Progenitor Dispersal and the Origin of Early Neuronal Phenotypes in the Chick Embryo Spinal Cord

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Using DiI and fluorescent dextrans, we have created fate maps of the neural plate and early neural tube describing the extent of progenitor cell dispersal and the spatial origin of morphologically distinct neuronal cell types along the dorsoventral axis of the developing chick spinal cord. Nonuniform dispersal and mixing of progenitors occur within the early neuroepithelium, with the degree of dispersal being determined by the initial position of the cells along the mediolateral axis of the neural plate. Dispersal is greatest in the midregions of the ventricular epithelium and decreases toward the dorsal and ventral midlines. Phenotypically diverse classes of neurons are born at specific dorsoventral locations in the neural tube. Motor neurons are the most ventral cell type generated followed, at progressively more dorsal positions, by distinct classes of interneurons. Several genes show dorsoventrally restricted patterns of expression within the neural tube and the fate maps were used to investigate the relationship between one of these genes, Pax3, and progenitor cell dispersal and fate. The results indicate that the dorsoventral pattern of Pax3 expression is not maintained by restrictions to cell mixing and are consistent with a role for this transcription factor in specifying the identity of neurons with contralateral descending axons.

INTRODUCTION

The patterning and morphogenetic events underlying the development of the spinal cord are currently the focus of intense investigation. Considerable progress has been made in identifying the mechanisms and molecules underlying the generation of cell diversity in this region (see Tanabe and Jessell, 1996, for review), yet, with few exceptions, there is surprisingly little detailed information about the early patterns of behavior of the undifferentiated progenitor cells on which these signals act. Moreover, although fate maps relating to broad regions of the neural plate have been produced (Schoenwolf et al., 1989; Schoenwolf and Sheard, 1990; Schoenwolf, 1992; Woo and Fraser, 1995; Catala et al., 1996), maps of the neural plate and early neural tube revealing the origin of the different neuronal types are not available. By the time their phenotype is recognizable, many newborn neurons can have migrated considerable distances away from their place of birth (e.g., Moody and Heaton, 1983; Tan and Le Douarin, 1991; Markham and Vaughn, 1991; Simon and Lumsden, 1993; Walsh and Cepko, 1993; Leber and Sanes, 1995; O’Rourke et al., 1995, 1997; Phelps and Vaughn, 1995; Clarke et al., 1998); thus, the position of the neurons themselves cannot be used as an indicator of their site of origin. A reliable fate map of the early neuroepithelium, combined with a description of progenitor cell movements during this time, would be a significant advance in this area and aid our interpretation of the factors underlying the early patterning of the nervous system (Clarke et al., 1998).

The spinal cord, like other regions of the central nervous system, develops from the initially morphologically homogeneous epithelium of the neural tube and is patterned along its dorsoventral axis by local environmental signals. Using classical embryological techniques and tissue culture experiments, the notochord and floor plate have been demonstrated to be the source of a diffusible ventralizing signal,
most likely Sonic hedgehog protein, which at different thresholds induces the formation of motor neurons and two molecularly distinct classes of ventral interneurons (Yamada et al., 1991, 1993; Placzek et al., 1993; Roelink et al., 1994, 1995; Marti et al., 1995; Chiang et al., 1996; Ericson et al., 1996, 1997). Similarly, dorsal cell fates, such as neural crest and dorsal interneurons, appear to be conferred by short-range signals emanating from the overlying surface ectoderm and dorsal midline of the neural tube (Dickinson et al., 1995; Liem et al., 1995, 1997; Selleck and Bronner-Fraser, 1995).

Several members of the Pax gene family are initially expressed throughout the entire neural plate but, in response to notochord-derived signals, rapidly become restricted to specific domains along the dorsoventral axis of the developing spinal cord. Transcripts of Pax3 and Pax7 are limited to the dorsal half of the neuroepithelium, whereas the expression of Pax6 is confined to the midlateral region.
TABLE 1
Diameter of Labeled Region Immediately Following Application of DiI or DiA

