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Original Article

Coexpression of HNF-3ß and Isl-1/2 and mixed distribution of ventral cell types in the early neural tube

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ABSTRACT Pattern formation in the vertebrate ventral neural tube has been proposed to depend on the ability of notochord-derived signals to induce the differentiation of distinct cell types in a distance-dependent manner. To determine whether the distribution of early differentiating cell types in the ventral neural tube is consistent with the operation of such a mechanism *in vivo*, the early localization of Isl-1/2$^+$ motor neurons in relation to HNF-3ß$^+$ floor plate cells has been investigated. In the most immature regions of the caudal spinal cord of early rat and chick embryos, an initial mixed distribution of cell types was detected that develops into a distribution characteristic of the final embryonic pattern where different cell types are found at distinct locations. In addition, a number of cells that coexpress both markers are detected within the floor plate. At later stages, coexpression is also detected in the hindbrain in the boundary region between HNF-3ß$^+$ ventricular cells and Isl-1/2$^+$ motor neurons. The mixed distribution of early differentiating cells expressing HNF-3ß and Isl-1/2 and the coexpression of these markers in single cells within the floor plate suggest that secondary cellular interactions are important in the generation of the final embryonic pattern of the neural tube.

KEY WORDS: *HNF-3β, Isl-1/2, floor plate, motor neuron, neural tube, pattern formation*

Introduction

Several mechanisms have been proposed for the formation of pattern during animal development. Special attention has been given to the possibility that inducing molecules act as morphogens by affecting cell fate directly at a distance from their source, with cells responding with strict thresholds. Recent experiments in frogs and flies (Green and Smith, 1990, 1994; Gurdon *et al.*, 1994; Wilson and Melton, 1994; Lecuit *et al.*, 1996; Nellen *et al.*, 1996) highlight the difficulty in assessing the contributions of different mechanisms to pattern formation *in vivo*. These and other studies point to the necessity of determining the normal distribution of induced cell types soon after induction in order to assess the feasibility of different mechanisms.

The patterning of the vertebrate neural tube begins at early neural plate stages when independent mechanisms appear to establish pattern first along the anteroposterior (A-P) and later along the mediolateral, future dorsoventral (D-V), axes (Roach, 1945; Jacobson, 1964; Ruiz i Altaba, 1992; Ericson *et al.*, 1995; Simon *et al.*, 1995). Along the D-V axis of the neural tube distinct cell types are found in a stereotyped arrangement. For example, in the hindbrain, discrete populations of floor plate cells, serotonergic neurons and motor neurons are detected sequentially from the ventral midline towards more dorsal regions. The mechanism by which this orderly array of ventral cell types is established is not clear.

Embryological manipulations and *in vitro* culture assays using cell type-specific markers have shown that the induction of different ventral neural tube cell types depends on the same inductive signal initially derived from the notochord and later from the floor plate (van Straaten *et al.*, 1988; Smith and Schoenwolf 1989; Placzek *et al.*, 1990, 1993; Clarke *et al.*, 1991; Hatta *et al.*, 1991; van Straaten and Hekking, 1991; Yamada *et al.*, 1991, 1993; Ruiz i Altaba, 1992; Goulding *et al.*, 1993; Hynes *et al.*, 1995b). These results, together with the lack of strict lineage restrictions in cell fate within the embryonic spinal cord and hindbrain (Leber *et al.*, 1990; Lumsden *et al.*, 1994) have suggested a model for ventral patterning of the neural tube where a diffusible signal derived initially from the notochord and later from the floor plate would act to specify distinct cell types in a concentration-dependent manner at constant positions from the ventral midline (Yamada *et al.*, 1991).

The signal molecule sonic hedgehog (shh) is first secreted by the notochord and then by both the notochord and floor plate and can induce the differentiation of ventral neural tube cell types (Echlard *et al.*, 1993; Krauss *et al.*, 1993; Riddle *et al.*, 1993;

Abbreviations used in this paper: Isl-1/2, Islet-1/2; HNF-3ß, hepatocyte nuclear factor-3ß; shh, sonic hedgehog.

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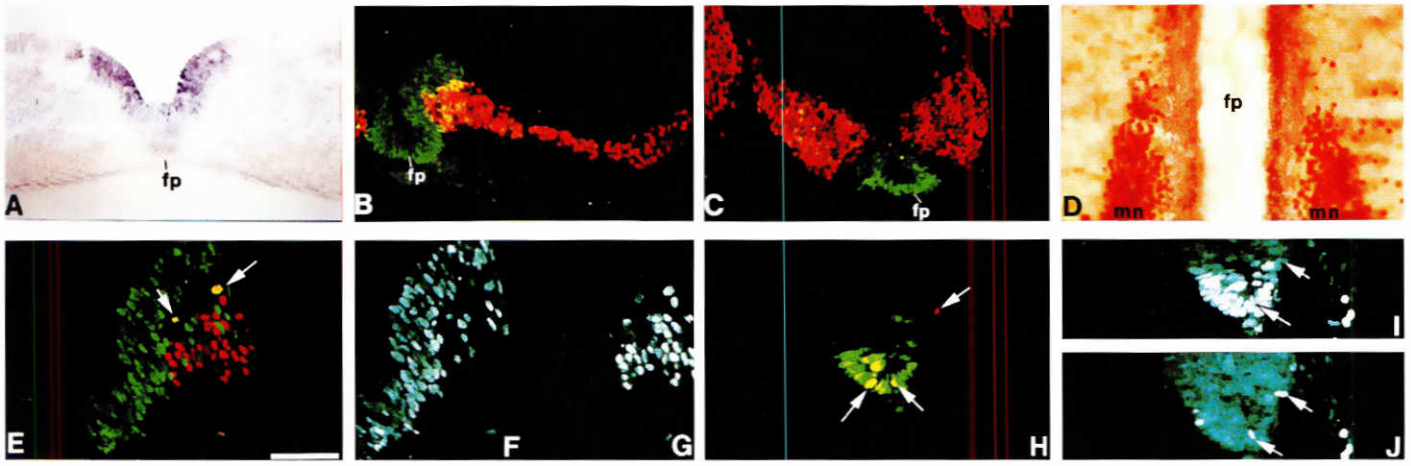


