INTRODUCTION

The nervous system contains many neural cell types which are characterized by their morphological and functional properties and by their specific positions along the body axes. In vertebrates, spinal somatic motoneurons can be divided into two large subpopulations. One innervates axial muscles and forms the medial motor column (MMC) which runs through all levels of the spinal cord. The other innervates limb muscles and forms the lateral motor column (LMC) in the brachial and lumbar segments. Limb motoneurons can be further subdivided into lateral and medial groups (LMCl and LMCm) based upon the positions of cells in the LMC. LMCl and LMCm motoneurons innervate muscles derived from dorsal and ventral muscle masses, respectively. Furthermore, the LMCl and LMCm are subdivided into individual motoneuron pools, which are localized in highly characteristic positions in both the anterior-posterior (A-P) axis and transverse plane of the spinal cord with only slight individual variation (Landmesser, 1978; Hollyday, 1980).

In the chick embryo, spinal motoneurons initially form ventrolateral structures as continuous, even columns along the A-P axis, then motoneuron subclasses become evident during development. Prior to the formation of distinct motor nerves and the segregation of motoneurons into columns, the combinatorial expression of four members of the LIM homeobox gene family, Islet-1, Islet-2, Lim-1 and Lim-3, distinguished subclasses of motoneurons (Tsuchida et al., 1994). Furthermore, by surgical manipulations in chick embryos the cellular basis of motoneuron regional differentiation has been analyzed in detail. Growth cones of motoneurons that innervate limb muscles, select their course within the plexus and reach the appropriate muscles after the positional relation between their somata and targets has been altered by spinal cord reversal (Lance-Jones and Landmesser, 1980) and by limb bud reversal (Ferguson, 1983). Motoneurons in the thoracic spinal cord transplanted to the lumbar region survive and initially differentiate in the same manner as those in the thoracic cord (O’Brien and Oppenheim, 1990). Also, the stereotypic movement seems similar: embryos in which the lumbosacral cord has been replaced by a brachial transplant show simultaneous leg movements that in several respects resemble wing flapping (Narayanan and Hamburger, 1971). These findings suggest that at the operative stages the motoneuron progenitors in the different positions along the A-P axis possess intrinsic differences that permit them to develop functionally appropriate synaptic connections. But the molecular mechanisms that give spinal motoneurons their regional specification are not yet clear.

Along the dorsoventral (D-V) axis, different cell types arise

SUMMARY

Cek8 and low affinity NGF receptor (LNGFR) are expressed at high levels on the chick spinal motoneurons of the brachial and lumbar segments from embryonic day (E) 5 to E7, but weakly on the motoneurons of the non-limb-innervating segments. We determined by means of heterotopic neural tube transplantation, that the expression of these molecules was already intrinsically determined at E2. We used these spatiotemporal specific molecules as markers of motoneuron subpopulations. To analyze how motoneurons acquire regional specification along the anterior-posterior (A-P) axis and in the transverse plane, we observed the expression of these molecules on ectopic motoneurons induced by implanting a supernumerary notochord or floor plate at E2. The ectopic motoneurons induced by the graft obtained from either the thoracic or lumbar segments had the same expression profile as the normal motoneurons at each A-P level. These findings suggest that regional specification of motoneurons, at least of Cek8 and LNGFR expression, is independent of the notochord and the floor plate and that the whole neural tube appears to be committed to differentiate into the motoneuron subtypes along the A-P axis at the operative stages.

Key words: chick, spinal motoneuron, differentiation, A-P axis, motoneuron subpopulation, Cek8, LNGFR
from different portions of the neural tube. The dorsoventral polarity of the spinal cord becomes apparent soon after its closure. During the early stages, elimination of the notochord and floor plate prevents the differentiation of motoneurons and other ventral neuronal types (Placzek et al., 1990; Hirano et al., 1991; van Straaten et al., 1991; Yamada et al., 1991; Ericson et al., 1992; Goulding et al., 1993). Also, grafting the notochord or the floor plate to the dorsal midline of the neural tube induces motoneurons in an ectopic dorsal position and suppresses the expression of some dorsal markers (van Straaten et al., 1985, 1989; Placzek et al., 1990, 1991; Yamada et al., 1991; Ericson et al., 1992; Goulding et al., 1993). The differentiation of motoneurons appears to involve the action of inductive signals initially from the notochord and later, from the floor plate (Placzek et al., 1990, 1993; Yamada et al., 1993). However, it has not been analyzed, whether the inductive signals from the notochord and/or floor plate determine motoneuron subclasses.