<table>
<thead>
<tr>
<th>Dorsolateral position</th>
<th>n</th>
<th>Mean (± SEM) diameter (μm)</th>
<th>Range (μm)</th>
</tr>
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<tbody>
<tr>
<td>Ventral</td>
<td>4</td>
<td>12 ± 2</td>
<td>7–18</td>
</tr>
<tr>
<td>Ventrolateral</td>
<td>12</td>
<td>12 ± 1</td>
<td>7–19</td>
</tr>
<tr>
<td>Lateral</td>
<td>13</td>
<td>13 ± 1</td>
<td>8–19</td>
</tr>
<tr>
<td>Dorsal</td>
<td>10</td>
<td>13 ± 1</td>
<td>9–16</td>
</tr>
<tr>
<td>All regions</td>
<td>49</td>
<td>13 ± 1</td>
<td>7–21</td>
</tr>
</tbody>
</table>

Note. n, number of measurements.

of the neural tube (Goulding et al., 1993; Liem et al., 1995; Ericson et al., 1997). The restricted expression of these genes proceeds the onset of cellular differentiation, suggesting that these factors play an important role in specifying regional identity. Indeed, recent evidence indicates that Pax6 may be involved in controlling the generation of distinct ventral cell types (Burrill et al., 1997; Ericson et al., 1997). The relationship between cell behavior and the establishment and maintenance of these expression patterns is not known. In particular, it remains unclear whether the sharp dorsoventral expression boundaries of these genes are maintained by limitations to progenitor cell movement or modulation of gene transcription.

Using the lipophilic dyes DiA and DiI, we have followed progenitor cell dispersal and mixing during the first 48 h of spinal cord neurogenesis in the chick embryo and created fate maps of the early neuroepithelium detailing the formation of the neural tube and the spatial origin of different classes of neurons. The relationship between these parameters and the Pax3 expression domain also was investigated. The early development of the dorsoventral axis of the neural tube involves extensive cell mixing, both within and between gene expression domains, and nonuniform expansion of the ventricular epithelium. Morphologically distinct neuronal cell types are born in specific dorsoventral regions of the neural tube which, in the case of one class of interneurons, includes the Pax3 domain.

A preliminary report has appeared in abstract form (Erskine and Clarke, 1996).

MATERIALS AND METHODS

Chick eggs were incubated at 37°C for 32-56 h until they reached Hamburger and Hamilton stages 8 to 15 (4–24 somites; Hamburger and Hamilton, 1951) and windowed and a small hole was made in the vitelline membrane over the area of interest. To aid visualization, a dilute solution of technical drawing ink (4% in Howard’s Ringer; 123 mM NaCl, 2 mM CaCl2, 5 M KCl, pH 7.2) was injected under the blastoderm.

Cell Labeling with DiI or DiA

Using iontophoresis, small groups of neuroepithelial cells in the presumptive spinal cord of stage 8–10 embryos were labeled in ovo with the lipophilic dye DiI [DiIC18(3); Molecular Probes D-282] or DiA (4-Di-16-Asp; Molecular Probes D-3883). Microelectrodes with a tip diameter of approximately 1 μm were tip-filled with either dye (3 mg ml−1 in dimethyl formamide), back filled with 1 mM LiCl, and inserted into an electrode holder connected to the positive pole of a 9-V battery. Using a micromanipulator, the electrode was carefully moved into the required position in the posterior neuropore and placed in contact with the tissue and a few cells were labeled by completing the circuit with a silver electrode placed in the egg and connected to the negative pole of the battery. Approximately 2–3 s was sufficient to label 5–10 cells. Successful injections were checked on an upright epifluorescent microscope (Nikon) fitted with an extralong working distance 20× objective and imaged using a cooled CCD camera (Photonic Science CoolView) connected to Biovision software on a Power Macintosh. Initial cluster size was measured from these images.

To label progenitor cells in older embryos (stages 13–15) in which the neural tube had already closed, a small split was made in the roof plate at the level of the 12th somite and the electrode was manipulated into position on the ventricular surface through the gap.

Dextran Labeling

Intracellular electrophoresis of lysinated rhodamine dextran (LRD) has been widely used to label single progenitor cells within the developing nervous system (e.g., Fraser et al., 1990). In this analysis, by making repeated injections at the same position within the neural plate of stage 8–10 embryos, two to four neighboring progenitors were marked. Thin-walled aluminosilicate microelectrodes (A-M Systems) were tip-filled with LRD (100 mg ml−1; Molecular Probes) and back-filled with 1 mM KCl. The electrode was positioned on the neural plate and a small group of neighboring cells was labeled by repeatedly ringing the tip two to three times with the negative-capacitance control. The number of cells injected and the quality of the label were determined using an upright epifluorescent microscope and the embryos were imaged using a cooled CCD camera as above.