Fig. 1. Comparison of the distribution of cells expressing HNF-3ß and Isl-1/2 in the rat ventral neural tube. (A) *Nomarski micrograph of a cross section through the hindbrain of an E12 rat embryo showing the expression of HNF-3ß mRNA (in blue) after in situ hybridization in the floor plate (fp) and adjacent ventral ventricular zone. There is little labeling in the basal region of floor plate cells as their nuclei occupy this position.* **(B,C)** *Fluorescent optical micrographs of cross sections through the hindbrain* **(B)** *and spinal cord* **(C)** *of an E12 rat embryo showing the distribution of HNF-3ß protein (in green) in the nuclei of floor plate cells (fp) and adjacent ventral ventricular zone cells and Isl-1/2 protein (in red) in post-mitotic motor neurons (Ericson et al., 1992). The distribution of HNF-3ß protein* **(B)** *and mRNA* **(A)** *is coincident. At hindbrain levels* **(B)** *there is a striking apposition between the domains of HNF-3ß expression in the ventral ventricular zone and of Isl-1/2 in medial cells. Note that the coincidence in the dorsal boundaries of these two cell populations. At spinal cord levels* **(C)** *the apposition between the domains of expression of HNF-3ß in the ventral ventricular zone and that of Isl-1/2 in medial cells is less striking although the most medial, and therefore possibly the youngest, Isl-1/2 cells are found adjacent to the ventral ventricular zone that expresses HNF-3ß.* **(D)** *Nomarski micrograph of the ventral zone of the hindbrain-midbrain region of an E12 rat embryo showing the similar expression pattern of HNF-3ß in ventral ventricular zone cells (gray) in regions displaying Isl-1/2 expressing motor neurons (reddish brown) and in regions in which Isl-1/2 positive motor neurons are absent. In this micrograph floor plate (fp) cells are out of focus.* **(E-G)** *Fluorescent confocal micrographs of cross sections through the hindbrain of an E12 rat embryo showing the expression of HNF-3ß (in green in* **E,F***) and Isl-1/2 (in red in* **E,G***).* **(E)** *shows the combined expression patterns whereas* **(F,G)** *show single expression patterns. Isl-1/2 cells are found immediately adjacent to the dorsal region of the ventral ventricular zone expressing HNF-3ß. A different neuronal population is likely to occupy a similar position adjacent to the ventral region of the ventral ventricular zone. Cells coexpressing HNF-3ß and Isl-1/2 appear yellow in* **(E)**. *See also Figure 3.* **(H-J)** *Fluorescent confocal micrographs of cross sections of the posterior spinal cord of an E10 rat embryo showing the expression of HNF-3ß (in green in* **H,I***) and Isl-1/2 (in red in* **H,J***).* **(H,I)** *show the combined expression patterns whereas* **(J)** *shows the expression of Isl-1/2 only. In the spinal cord shortly after neural tube closure HNF-3ß expression is detected in a wide ventral domain in which all cells express this transcription factor (not shown). The first motor neurons expressing Isl-1/2 (arrows in* **H-J***) appear in an apparently scattered fashion in the ventral neural tube, including the ventral HNF-3ß domain in which they coexpress these two factors* **(H,I)**. *In all panels except* **(D)** *dorsal side is up.* **(D)** *shows a dorsal view with anterior end up.* **(B, E-G)** *show one half of the neural tube only. Scale bar: A-C, 80 µm; D, 50 µm; E-J, 50 µm.*

Roelink *et al.*, 1994, 1995; Ekker *et al.*, 1995; Hynes *et al.*, 1995a; Martí *et al.*, 1995a,b; Ruiz i Altaba *et al.*, 1995b; Tanabe *et al.*, 1995). Analysis of the response of induced cells to different concentrations of shh *in vitro* shows that high levels of shh can induce floor plate cells whereas low levels induce motor neurons but not floor plate cells (Roelink *et al.*, 1995). In midbrain explants, high concentrations can also induce dopaminergic neurons (Hynes *et al.*, 1995a).