In this study, in order to understand how spinal motoneurons acquire regional specification, especially in relation to the inductive influences from the notochord and floor plate, we examined the expression of the spatiotemporal specific molecules, Cek8 and low affinity NGF receptor (LNGFR; mAbs M7412 and M7902 antigen, see Tanaka et al., 1989). Cek8 is a chick homologue of Sek originally identified in mouse as a segmentally expressed receptor type tyrosine kinase (Nieto et al., 1992; Ohta et al., 1995). Cek8 and LNGFR are expressed at high levels on the chick spinal motoneurons of the brachial and lumbar segments, but weakly on the non-limb-innervating segments. In addition, Cek8 is expressed at high levels by motoneurons at the LMC through lumbosacral segments (LS) 1-4 and by whole cells in the LMC through more posterior lumbosacral region at St. 27. Initially, to determine whether these spatial specifications are influenced by the spinal cord intrinsically or extrinsically on and after E2, we examined the expression patterns after heterotopic transplantation of the neural tube, neural plate or the somatopleure. The results suggested that the expression of these regional specific molecules was already specified by E2. Subsequently, by implanting a supernumerary notochord or floor plate, ectopic motoneurons were induced and the expression of the spatiotemporal-specific molecules was observed. The ectopically induced motoneurons had the same properties of Cek8 and LNGFR expression as the normal motoneurons at each A-P level. The graft obtained from either the thoracic, or the lumbar region identically influenced the expression of the induced motoneurons. Therefore, the regional specification of motoneurons along the A-P axis, in terms of Cek8 and LNGFR expression, appeared to be independent of the notochord and floor plate and was present in the whole neural tube at least after the operative stages.

MATERIALS AND METHODS

Animals
Chick eggs (White Leghorn) were incubated at 38°C in a humidified forced air incubator, and the embryos were staged according to Hamburger and Hamilton (1951).

Surgical procedures
Transplantation of the somatopleure
Surgical manipulations were performed using a modification of the method of Lance-Jones and Dias (1991). Lumbosacral and thoracic somatopleure were obtained from St. 13-15 embryos. By St. 14-15, somatic segmentation has only proceeded to somatic level 24-27, and at St. 13-14 to somatic level 19-24. The more posterior level was defined by measuring roughly equivalent lengths of somatic mesoderm. In the host embryos at St. 13-14, one side of the thoracic somatopleure adjacent to somatic level 19-25 was excised with a tungsten needle. The graft of the lumbosacral somatopleure (axial levels 26-33) was removed from St. 14-15 embryos and transferred to the position of the excised thoracic somatopleure. In others, the graft of thoracic somatopleure was transferred into the lumbosacral region. The host embryos were incubated until about St. 27.

Neural tube transplantation
Host embryos were St. 13-14. About four of the presumptive thoracic neural tube segments (adjacent to somatic level 19 to 25) of the host embryo, which were at the level of the unsegmented mesoderm region at this stage, were excised using a tungsten needle. The underlying notochord was left intact. Donor embryos were incubated until they reached St. 15-16. The piece of neural tube to be transplanted was removed from the presumptive lumbar neural tube (adjacent to somatic numbers 25 to 32) at the level of the unsegmented mesoderm region. The lumbar transplant was transferred into the appropriate thoracic cord region of the host embryo. The A-P and D-V polarities of the transplant were maintained. Before grafting, the lumbar cord was dissociated from adjacent tissues with 0.1% dispase in Tyroe’s solution and rinsed well with the same solution.

Neural plate implantation
Neural plate grafts with the underlying notochord (about 3 somites long) were obtained from St. 10-13 donor embryos. The grafts were dissociated gently and mechanically from adjacent tissues, then inserted into the St. 13-16 host embryos in the unsegmented mesoderm at the presumptive lumbar or thoracic region. Prior to inserting the neural plate, an incision was made in the surface ectoderm of the host embryo to expose the unsegmented mesoderm at the operative region and a small slit was made in the unsegmented mesoderm to implant the graft. After the excision, the donor embryos were also incubated and whether the excised region was the lumbar or thoracic region was recorded. The host embryos were incubated until the donor embryos reached St. 27.

Notochord implantation
Notochord grafts (2-4 somites long) were obtained from St. 10-13 chick embryos, when the notochord has the ability to induce motoneurons (Yamada et al., 1993). The thoracic or lumbar region was dissected out of the embryo. The notochords were isolated from surrounding tissue with 0.1% dispase in Tyrode’s solution for 5 minutes and rinsed several times with the same solution. The host eggs were incubated until the embryos reached St. 10-13. An incision was made in the ectoderm and underlying mesoderm to expose one side of the neural tube at the level of the unsegmented mesoderm in the presumptive thoracic or lumbar level. A donor notochord was placed in the vicinity of the incision. Using a tungsten needle, the graft was inserted between the dorsal neural tube and the somitic mesoderm. The host embryos were incubated until about St. 27.

