox* gene expression appear, however, to be subordinate to the patterning information provided later by paraxial mesoderm, at least in the context of MN differentiation.

Signals from paraxial and/or intermediate mesoderm have also been implicated in the induction of limb differentiation in cells of the lateral plate mesoderm (Stephens et al., 1991; Crossley et al., 1996; Ohuchi et al., 1997; Fernandez-Teran, 1997). The induction of LMC neurons by limb level paraxial mesoderm thus raises the possibility that the paraxial mesoderm controls, in a coordinated manner, the differentiation of limb mesenchyme and the generation of MN subtypes whose axonal projections to and within the limb depend on limb mesenchymal cells (Landmesser, 1980; Lance-Jones and Dias, 1991).

Coordination of rostrocaudal and dorsoventral signaling in the generation of motor neuron diversity

At hindbrain levels of the neuraxis the rostrocaudal identity of neural cells appears to be established prior to the specification of dorsoventral cell fates (Simon et al., 1995). Our results suggest that at spinal cord levels progenitor cells in the ventral neural tube are still sensitive to both rostrocaudal and dorsoventral patterning signals. Graded Sonic Hedgehog signaling along the dorsoventral axis appears to establish several distinct progenitor cell populations, notably those defined by expression of Pax6 and Nkx2.2 (Ericson et al., 1997). In the caudal hindbrain, the specification of these two progenitor cell populations by Sonic Hedgehog appears to direct the generation of somatic and visceral MNs (Ericson et al., 1997). In the spinal cord, however, Pax6 and Nkx2.2 are expressed uniformly along the rostrocaudal axis and virtually all MNs appear to derive from Pax6 progenitors (Ericson et al., 1997; unpublished observations).

Thus, in the spinal cord the interpretation of rostrocaudal patterning signals from the paraxial mesoderm is likely to involve the activation of a transcriptional program distinct from that controlling dorsoventral pattern. The identity of relevant factors remains unknown but since *Hox* genes are expressed in restricted domains along the rostrocaudal axis of the spinal cord (Kessel and Gruss, 1991; Krumlauf, 1994), they could be involved in the interpretation of signals provided by the paraxial mesoderm. In support of this, the ectopic expression or targeted inactivation of *Hox* genes at hindbrain levels has been shown to result in changes in cranial MN identity (Zhang et al., 1994; Goddard, et al., 1996; Studer et al., 1996).

In conclusion, the present studies indicate a role for the paraxial mesoderm in initiating the diversification of MN subtypes, but the source of signals involved in many subsequent steps in the acquisition of MN subtype identity remain to be resolved. For example, within a single limb-level segment of the spinal cord it is still unclear how the distinctions between MNs of the MMC and LMC or of the medial and lateral subdivisions of the LMC are defined.

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