Here, the early pattern of ventral cell types has been examined by analyzing the earliest expression of transcription factors that mark specific groups of neural cells. These are the LIM-homeodomain proteins Isl-1 and Isl-2 and the winged-helix protein HNF-3ß. Isl-1 and Isl-2 are expressed by post-mitotic embryonic motor neurons (Ericson *et al.*, 1992; Tsuchida *et al.*, 1994; Appel *et al.*, 1995; Pfaff *et al.*, 1996) and Isl-1, which is expressed before Isl-2, is required for motor neuron differentiation (Pfaff *et al.*, 1996). HNF-3ß is expressed by floor plate cells (Ang *et al.*, 1993; Bolce *et al.*, 1993; Monhagan *et al.*, 1993; Ruiz i Altaba *et al.*, 1993b, 1995a; Sasaki and Hogan, 1993; Strähle *et al.*, 1993) and transiently by adjacent ventricular zone cells (Ruiz i Altaba *et al.*, 1993b; Hynes *et al.*, 1995a; Martí *et al.*, 1995b). Widespread expression of HNF-3ß and that of its early functional homolog in frogs, Pintallavis, drives ectopic floor plate differentiation (Ruiz i Altaba *et al.*, 1993a,1995b; Roelink *et al.*, 1994; Sasaki and Hogan, 1994). The requirement for HNF-3ß in floor plate differentiation, however, has not yet been tested as it is first necessary for notochord development (Ang and Rossant, 1994; Weinstein *et al.*, 1994).

In this paper it is shown that at early stages in the spinal cord there are Isl-1/2$^+$ cells within the floor plate that coexpress HNF-3ß. In the hindbrain of older embryos cells that coexpress Isl-1/2 and HNF-3ß are also detected lateral to the ventral ventricular zone. These observations contrast with the regionalized and exclusive expression of these markers at late stages, when HNF-3ß marks the floor plate and Isl-1/2 marks the motor neurons pools in the ventral horns. The significance of this shift in the organization of cell types in the ventral neural tube is discussed in the context of pattern formation.

Results

HNF-3ß$^+$ cells in the ventral ventricular zone may comprise neuronal progenitors

The domain of HNF-3ß expression in the ventral neural tube is transiently larger than that of the floor plate extending into the

ventral ventricular zone. All cells in these regions, including mitotically active cells, express HNF-3ß (Ruiz i Altaba, *et al.*, 1993a, 1995b; Hynes *et al.*, 1995a; Martí *et al.*, 1995b and not shown) raising the possibility that HNF-3ß is expressed by ventral neuronal progenitor cells (Ruiz i Altaba *et al.*, 1993b). Consistent with this, HNF-3ß$^+$ cells in the ventral ventricular zone of the midbrain have been proposed to include progenitors of dopaminergic neurons (Hynes *et al.*, 1995a,b).

To investigate whether HNF-3ß$^+$ cells in the ventral ventricular zone also comprise motor neuron precursors, the expression patterns of HNF-3ß and Isl-1/2 were compared. At E12 the expression of Isl-1/2 in cells closest to the ventricular zone occurred in a domain adjacent to that of HNF-3ß in the hindbrain (Fig. 1B) and spinal cord (Fig. 1C). The juxtaposition of the domains of HNF-3ß and Isl-1/2 expression was clearest at hindbrain levels where the dorsal limits of the domains of HNF-3ß and Isl-1/2 were coincident whereas in the spinal cord there was a small gap in between these two cell populations. Thus, some HNF-3ß$^+$ ventricular cells in the hindbrain may be motor neuron precursors. The expression of HNF-3ß, however, is not an obligate step in the pathway of motor neuron differentiation. A diffusible motor neuron-inducing factor from the notochord and floor plate (Yamada *et al.*, 1993; Tanabe *et al.*, 1995), and purified shh, can induce Isl-1/2$^+$ cells in the absence of HNF-3ß expression at any time (Roelink *et al.*, 1995; Tanabe *et al.*, 1995).

In contrast with the situation in the hindbrain, in the anterior midbrain, HNF-3ß$^+$ cells were not adjacent to embryonic motor neurons expressing Isl-1/2 (Fig. 1D). Along the A-P axis of the neural tube, HNF-3ß$^+$ ventricular cells are likely to include progenitors of cell types other than motor neurons, such as dopaminergic neurons in the midbrain (Hynes *et al.*, 1995a,b). Similarly, along the D-V axis, HNF-3ß$^+$ ventricular cells may represent progenitors of different cell types. For example HNF-3ß$^+$ ventricular cells in the hindbrain could include progenitors of serotonergic neurons, a cell type found in between the floor plate and the motor neuron pools (see Yamada *et al.*, 1991), and in the spinal cord they could include oligodendrocyte progenitors (Pringle and Richardson, 1993; Yu *et al.*, 1994).

A limited number of ventral neural tube cells transiently coexpress HNF-3ß and Isl-1/2

E10-12 rat embryos

To determine whether single cells coexpressed HNF-3ß and Isl-1/2, double-labeled sections were examined by confocal microscopy. In the hindbrain of E12 rat embryos, cells expressing HNF-3ß or Isl-1/2 formed two distinct groups but there was cell mixing at the boundary between the two groups (Fig. 1E-G). Within each group there was expression at high and low levels (Figs. 1E,F, 2). The majority of cells expressing HNF-3ß at high levels were located closest to the ventricular zone and the majority of cells expressing Isl-1/2 at high levels were furthest from it. Most cells expressing either of these transcription factors at low levels were found intermixed in the border zone between the two populations (Fig. 2). Of all the labeled cells in three different hindbrain sections 49% expressed HNF-3ß at high levels and 14% at low levels. 24% expressed Isl-1/2 at high levels and 8% at low levels. In addition, 4% coexpressed both HNF-3ß and Isl-1/2 at low levels (n= 426; Fig. 2). Of all the cells expressing these factors at low levels,