Floor plate implantation
The grafts of the floor plate were taken from St. 17 or 24 chick embryos, when the floor plate has the ability to induce motoneurons (Yamada et al., 1993). Grafts, 2-4 somites long, were obtained from the thoracic or lumbar region. The site of the neural tube was isolated from surrounding tissue with 0.1% dispase as described above and the floor plate graft was cut with a tungsten needle. Host embryos were implanted as described for the notochord.
Histology
Control and operated embryos were fixed overnight with 4% paraformaldehyde (PFA) in PBS (137 mM NaCl, 9.0 mM Na2HPO4, 2.9 mM NaH2PO4, pH 7.2, treated with 0.1% diethylpyrocarbonate) at 4°C. The embryos were transferred to 0.5 M sucrose/4% PFA and gently shaken on a platform for 3 hours at 4°C. After briefly washing with PBS, they were transferred to 0.5 M sucrose in PBS and gently shaken on the platform for 2 hours at 4°C. The embryos were excised, embedded in Tissue-Tek OCT compound (Miles) and frozen. Sections cut at 10 µm on a cryostat microtome were alternately collected for in situ hybridization and immunohistochemistry. In others, to define the Cek8 expression profiles in more detail at the lumbar cord, laminectomy was performed on the embryos and the lumbosacral ventral roots were exposed. The roots of LS 1,3 and 5 were marked with DiI solution to identify the A-P level. The embryos were excised, embedded, then immediately frozen. Sections were alternately collected for in situ hybridization of Cek8 and Islet-1.

In situ hybridization
Molecular probe
The Cek8 RNA probe for in situ hybridization was prepared by transcribing an 889 base pair BamHI-EcoRI fragment from the 3’ untranslated region of Cek8 cDNA beginning at nucleotide 2711 (Ohta et al., 1995). Islet-1 RNA probe was generated by transcribing nucleotides 317-1308 of reported chick Islet-1 cDNA (Tsuchida et al., 1994), which was prepared by reverse transcription polymerase chain reaction. T3, T7 or SP6 RNA polymerase were used in a reaction mix containing 0.83 mM digoxigenin (DIG)-11-UTP (Boehringer Mannheim).

Hybridization
In situ hybridization was performed by a modification of the method of Ohta et al. (1995). Sections were digested with 1 µg/ml proteinase K for 10 minutes at 37°C. Hybridization proceeded for 16 hours at 55°C in hybridization solution containing 1 µg/ml of each DIG-labeled RNA probe. After washing, sections were blocked with 10% heat-inactivated sheep serum, then incubated overnight at 4°C with a 1:1000 dilution of alkaline phosphatase-conjugated anti-DIG antibody (Boehringer Mannheim). The antibody was absorbed against St. 27 embryo fragments prior to use. An alkaline phosphatase-mediated color reaction proceeded using 4-nitroblue tetrazolium chloride (Boehringer Mannheim) and 5-bromo-4-chloro-3-indolyl phosphate (Boehringer Mannheim). Specimens were developed then stopped at the appropriate time.

Immunohistochemistry
The monoclonal antibody (mAb) M7902 against chick LNGFR was a hybridoma supernatant (Tanaka et al., 1989), mAb SC1 for the motoneuron marker was purified from ascites. Since both mAbs SC1 and M7902 were mouse IgG, mAb SC1 was biotinylated with NHS-LC-Biotin (Pierce) for double staining. After washing with PBS, sections were incubated with mAb M7902 (1:5 dilution) for 30 minutes at room temperature (RT). The second antibody, rhodamine conjugated rabbit anti-mouse IgG F(ab)2 (Chemicon International Inc., diluted 1:200), was added for 30 minutes at RT. After washing, the sections were blocked with mAb M 7902 overnight at RT. After washing, the sections were incubated with biotinylated mAb SC1 for 30 minutes at RT and washed, then a goat anti-biotin antibody (Pierce, diluted 1:200) was applied for 30 minutes at RT. FITC-conjugated rabbit anti-goat IgG F(ab)2 (Chemicon International Inc., diluted 1:70) was then added for 30 minutes at RT. The sections were mounted and photographed using an Olympus microscope with Kodak Ektachrome 400.

RESULTS
Expression profiles of Cek8 and LNGFR in the chick embryo spinal cord
The expression profiles of Cek8 were examined in the chick embryo spinal cord from St. 23 to St. 27 by in situ hybridization. Sections immediately adjacent were stained with mAbs SC1 and anti-LNGFR or examined by in situ hybridization of Islet-1. mAb SC1 recognizes a membrane protein, SC1, which is present in motoneurons, dorsal root ganglion neurons, the notochord and floor plate cells of the chick embryo at the early developmental stages (Tanaka and Obata, 1984) and has been used as a motoneuron marker (Yamada et al., 1991; Ericson et al., 1992; Hirano and Tanaka, 1994). Islet-1 is a member of the LIM homeobox gene family and the earliest marker of developing motoneurons (Ericson et al., 1992). In the LMC of the St. 27 embryo, Islet-1 mRNA is expressed only in the LMCm (Tsuchida et al., 1994).