Analysis

Embryos were fixed 24–48 h after labeling. The roof plate of the hindbrain and spinal cord was split and the embryo was placed in 3.5% paraformaldehyde in PBS overnight. Some embryos were examined whole to enable the anterioposterior position of the labeled cells to be determined but mostly the spinal cord was dissected free of surrounding tissues and prepared as a flat whole mount, ventricular side up, in 90% glycerol, 2.5% DABCO (Sigma) in PBS. The position of the labeled cells in these specimens was drawn and measured on a standard epifluorescent microscope (Nikon) with the aid of an eyepiece graticule. To identify the migrating neural crest the somites were dissected and mounted flat in 90% glycerol.

For specimens labeled at stages 13–15, which frequently contained brightly labeled neurons, a Leica confocal microscope was used to obtain two optical sections of each polyclone, one close to the ventricular surface to visualize the progenitors and one at the pial surface to record the neurons. A transmitted light image was made in order to relate the position of the progenitors to morpho-
logical landmarks such as the floor plate and enable accurate measurement of their location.

**Pax-3 Expression**

Whole-mount in situ hybridization was performed as described by Nieto et al. (1996). Briefly, embryos were dissected in PBS and fixed overnight in 4% paraformaldehyde. Embryos were dehydrated in a methanol series and subsequently rehydrated. After a brief proteinase K treatment (1 min per stage with 20 μg ml⁻¹ proteinase K) embryos were refixed and prehybridized for a minimum of 2 h at 65°C. Chick Pax3 clone corresponding to nucleotides 468–1113 was a gift of Martin Goulding (Salk Institute, CA). DNA was transcribed by T3 RNA polymerase using digoxigenin. The probe was used at a concentration of 50 ng ml⁻¹ at 65°C for a minimum of 12 h and then detected as described by Nieto et al. (1996). After staining, either the spinal cord was removed and flat mounted as above or the whole embryo was embedded in 7.5% gelatin in PBS, frozen at −70°C, and sectioned transversely at 30 μm on a cryostat.

**RESULTS**

**Progenitor Cell Dispersal during the First 48 h of Spinal Cord Neurogenesis**

Small groups of neighboring neuroepithelial cells were labeled with DiI or DiA in one of five domains (see Fig. 1A) along the mediolateral axis (future dorsoventral axis) of the folding cord neural plate (Figs. 1B and 1C) and the position and dispersal of the labeled descendants were determined 48 h later (Figs. 1D–1F). Initial cluster size was similar in each region, measuring approximately 13 μm in diameter and containing between 5 and 10 cells (Table 1).

After 48 h, when the embryos had reached stages 17–20, all labeled cells were located in the cervical and brachial region of the cord (level 7–16 somites) and consisted of both neurons, identified by their axon and location close to the pial surface, and undifferentiated progenitors, recognized by their lack of processes and columnar morphology spanning the depth of the neuroepithelium. Only the position of these latter cells was analyzed. Each progenitor cell cluster was drawn onto a standardized spinal cord outline (Fig. 2) and, using an eyepiece graticule, the distance was measured on a microscope from the midline to the most medially and most laterally labeled progenitor. To allow comparison between specimens of different size, the distance between

**FIG. 2.** Standardized diagrams of flat-mounted stage 20 spinal cords showing examples of the position, size, and shape of progenitor cell clusters derived from application of DiI to the ventral (A), ventrolateral (B), lateral (C), dorsolateral (D), or dorsal (E) domains of the neural plate. For clarity, any neurons present in the clusters have been omitted. The dotted lines indicate the edges of the roof plate. The left side of each diagram shows labeled cells on the ipsilateral side of the spinal cord, the right side those located contralateral to the site of dye application. Bar, 100 μm.
the midline and the dorsal edge of the neuroepithelium also was measured and the results were expressed as a percentage of this parameter.

On the side of the cord which was initially labeled, the progenitor cells and their descendants were found grouped together within a single, irregularly shaped cluster. Few, if any, outlying cells were observed (Figs. 1D–1F and 2). Most had dispersed to a similar extent along both the antero-posterior and dorsoventral axes. The exception to this was clusters which contained either floor plate or roof plate cells; cells in these regions spread almost exclusively in an anteroposterior direction (Figs. 1 and 2). In several cases, labeled cells were also found on the side of the cord contralateral to the original label (e.g., Fig. 1D). These cells will be discussed separately later.