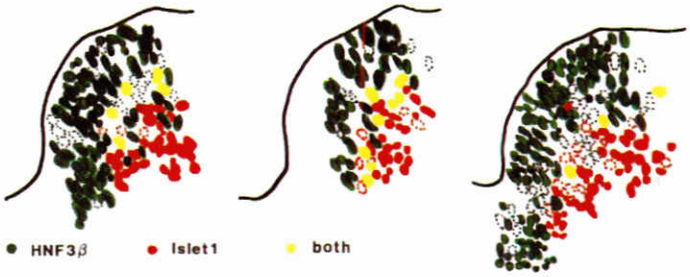


Fig. 2. Distribution and coexpression of HNF-3ß and Isl-1/2 cells in the rat ventral hindbrain. *The drawings show tracings of the contours of three unilateral confocal images of the ventral region of cross sections from E12 rat hindbrains showing the expression of HNF-3ß (in green), Isl-1/2 (in red) and the coexpression of both factors (in yellow). The data used to generate these drawings was taken from confocal images similar to that shown in Figure 1E. Colored areas represent labeled nuclei. Filled nuclei represent high expression of HNF-3ß or Isl-1/2. Unfilled nuclei with dashed borders represent lower expression of HNF-3ß or Isl-1/2. Yellow nuclei represent the coexpression of HNF-3ß and Isl-1/2 at low levels. Only the nuclei of cells expressing HNF-3ß or Isl-1/2 near the boundaries between the two populations have been traced. See text for further details.*

double-expressing cells represented 23% of all cells expressing HNF-3ß (n=78) and 33% of all cells expressing Isl-1/2 at low levels (n=54).

The analysis described above for E12 embryos raised the possibility that cells expressing only Isl-1/2 at high levels at this late stage could have coexpressed Isl-1/2 and HNF-3ß at earlier stages. Analysis of the earliest expression of Isl-1/2 in the ventral region of the caudal spinal cord of E10 rat embryos showed that the first Isl-1/2 positive cells were found scattered throughout the ventral neural tube (arrows in Fig. 1H, J and not shown). Moreover, cells expressing Isl-1/2 were often found at the ventral midline, within the floor plate area, coexpressing HNF-3ß (Fig. 1H-J).

Stage 15 chick embryos

A similar analysis of the position of the most caudal, and thus the most immature or most recently having become post-mitotic, Isl-1/2$^+$ cells in the spinal cord of stage 15 chick embryos showed also that some Isl-1/2$^+$ cells were found within the domain of expression of *HNF-3ß* (Fig. 3A,B). In addition, a limited degree of scattering was observed for the first cells expressing HNF-3ß in ventral regions of the posterior spinal cord of younger (stage 10) chick embryos (not shown; Ruiz i Altaba *et al.*, 1993b).

An analysis of the distribution of the most caudal Isl-1/2$^+$ cells of the spinal cord (in the prospective lower thoracic region) was also carried out in stage 15 chick embryos. The analysis was performed by scoring the position of expressing cells in cross sections by confocal microscopy as being within the HNF-3ß$^+$ floor plate or lateral to the floor plate (Table 1). About 90% of the most caudal Isl-1/2$^+$ cells in the spinal cord (n= 11) were found within the floor plate area with 1 or less than 1 Isl-1/2$^+$ cell per 10-12 µm section on average (n= 10; 15 sections counted; Fig. 4A,C).

In contrast, in more anterior (upper thoracic) regions, less than 1 Isl-1/2$^+$ cell was found within the floor plate on average per section (n= 4; 15 sections counted; Fig. 4C) representing ~7% of all positive cells (n= 57). Here, an average of 5-6 Isl-1/2$^+$ cells per section were found adjacent to the floor plate in the future basal plate area where the motor neuron pools will form (Table 1). This

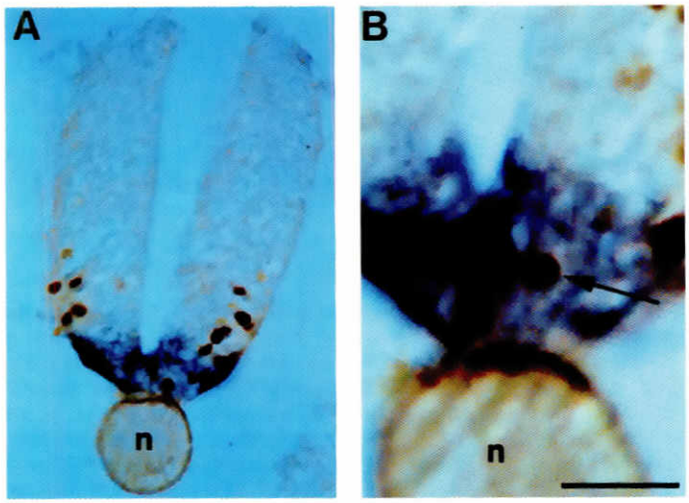


Fig. 3. Distribution of HNF-3ß mRNA and Isl-1/2 protein expression in the ventral neural tube of chick embryos. (A,B) *Nomarski micrographs of cross sections through the posterior spinal cord of stage 15 chick embryos showing the expression of HNF-3ß mRNA (in blue) in the floor plate and the expression of Isl-1/2 protein (in reddish brown) in the first differentiating motor neurons. Isl-1/2 cells are detected in regions lateral to the floor plate* **(A)** *as well as in the domain of HNF-3ß expression, including the ventral midline* **(A,B)** *adjacent to the notochord (n).* **(B)** *shows a high magnification of the ventral midline. In* **(A,B)** *dorsal side is up. Scale bar: A, 100 µm; B, 40 µm.*

suggests that the majority of Isl-1/2$^+$ cells do not derive from cells that were originally located within the floor plate.