In the chick embryo spinal cord, Cek8 transcripts were first detected in motoneurons and the ventricular cell layer at St. 24 when motoneuron axons enter the limb (Tosney and Landmesser, 1985). The expression level of the motoneurons in the brachial and lumbar regions progressively increased. At St. 27, Cek8 was expressed at high levels by motoneurons in the LMC of the lumbar (Fig. 1D) and brachial regions (data not shown) but not in the thoracic region (Fig. 1C). Sections adjacent to those were immunostained with the mAb SC1 (Fig. 1A,B). The immunoreactivity of mAb SC1 was intense in the entire motor column throughout the spinal cord. The expression of Cek8 was restricted to the lateral region of the LMC at the anterior lumbosacral region. In the LMC at St. 1-4, motoneurons that intensely expressed Cek8 were located laterally adjacent to motoneurons that expressed Islet-1 in the LMCm. Motoneurons that expressed Cek8 were entirely separate from Islet-1-positive motoneurons (Fig. 2A,B). At the more posterior lumbosacral region, motoneurons in the whole LMC expressed Cek8, and then motoneurons in the LMCm co-expressed Cek8 and Islet-1 (Fig. 2C,D). The expression of Cek8 was developmentally transient and the intense expression in the LMC gradually decreased after reaching a peak at St. 27-29, becoming undetectable at St. 35 (Ohta et al., 1995). Control experiments using a sense probe resulted in no apparent signals (data not shown).

At St. 24, anti-LNGFR mAb weakly stained the whole spinal cord with the exception of the ventricular cell layer, roof and floor plates. At St. 27, the immunoreactivity of LNGFR in the entire LMC became more intense than elsewhere (Fig. 1F). The LNGFR expression in the LMC was also developmentally transient and the staining intensity gradually decreased after reaching a peak at St. 28 (Tanaka et al., 1989). The staining intensity in axial motoneurons was low throughout development (Fig. 1E).

Cek8 and LNGFR expression by thoracic and lumbar motoneurons following exchange of their targets
The thoracic somatopleure was excised and the lumbar somatopleure obtained from the another embryo was implanted into the site, thus the embryo had three hindlimbs. One was situated in the right thoracic region and the normal limbs were in the usual position (3 embryos) (Fig. 3A). In a series of transverse
sections through the supernumerary limb, mAb SC1 stained many thoracic motoneuron axons that entered the limb. Motoneurons on both the experimental (adjacent to the supernumerary hindlimb) and the control (contra lateral) thoracic sides expressed very low levels of Cek8 (Fig. 3B) and LNGFR (data not shown). After the lumbar was replaced with the thoracic somatopleure, the embryo lacked a hindlimb on the operative side (3 embryos) (Fig. 3C). Motoneurons located in the lumbar region on the side of the missing limb, expressed Cek8 and LNGFR at the same intensity as the control side (Fig. 3D; LNGFR data not shown). These observations suggested that the expression of Cek8 and LNGFR in motoneurons is independent of their peripheral targets. Following removal of the limb bud, cell counts of the LMC motoneurons on the operative side were reduced in the St. 27 embryo (Oppenheim et al., 1978). Significant motoneuron loss was not detected in our histological analysis.

**Cek8 and LNGFR expression by motoneurons following heterotopic transplantation of neural tube and neural plate**

The observations of the hindlimb transplantation and removal suggested that regional specification, the expression profiles of Cek8 and LNGFR, could be intrinsic properties of motoneurons or influenced by the environment, other than their targets. To change the microenvironment of the cord along the A-P axis we performed neural tube heterotopic transplantation and neural plate implantation. First, we analyzed the expression profiles in motoneurons following neural tube heterotopic transplantation. The lumbar neural tube was heterotopically transplanted in ten embryos, and of these four survived until St. 27. The transplanted lumbar cords that remained, apparently survived and differentiated quite normally when placed into the thoracic region. Similar to the normal lumbar cord, motoneurons of the grafted lumbar cord that had been transplanted into the thoracic region expressed very high levels of Cek8 (Fig. 3E,F) and LNGFR (data not shown). In 3 embryos, the lumbar cord was replaced by a thoracic cord, three to four segments in length, using the procedure described above. In these embryos, the expression intensity of Cek8 and LNGFR in motoneurons was similar to that in the normal thoracic cord (data not shown). These observations indicated that the Cek8 and LNGFR expression patterns of motoneurons are intrinsic properties of the neural tube.