Confocal analysis of the expression of HNF-3ß and Isl-1/2$^+$ cells in the caudal spinal cord showed that ~75% of Isl-1/2$^+$ cells found within the floor plate coexpressed HNF-3ß (n= 8; Fig. 4A-C). Isl-1/2$^+$ cells found lateral to the floor plate and distal from the ventricle never expressed HNF-3ß (n= 12; Fig. 4C).

Together, these observations point to two important conclusions: first, the earliest differentiating Isl-1/2$^+$ motor neurons are detected throughout a wide domain in the ventral neural tube, including the floor plate. Second, single ventral midline cells coexpress HNF-3ß and Isl-1/2.

Discussion

Early mixed distribution of ventral cell types

A prediction from *in vivo* transplantation (Yamada *et al.*, 1991) and *in vitro* explant (Martí *et al.*, 1995a; Roelink *et al.*, 1995) studies is that the differentiation of distinct ventral neural tube cell types would occur at defined positions from the ventral midline. The analysis of the position of early differentiating cells expressing Isl-1/2 and HNF-3ß in the caudal spinal cord presented here shows that Isl-1/2$^+$ cells display a wider distribution than expected. In contrast with the defined localization of Isl-1/2$^+$ motor neurons to the ventral horns at later stages (Ericson *et al.*, 1992; Pfaff *et al.*, 1996), a number of Isl-1/2$^+$ cells are found intermingled with HNF-3ß$^+$ cells both in the early caudal spinal cord and in the later hindbrain. In the early spinal cord, this mixing occurs within the floor plate area whereas later in the hindbrain it occurs in the border region between the ventral ventricular zone and the motor pools.

Early mixing of cell types, as observed for HNF-3ß$^+$ and Isl1/2$^+$ cells, may be a general phenomenon. For example, cells expressing the homeobox gene *Nkx2.2* in the ventral neural tube are first found at the ventral midline becoming restricted to a region flanking the floor plate at later stages (e.g. Barth and Wilson, 1995; Pfaff *et al.*, 1996). The mixing of cells expressing Isl-1/2 and HNF-3ß was also observed within an ectopically induced floor plate by a notochord graft at a time when the endogenous floor plate and motor neuron pools already displayed separate populations (Ruiz i Altaba *et al.*, 1995a). The delay in the resolution of floor plate and motor neuron cell groups in the ectopic versus the endogenous ventral regions may be a consequence of the delayed induction by the notochord graft in comparison with the endogenous notochord. Similarly, dorsal neurons appear to be mixed with ectopic floor plate cells induced by a dorsal notochord graft at early but not late stages following grafting (Artinger and Bronner-Fraser, 1992).

The mechanisms by which the coexpression of Isl-1/2 and HNF-3ß and the mixed distribution of cell types occurs is not known. Signals from the notochord could initially induce distinct cell types in a mixed fashion within a ventral domain. In this case, neighboring cells could respond differently to identical concentrations of inducer. Alternatively, cells of different phenotypes induced at distinct D-V positions may mix shortly after being induced. For example, Isl-1/2$^+$/HNF-3ß$^-$ motor neurons induced lateral to the floor plate could move to the ventral midline. However, this would appear to be unlikely since some cells in the floor plate coexpress HNF-3ß and Isl-1/2.

Cell lineage analyses are consistent with either possibility. Retroviral marking of single progenitor cells in the embryonic spinal cord shows that these can give rise to different kinds of neurons and glia located at varying positions within the neural tube, characteristic of the cell type in question, such as motor neurons in the ventral horns (Leber *et al.*, 1990). This suggests that dispersion of cell types originally induced at defined positions is indeed possible. In addition, dye marking studies in the embryonic hindbrain have shown that different types of neurons can be found intermixed, occupying the same position within the neural tube in relation to the midline (Lumsden *et al.*, 1994) raising the possibility of a mixed induction of progenitor cells or a mixing of differentiated cell types previously induced at defined positions.

The resolution of the final D-V pattern of the neural tube could involve three possible mechanisms. First, extinction of the expression of inappropriate markers. Because Isl-1 and Isl-2 are expressed in post-mitotic neurons, it is unlikely that Isl-1/2$^+$ cells within the floor