To examine when neural tube acquired these properties we examined the expression profiles of motoneurons after neural plate implantation. Following the excision of the neural plate with the underlying notochord in St. 11 embryos with 13 somites, the donor embryos lacked the thoracic cord at the later stages. Grafts of thoracic neural plate were inserted into the lumbar unsegmented mesoderm in host embryos (3 embryos). The implanted graft and the host mesoderm formed a spinal cord-like structure with dorsal root ganglia, dermomyotome

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*Fig. 1. Expression of SC1, Cek8 and LNGFR in the chick spinal cord of a St. 27 chick embryo. Transverse sections through the thoracic (A,C,E) or lumbar cord (B,D,F). A,E, and B,F were double immunostained. Adjacent sections to those processed for immunohistochemistry were analyzed for Cek8 expression by in situ hybridization (C,D). (A,B) SC1 expression. Floor plate and motoneurons express the SC1 antigen. Dorsal root ganglion neurons and their afferent fibers in the dorsal funiculus are also labeled. Motoneurons of the thoracic (A) and lumbar cords (B) were equally stained with SC1 antibody. (C,D) Cek8 transcription. Cek8 is expressed weakly in the ventricular cell layer of the thoracic and lumbar cords. The motoneurons express low levels of Cek8 in the thoracic cord (C), and high levels in the lumbar cord (D). (E,F) LNGFR expression. LNGFR antibody staining of the LMC in the lumbar cord (F) is brighter (yellowish) than the axial motor column in the thoracic cord (E). LNGFR antibody stains dorsal root ganglia and the cells surrounding the spinal cord, myotome and other cord cells except for the ventricular cell layer, roof and floor plates. Scale bar, 180 μm.*
and sclerotome on both sides, then appeared to differentiate almost normally in the lateral position to the normal cord (Fig. 4B-D). Motoneurons that derived from the thoracic neural plate, expressed high levels of SC1 (Fig. 4C), but not Cek8 (Fig. 4B) or LNGFR (Fig. 4D). Following the excision of the neural plate in St. 12 embryos with 18 somites, the donor embryos lacked the lumbar cord. Lumbar neural plates with the underlying notochord were inserted into the thoracic or lumbar mesoderm region of the host embryos (2 embryos each). Under either condition, the motoneurons derived from the lumbar neural plate expressed high levels of SC1, Cek8 (Fig. 4A) and LNGFR in the thoracic or lumbar region of the host embryos. Prior to St. 11, we could not confirm whether the origin of the graft was the thoracic or lumbar plate, therefore we did not perform further analyses. The results revealed that the neural plate already had the regional differences in expression profiles of Cek8 and LNGFR in motoneurons along the A-P axis by the operative stages.

The influence of notochord and floor plate on the motoneuron specification

The differentiation of motoneurons seems to depend on inductive signals emanating from the notochord and/or the floor plate. During early development, motoneurons are induced in the ectopic dorsal portion by implantation of the notochord or floor plate (van Straaten et al., 1985; Placzek et al., 1991; Yamada et al., 1991, 1993; Ericson et al., 1992). Heterotopic transplantation of the neural tube indicated that a regionally different notochord does not affect the specification of motoneuron subtypes. However, it remains possible that the transplanted neural tube was already committed and was not affected by the ectopic notochord, even though the notochord had determinative effects on motoneuron subtype specifica-
tion. To clarify whether the notochord determines the subtype of motoneurons, we analyzed the expression of Cek8 and LNGFR by the ectopic motoneurons induced by notochord implantation. The graft site was examined morphologically to determine the effect of the notochord on the spinal cord. Transverse sections through the region of the graft were examined for the expression of Cek8, SC1 and LNGFR. Although the induced floor plate was stained with mAb SC1, it was also assessed by morphological changes and staining with mAb FP1 (Yamada et al., 1991).

Fig. 5A-F shows examples in which the ectopic notochord obtained from the thoracic region was placed dorsally in the lumbar region. By placing an extra notochord at various positions along the D-V axis, motoneurons were induced at various sites along the D-V axis in the spinal cord. Fig. 5A shows that the area of the SC1-positive ectopic motoneurons was localized to the dorsal spinal cord and completely separated from the host LMC (6 embryos). In seven other specimens, the region of ectopic motoneurons fused to the native LMC, resulting in an enlarged LMC (Fig. 5D). The expression of Cek8 and LNGFR within the SC1-positive ectopic motoneurons was as intense as that in motoneurons in situ (Fig. 5B,C,E,F). The expression of Cek8 and LNGFR on the ectopically induced motoneurons was not affected by their location along the D-V axis. In addition, the ectopic motoneurons induced in the lumbar cord by the extra notochord obtained from either the thoracic or lumbar region were identical (data not shown). However, when the ectopic motoneurons were induced in the thoracic region by a second notochord obtained from the lumbar region (6 embryos), SC1 was expressed (Fig. 5G), whereas Cek8 and LNGFR were expressed at low levels (Fig. 5H,I). The expression levels of Cek8 and LNGFR were weaker than those in the lumbar region, similar to the in situ motoneurons in the thoracic region. Our results revealed that the second notochord could induce motoneuron subpopulations ectopically, but did not influence their regional specification, in terms of the expression patterns of Cek8 and LNGFR.

In the heterotopic transplantation of the neural tube and

![Fig. 4. Cek8, SC1 and LNGFR expression in motoneurons following neural plate implantation. In the transverse sections through the graft, Cek8 expression was analyzed by in situ hybridization, whereas SC1 and LNGFR expression was analyzed by immunohistochemistry. (A) The lumbar neural plate obtained from the St. 12 embryo was implanted in the middle of the unsegmented mesoderm in the lumbar region. The cord like structure (*) derived from the lumbar neural plate is adjacent to the original lumbar cord. The second cord appears to maintain the original D-V arrangement and has motor columns. The motoneurons in the columns express Cek8 intensely, like those in the original LMC. The notochord implanted with the neural plate has fused with the original notochord (n). The axial structures of the dermomyotome and sclerotome appear to develop normally around the second cord. (B-D) The thoracic neural plate obtained from the St. 11 embryo was implanted into the lumbar region. (B) The second cord (*) derived from the thoracic neural plate is located laterally to the original lumbar cord. The motor columns in the second cord do not express high levels of Cek8, while the ventricular cells express low levels, like those of the host embryo. The motor columns in the in situ lumbar cord express high levels of Cek8. n’, notochord implanted with the neural plate. (C) The transverse section adjacent to B was stained with mAb SC1. The second cord appeared to maintain the original D-V arrangement and has SC1-positive motor columns, a floor plate and apparently normal (right) and elongated (left) dorsal funiculi. Dorsal root ganglia (DRG) derived from the host and transplant were formed normally, but axons of the DRG and motoneurons between the two cords grew abnormally. (D) LNGFR expression in the same section as C. The motoneurons in the host LMC intensely express LNGFR, whereas the levels in the transplant are low. Scale bar, (A,B) 300 μm; (C,D) 180 μm.
plate, and the transplantation of an extra notochord obtained from different A-P axes, the original floor plate might have predominantly influenced motoneuron regional specification. To analyze the floor plate influence, we also performed floor plate transplantation. Ectopic motoneurons induced by the floor plate in the lumbar cord expressed high levels of Cek8 and LNGFR (Fig. 5J-L). Motoneurons induced in the thoracic cord weakly expressed Cek8 and LNGFR (data not shown). The

**Fig. 5.** Cek8, SC1 and LNGFR expression in ectopic motoneurons induced by implantation of the notochord (A-I) and the floor plate (J-L). SC1 (A,D,G,J) and LNGFR (C,F,I,L) expression was analyzed in the transverse section by double immunohistochemistry. Cek8 expression was examined in the adjacent section by in situ hybridization (B,E,H,K). (A-F) The ectopic column was induced in the lumbar region by the notochord obtained from the thoracic region. (A) The ectopic notochord (n') induced ectopic SC1 expression in the dorsal spinal cord. These SC1-positive cells are separated from the original LMC, and are ectopic motoneurons, but from histochemical evidence they are not considered to be floor plate cells. (B) Adjacent section to A. The motoneurons in the dorsal ectopic motor column express high levels of Cek8. (C) The same section as A. The motoneurons in the dorsal ectopic motor column express high levels of LNGFR. (D) The supernumerary notochord was implanted more ventrally (n'). The induced SC1-positive motoneurons fused with the original LMC, which apparently extended the LMC. (E) Adjacent section to D. The motoneurons in the dorsal ectopic motor columns intensely express Cek8. (F) The same section as D. The motoneurons in the dorsal ectopic motor column, intensely express LNGFR. (G-I) The ectopic column was induced in the thoracic region by the notochord obtained from the lumbar region. (G) The ectopic notochord (n') induced SC1-positive ectopic motoneurons, and the ectopic motoneurons are separated from the original LMC. (H) Adjacent section to G. The SC1-positive ectopic motoneurons express Cek8 as weakly as the original motoneurons. The ventricular layer expresses Cek8 weakly in both the implanted notochord and the opposite sides. (I) The same section as G. High levels of LNGFR expression were undetectable, in the SC1-positive ectopic motoneurons. The expression level of LNGFR in the ectopic and original thoracic motoneurons is identical. (J-L) The ectopic column was induced in the lumbar region by the floor plate obtained from the thoracic region. (J) The implanted floor plate (f') stained with the SC1 antibody, induced ectopic SC1 expression in the dorsal spinal cord. These SC1-positive cells fused with the original LMC, and then the LMC appears to be extended. (K) Adjacent section to J. The ectopic Cek8 expression is as intense as that in the original motoneurons in the lumbar cord. (L) The same section as J. The ectopic region, stained with LNGFR antibody, is coextended with induced ectopic SC1 expression. The intense level of the LNGFR ectopic expression is equal to that in the original lumbar motoneurons. Arrowhead, DRG. Scale bar, 140 μm.
expression was independent of the site of origin of the floor plate, either lumbar or thoracic, and of the developmental stage (5 embryos each, data not shown). In conclusion, like the results of the notochord implantation, the second floor plate at the operative stages induced the motoneuron subpopulations and did not influence their regional differentiation along the A-P axis, at least in terms of expression of Cek8 and LNGFR by spinal motoneurons.