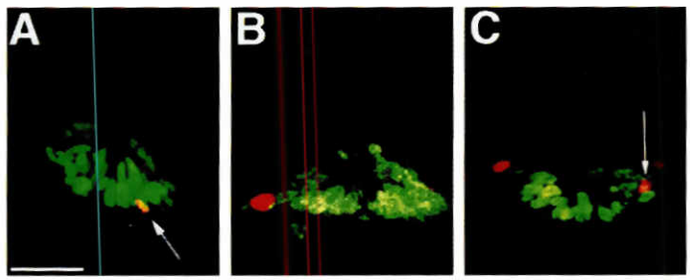
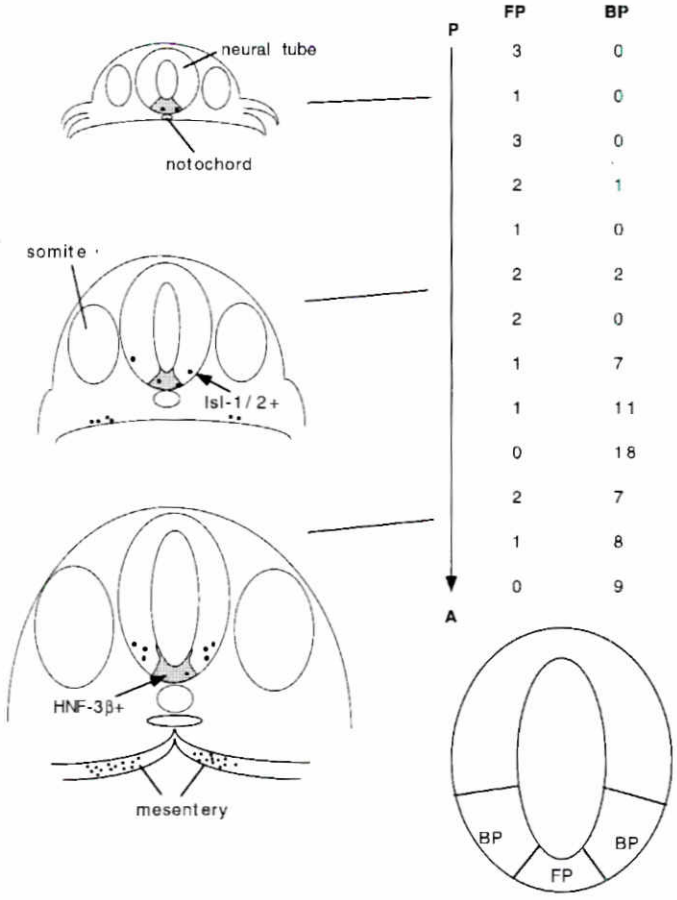


Fig. 4. Localization of the most caudal Isl-1/2$^+$ cells within the chick ventral spinal cord. (A-C) *Cross sections of the spinal cord of stage 15 chick embryos showing the ventral area. All three panels show expression of HNF-3ß protein (in green) and Isl-1/2 protein (in red). Coexpressing cells* **(A,C)** *are marked by arrows (yellow). Panels* **A** *and* **B** *are more caudal than panel* **C**. *Note the presence of two Isl-1/2$^+$/HNF-3ß$^-$ cells in panel* **C** *located in the basal plate flanking the floor plate. Scale bar, 25 µm*

TABLE 1

QUANTITATION OF THE DISTRIBUTION OF Isl-1/2$^+$ CELLS WITHIN THE CHICK CAUDAL SPINAL CORD

Isl-1/2 $^+$ cells in the caudal spinal cord of st.15 chick embryos



	FP	BP
P		
	3	0
	1	0
	3	0
	2	1
	1	0
	2	2
	2	0
	1	7
	1	11
	0	18
	2	7
	1	8
	0	9
A		

The position of Isl-1/2$^+$ cells (black dots) was determined in labeled cross sections. The diagrams on the left column show three representative sections of the axis of a stage 15 chick embryo from posterior (upper) to more anterior (lower) regions. The presence of Isl-1/2$^+$ cells in the mesentery is shown as guide for anteroposterior (A-P) position. The expression of HNF-3ß in floor plate cells and in the adjacent ventral ventricular zone is indicated by shading. Each row on the right shows the number of positive cells in three sections taken at ~120 µm intervals. The first row includes the first section (~10 µm) that contained a labeled cell. The position of Isl1/2+ cells along the D-V axis of the neural tube (diagram at the bottom right) is shown as divided into the floor plate (FP) and the basal plate (BP).

plate change fate and become floor plate cells. Second, selective death of cells located in inappropriate areas. In chick embryos programmed cell death has been shown to occur in the floor plate at times (stage~18; Homma *et al.*, 1994) subsequent to the period when mixed cell types are detected (stage~15). Third, cell sorting involving migration. However, the movement of young motor neurons or their precursors along the D-V axis has not been demonstrated.

Induction and pattern formation

The direct establishment of cellular pattern by an inducing molecule is thought to depend on its ability to act at a distance and to specify distinct fates at different concentrations. The best evidence for the patterning ability of inducers acting as morphogens in vertebrate development derives from the analysis of gene expression induced by activin during mesodermal induction in frog animal cap explants (Smith, 1987; Gurdon *et al.*, 1994) and by shh during neural differentiation in neural plate explants. *In vitro*, high levels of shh can induce the differentiation of floor plate cells and motor neurons whereas low levels can induce motor neurons but not floor plate cells (Roelink *et al.*, 1995). Similarly, different activin concentrations have been shown to induce different mesodermal cell types and positional markers (Smith, 1987; Green and Smith, 1990; Gurdon *et al.*, 1994). However, Shh protein has only been detected *in vivo* in cells that synthesize it (Martí *et al.*, 1995b; Roelink *et al.*, 1995) and sharp threshold responses to different concentrations of activin are not present at the time of mesoderm induction (Green *et al.*, 1994; Wilson and Melton, 1994).