**The subdivision of ectopic motor column into Cek8 and Islet-1 expressing regions**

We compared Cek8 expression in the ectopic motoneurons with that of Islet-1. When the notochord was implanted at LS 1-4 where Cek8 and Islet-1 expression segregate in a normal LMC at St. 27 (Fig. 2), their expression also segregated in the ectopic motor columns (Fig. 6A-D). When the dorsal ectopic motor column was divided from the in situ LMC, Cek8 was expressed by motoneurons located in the dorsolateral position (Fig. 6A) and Islet-1 was expressed by motoneurons located in the ventromedial position in the ectopic column (Fig. 6B). The ventral LMC on the side of the second notochord was larger than the LMC on the control side due to fusion with the ectopic motoneurons. The ectopic expression of Cek8 and Islet-1 were similar in the mediolateral segregation around the second notochord. It seemed to be the second midline axis. When the ectopic columns fused with the original LMC, Cek8 was expressed in the lateral region and Islet-1 was expressed in the medial region (Fig. 6C,D). Furthermore, when the ectopic motor column was induced at the region where Cek8 was expressed in the whole LMC at the more posterior levels, the expression profiles of the ectopic motoneurons were similar to those of the in situ LMC. Islet-1 was still expressed in the medial region and Cek8 seemed to be expressed in all the ectopic motoneurons (Fig. 6E,F). These results revealed that motoneurons in the ectopic column are subdivided on the basis of Cek8 and Islet-1 expression as they are in LMC in situ and that the second notochord could be the new midline axis of mediolateral patterning in the ectopic column.

**DISCUSSION**

Spinal somatic motoneurons are divided into two large subpopulations, axial and limb motoneurons. Limb motoneurons form the LMC in the brachial and lumbar segments and these axons form the plexuses. The limb motoneurons are further divided into each motor pool innervating a single muscle (Landmesser, 1978; Hollyday, 1980). The motoneuron axons of each pool destined to innervate a distant limb target make a number of complex pathway choices as they traverse the distal environment (Landmesser, 1984). The limb connective tissues influence this process (Lance-Jones and Dias, 1991). The motoneurons in each pool must have unique characteristic molecules which enable them to innervate unique target muscles and to receive unique synapses on their somata. Although no such molecules have been identified, the LIM homeobox genes distinguish the subtypes of spinal motoneurons (Tsuchida et al., 1994). We found that Cek8 (Ohta et al. 1995) and LNGFR (Tanaka et al., 1989) are expressed at high levels in the LMC of the brachial and lumbar cord during E5 and E7. Coincident with our findings of different molecular expression between thoracic and lumbar motoneurons, those motoneurons committed to innervate limb and axial muscles have different survival requirements in vivo (O’Brien and Oppenheim; 1990). Using these molecules as markers of motoneuron subpopulations in limb-innervating segments, we
analyzed the generation of motoneuron subpopulations after embryonic manipulation.

Our first conclusion obtained from this study is that the regional specification of motoneurons expressing Cek8 and LNGFR along the A-P axis is already determined intrinsically at the earliest operative stage, St. 12. Studies of cord reversal (Lance-Jones and Landmesser, 1980) and wing or thoracic cord implantation into the lumbar region (Narayanan and Hamburger, 1971; O’Brien and Oppenheim, 1990) have suggested that the spinal motoneuron progenitors are already committed to differentiate into the specific motoneuron subtypes when the operations were performed at St. 15-17, because motoneurons have not yet been born at those stages. Motoneuron progenitors are still in the mitotic cycle and are multipotential, producing different cell types at these stages (Leber et al., 1990). The conclusion, that the commitment occurs before the neuronal differentiation was obtained from functional analysis. We reached an equivalent conclusion using endogenous markers, Cek8 and LNGFR.

Preliminary reports of the cord reversal and retrograde labeling of motor pools indicated that motoneurons are not yet committed to innervate specific targets at St. 13-14 (Matise and Lance-Jones, 1992), whereas motoneurons are apparently committed at St. 15-16 (Lance-Jones and Landmesser, 1980). By contrast, our results of neural tube heterotopic transplantation at St.13-15 indicated that motoneuron progenitors are committed to Cek8 and LNGFR expression. This discrepancy might be due to the differences in the cell populations examined in each case. The study of cord reversal was a detailed investigation of a single motor pool, whereas we characterized the whole of the LMC. The stereotypic hindlimb movement controlled by the transplanted wing cord in the lumbar region has some similarities with our results, because the behavior represents the whole cord neural circuit (Narayanan and Hamburger, 1971). It is possible that motoneurons are committed to become a member of the LMC before becoming committed to a motor pool. Our study of endogenous marker molecules for each motor pool have enabled us to clarify this.

The endogenous markers allowed ectopic motoneurons to be analyzed at any location, whereas the other studies required functional connections with muscles. This was a considerable advantage for our study as we could examine dorsally induced ectopic motoneurons as well as the lumbar cord in the thoracic region. In motoneurons ectopically induced by a supernumerary notochord and floor plate along the D-V axis, the expression of Cek8 and LNGFR is identical with that of the in situ motoneurons. The ectopic motoneurons can differentiate into the motoneuron subpopulations, but the notochord and floor plate cannot influence regional specificity along the A-P axis. The segregated expression of Cek8 and Islet-1 in the LS 1-4 in the ectopic motor column as well as in situ LMC (Fig. 6) is remarkable. Cek8 was expressed in LMCm and the most lateral region of the ectopic column, whereas Islet-1 was in LMCm and medial region of the ectopic column. At lumbar levels, most LMCi neurons are born at stages 18-20 and most LMCm neurons at stages 20-21 (Whitelaw and Hollyday, 1983). Although we do not know whether the Cek8-positive motoneurons were generated before those that were Islet-1 positive in the ectopic column, the identical mechanism of differentiation seems to govern both types of motoneurons.

Thus, our second major conclusion is that the notochord and floor plate do not influence the motoneuron regional specificity along the A-P axis, but cells along the whole neural tube appear to be committed to differentiate into a subtype of motoneurons if exposed to the appropriate signal.

As for the commitment, one important question is when the motoneuron progenitor is committed along the A-P axis. To clarify this, we tried to heterotopically transplant the neural plate as early as possible. The donor embryos of neural plate grafts were also grown to confirm the graft origin, because a short neural plate corresponded with longer segments at later stages. The embryos before St. 10 did not develop well after neural plate excision and the neural plates from embryos at St. 11-12 were used as the earliest analyzable rudiment of a thoracic or lumbar cord. However, the grafted neural plate became disorganised during and after manipulation and did not differentiate normally. Therefore, we transplanted the neural plate together with the underlying notochord. Under these conditions, the underlying notochord might influence the commitment of motoneuron regional specificity. We consider that this is not so, because we used the notochord derived from the neural plate in transplantation. Even this notochord did not influence regional specificity on the ectopically induced motoneurons. The other argument is that the transplantation was into the unsegmented mesoderm and not into the neural plate, because we could not replace the neural plate. The ectopic neural plate implantation also revealed that an environment including notochord, mesoderm, peripheral target (Fig. 3) and the supernumerary notochord or floor plate (Fig. 5) did not influence the differentiation of motoneuron subtypes. The remarkable result is that the isolated neural plate corresponding to 2-3 segments differentiated as normal thoracic or lumbar cord with regard to Cek8 and LNGFR expression. However, it is not possible to simply conclude that motoneuron progenitors in the neural plate are committed because it remains possible that the motoneurons differentiated from the thoracic neural plate express Cek8 and LNGFR at high levels after neural plate replacement in the lumbar region.

Another important question is how the motoneuron progenitors are committed along the A-P axis. In vitro studies using Xenopus have suggested that the anterior notochord can provide sufficient positional information along the A-P axis for the neuroectoderm, to result in the expression of a spatio-restricted molecular marker, engrailed-2 (Hemmati-Brivanlou et al., 1990). However, in chick embryos when Hensen’s node is surgically removed and the notochord is not formed, these embryos express engrailed-2 in a manner similar to that of the controls (Darnell et al., 1992). Our results suggested that the notochord and floor plate differentiate at the experimental stages used are neither required nor sufficient to provide motoneuron progenitors with regional specification. Therefore, notochord influences on A-P patterning of the developing neural tube have not yet been established. After notochord ablation, the floor plate and motoneurons can be formed in a delayed fashion in vivo and in vitro from isolated neural plates (Artinger and Bronner-Fraser, 1993). This suggests that either there is an early induction of the floor plate by the chordamesoderm of Hensen’s node, or that only limited interactions between the neural plate and notochord immediately after neurulation are required for floor plate determination. It is also undeniable that these limited induction signals can provide the spinal cord with
regional specification. Since the recent demonstration of clear planar induction of Hox gene expression in the rhombomere neuroepithelium (Grapin-Botton et al., 1995), this mechanism in the commitment of motoneuron differentiation should also be analyzed. Understanding how motoneurons obtain their unique characteristics during development will contribute to revealing the principles of how the body plan of the nervous system is laid down.

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