In normal development, if a notochord-derived signal shh induces ventral neural tube pattern in a concentration-dependent manner, two simple scenarios could be proposed for the early distribution of ventral cell types. First, if inductive signals from the notochord were to act rapidly it could be predicted that the onset of the differentiation of distinct ventral cell types requiring different concentrations of shh would occur in a region-specific manner. Floor plate cells would first appear at the midline and motor neurons would first develop exclusively in more lateral regions. Alternatively if a high level of inducer was only achieved after a period of time it could be predicted that all cells that respond to a high concentration would respond first to a low concentration. In this case, the early differentiation of ventral cell types induced by low concentrations of shh would occur first closest to the ventral midline and all cells induced by high levels of inducer would transiently express markers of fates induce by lower concentrations.

Two observations derived from the analysis of the distribution of early cell types in the ventral neural tube presented here suggest a more complex scenario. First, a fraction of cells within the floor plate coexpress markers of two distinct cell types: HNF-3ß and Isl-1/2. Second, distinct cell types do not always appear to develop at defined position from the midline since a number of HNF-3ß$^+$ and Isl-1/2$^+$ cells were initially mixed within the floor plate area. In addition, the expression of Isl-1/2, a marker induced by low levels of shh, was not detected in all cells that express HNF-3ß, a marker induced by high levels of shh, since not all floor plate cells transiently express Isl-1/2. Because Isl-1/2 expression is a relatively late marker of motor neurons, occurring in post-mitotic cells, the possibility remains that HNF-3ß$^+$ cells in the floor plate transiently express earlier, as yet unidentified, motor neuron fate markers. A result possibly consistent with this is that when the ventral midline is isolated from the caudal region of stage 10 chick embryos, before it is induced to become floor plate, and cultured *in vitro*, the differentiation of Isl-1/2$^+$ cells is detected (Yamada *et al.*, 1993). However, this result, is also consistent with a mixed induction of cell types and argues against the lateral origin of Isl-1/2$^+$ cells found at the ventral midline.

Independent of the mechanisms by which coexpression of markers and mixing of cell types occurs, these findings suggest that cellular interactions subsequent to induction by shh are involved in resolving the initial mixed distribution of cell types into separate stereotyped domains. Induction and pattern formation in the ventral neural tube may, therefore, not be the same process.

Similarly, the generation of distinct responses in mesodermal cells induced by different activin concentrations requires secondary interactions among the induced cells (Green *et al.*, 1994; Wilson and Melton, 1994).

Materials and Methods

Embryos

Rat embryos (Hilltop lab animals) were obtained at the desired embryonic day (E) of gestation with E0.5 counted as the morning after conception. E10 embryos had 10-12 somites with 5-6 somites forming every 12 h. Chick embryos (Spafas) were incubated in the laboratory and dissected at the desired stage (Hamburger and Hamilton, 1951). Rat and chick embryos were dissected in L-15 air media (Specialty Media) on ice.

Immunocytochemistry in sections

E10-E11 rat embryos were fixed in MEMFA (3.7% formaldehyde, 1 mM EGTA, 2 mM $MgCl_2$, 0.1 M MOPS pH 7.4) for 15-20 min at room temperature. Older embryos were fixed in fresh 4% paraformaldehyde in 0.2 M phosphate buffer pH 8 on ice for 1 h. After fixation the embryos were washed 1-2 times in PBS at room temperature before immersion in 15% sucrose, 0.2 M phosphate buffer at 4°C overnight. Embryos were then embedded in Tissue-Tek and sectioned in a cryostat. 10 μm sections were routinely used for immunocytochemistry. Sections were placed on Superfrost slides (Fisher) and air-dried. After washing with PBS plus 0.1% Triton X-100 (PBT) once, the sections were incubated with primary antibodies in PBT plus 10% heat-inactivated goat serum (HINGS) overnight at 4°C. After one wash with PBT at room temperature, sections were incubated with fluorochrome-coupled secondary antibodies (see below) in PBT plus 10% HINGS for 40 min at room temperature. Sections were then washed once in PBT and coverslipped with 50% glycerol, 0.1 M carbonate buffer pH 9.0 with p-phenylenediamine to retard bleaching. Labeled sections were kept covered at 4°C. For labeling of nuclei, sections were stained with Hoechst dye #33258 (0.01 mg/ml in PBT) for 5-10 min at room temperature and then washed once in PBT before coverslipping.

Sections were viewed with an Axiophot (Zeiss) microscope under fluorescent light or with an MRC 600 confocal microscope (BioRad) producing 2 μm optical sections. Pictures were taken with Kodachrome 100 ASA slide film.

Whole-mount antibody labeling

Whole mount-labeling was performed essentially as described by Dent *et al.* (1989) and Patel *et al.* (1989). Rat embryos younger than E11 were processed whole. Older embryos were dissected prior to fixation. For HNF-3ß and Isl-1/2 labeling, embryos were fixed in MEMFA for 20 min at room temperature. After fixation, embryos were dehydrated in methanol and bleached in methanol containing 10% hydrogen peroxide at 4°C overnight under bright fluorescent light. Double labeling was performed with sequential antibody incubations using peroxidase-coupled secondary antibodies. Embryos were rehydrated, washed in PBT and blocked in PBT plus 10% HINGS. Incubation with the first primary antibody was in PBT plus 10% HINGS overnight at 4°C. After 4-6 30 min washes in PBT, embryos were incubated in secondary antibodies coupled to horseradish peroxidase (see below) in PBT plus 10% HINGS at room temperature for 2 h and washed for another 2-4 h before starting the peroxidase reaction. The first peroxidase reaction was performed with 0.5 mg/ml diaminobenzidine and 0.003% hydrogen peroxide in the presence of a mixture of niquel and cobalt (0.003% $Ni(NH_4)_2SO_4$, 0.003% $CoCl_2$) to give a gray-black reaction product. Embryos were extensively washed in PBT and blocked in PBT plus 10% HINGS before proceeding to the second antibody round. Labeling with the second primary antibody and secondary antibodies were as described above. The second peroxidase reaction was performed in the absence of metals giving a red-brown precipitate product. Embryos were washed in PBT, dehydrated in methanol and viewed under Nomarski optics after clearing in benzyl alcohol/benzyl benzoate (1/2). Pictures were taken with Kodak 160 ASA tungsten-balanced slide film.

Antibodies

Polyclonal antibodies against the rat HNF-3ß protein were generated in mice. Mice were immunized intraperitoneally with a 30 amino acid peptide corresponding to the N-terminus of the protein, coupled by a C-terminal cysteine to activated keyhole lympet hemocyanin (Pierce). Tail bleeds of these mice provided small amounts of high-titer (IgG) polyclonal antisera. The polyclonal antisera was used at 1/5000.

A monoclonal antibody (mAb 4C7) against chick HNF-3ß protein (CP7; Ruiz i Altaba *et al.*, 1995a) was obtained from a mouse immunized with purified recombinant CP7 protein made *in vitro* using the His-Tag system (Quiagen). The injected protein derived from the region of the CP7 cDNA from bp 38 to bp 433 encoding a portion of the chick HNF-3ß protein from aa 37 to aa 171 which includes the N'terminal region and the first 16 aa of the 110 aa DNA-binding domain. This antibody was used as hybridoma supernatant at 1/1 dilution. MAb 4C7 crossreacts with mammalian HNF-3ß (not shown).

Rabbit antisera against the rat Isl-1/2 protein was used at 1/10,000 (Thor *et al.*, 1991; Ericson *et al.*, 1992) or at 1/1500 (K5; S.B. Morton *et al.*, unpublished; Tsuchida *et al.*, 1994). These antisera cross-react with chick Isl-1/2 (Ericson *et al.*, 1992). Mouse monoclonal antibody 4D5 (IgG; Tsuchida *et al.*, 1994; S.B. Morton *et al.*, unpublished) against rat Isl-1/2 was used at 1/10 to 1/100 as hybridoma culture supernatant. Both the rabbit polyclonal (K5) and the mouse monoclonal (4D5) antibodies recognize the Isl-1 protein as well as the highly related Isl-2 protein. However, because Isl-2 is expressed after Isl-1 and initially all Isl-2$^+$ cells also express Isl-1 protein (Tsuchida *et al.*, 1994; Pfaff *et al.*, 1996) we consider reactivity with K5 and 4D5 in early motor neurons a sign of Isl-1 expression.

Secondary antibodies were goat anti-rabbit or anti-mouse coupled to fluorescein, rhodamine, Texas Red (Boehringer Mannheim, TAGO or Molecular Probes), or horseradish peroxidase Boehringer Mannheim. When available, Fab' fragments were used. Secondary antibodies were used at 1/100-1/400.

In situ hybridization

Whole-mount *in situ* hybridization was performed essentially as described by Harland (1991) with minor modifications. Rat or chick embryos were dissected and fixed in MEMFA for 2 h at room temperature. Digoxygenin-labeled single stranded RNA probes were not hydrolyzed and the embryos were prehybridized for 10 min. Hybridization was visualized by labeling with secondary anti-digoxygenin antibodies coupled to alkaline phosphatase and reacted with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate to give a blue precipitate.

A digoxygenin-labeled antisense single stranded RNA probe for rat *HNF-3ß* were generated by transcribing the RP5 cDNA clone (Ruiz i Altaba *et al.*, 1993a) with SP6 RNA polymerase in the presence of digoxygenin-UTP after digestion with EcoRI. A digoxygenin-labeled antisense RNA probe for chick *HNF-3ß* was generated by transcribing the CP7 cDNA clone (Ruiz i Altaba *et al.*, 1995a) with SP6 RNA polymerase in the presence of digoxygenin-UTP after digestion with EcoRI. In both cases, a trace of ^{32}P-UPT was added to calculate incorporation.

After *in situ* hybridization, embryos were dehydrated through a methanol and xylene series and embedded in paraplast. 10-15 μm sections were cut in a microtome. Alternatively, after *in situ* hybridization and before sectioning in wax, the embryos were washed in PBT extensively after the alkaline phosphatase reaction, blocked in PBT plus 10% HINGS and labeled in whole mount with primary antibodies as described above. The epitopes recognized by the antibodies against Isl-1/2 but not those recognized by the antibodies against HNF-3ß survived the *in situ* procedure. Sections were dried, dewaxed in xylene, mounted with Permount (Fisher) and viewed under Normarski optics with an Axiophot (Zeiss) microscope.

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