**Relationship between Initial Labeling Position and Final Cluster Position and Spread**

After 48 h, the position of each ipsilateral cell cluster along the dorsoventral axis of the neural tube was related to the domain of the neural plate in which it was labeled (Figs. 1D–1F, 2, 3A, and B). No instances were found in which the labeled cells had, either randomly or in a coordinated manner, changed their relative positions within the neuroepithelium. As a result, floor plate cells were only found in clusters originating from the ventral region, roof plate cells from the lateral, dorsolateral, and dorsal regions, and neural crest cells from the dorsolateral and dorsal regions of the neural plate (Fig. 3A). However, there was considerable mixing between the descendants of cells initially located in different domains of the neural plate. Substantial overlap and mixing of progenitors derived from both adjacent and nonadjacent regions occurred (Figs. 3A and 3B).

The extent of dorsoventral expansion of the labeled clusters was nonuniform and strongly dependent on their site of origin and, thus, final position within the neuroepithelium (Figs. 3C and 3D). Dispersal was greatest in clusters located midway along the dorsoventral axis and decreased toward the dorsal and ventral midlines (Fig. 3D). Clusters derived from progenitors labeled in the lateral region of the neural plate spread 62 ± 4% of the width of the neuroepithelium (159 ± 12 μm; n = 9) compared to 32 ± 7% (93 ± 18 μm; n = 8) for clusters originating in the ventral region and 37 ± 5% (92 ± 16 μm; n = 9) for those in the dorsal region (Fig. 3C).

**Comparison of Progenitor Cell Behavior during Different Periods of Spinal Cord Neurogenesis**

Clusters labeled at stages 7–9 (see Fig. 1A) and examined 24 h later (stages 13–15) occupied the same relative positions along the dorsoventral axis and showed the same position-dependent pattern of expansion as those analyzed at stages 17–20 after 48 h (see above; Fig. 3B). By contrast, the dorsoventral expansion of clusters labeled at later stages (stages 14–15; Fig. 5A) and examined 24 h later (stages 17–20) was much more uniform and less dependent on their position within the neural tube (Fig. 5). Mean cluster size was also significantly smaller, cells spreading only 18 ± 1% (62 ± 4 μm; n = 47) along the dorsoventral axis compared to 47 ± 6% (92 ± 5 μm; n = 31; P < 0.001; Student’s t test) in the previous 24 h. Thus, widespread progenitor cell dispersal and mixing only occur during a very short period of neural development. A fate map summarizing these results and illustrating the degree of progenitor cell dispersal and mixing which occurs at different developmental stages is shown in Fig. 6.

**Origin of the Fluorescent Cells Located Contralateral to the Original Label**

In a significant number of embryos (61%; 47/77), analyzed 24–48 h after application of DiI to the neural plate, labeled cells were also found on the contralateral side of the spinal cord (Figs. 1D and 2–4). These cells occupied the same antero-posterior and dorsoventral position as those on the ipsilateral side, the two clusters usually separated by regions of unlabeled cells. At least two explanations can account for this result. First, a subpopulation of progenitor cells could be migrating to occupy specific locations on the contralateral side of the cord. Alternatively, the labeling of contralateral cells could be an artifact and the result of dye transfer across the lumen of the neural tube. During develop-
Development, the lumen of the spinal cord temporarily becomes totally occluded due to the close midline apposition of its lateral walls (e.g., Desmond and Schoenwolf, 1985) and, in whole embryos examined 12 h after labeling, labeled cells on the contralateral side of the spinal cord were seen frequently in very close contact with brightly labeled ipsilateral cells (Fig. 4C). Thus, although spread of DiI/DiA between cell membranes is not considered to be a serious problem (see Honig and Hume, 1989), it is possible that due to this close cell–cell contact some transcellular dye transfer could have occurred.

To determine the potential origin of the contralaterally labeled cells, the dispersal of progenitors labeled by intracellular microinjection of the cell autonomous marker LRD was determined and compared with the patterns of cell dispersal observed using DiI. Unlike DiI, LRD is contained in the cytoplasm rather than the cell membrane and, since it is too large to pass through gap junctions, cannot, under any circumstances, be transferred to neighboring cells.

LRD was injected into two to four adjacent neural plate cells and the position and dispersal of the descendant progenitors were determined 24 h later as above. This time point was chosen to minimize the likelihood of the dye being diluted to undetectable levels. Due to the significant

**FIG. 4.** (A, B) Position and spread of progenitor cell populations 24 h after labeling with either DiI (open symbols) or LRD (filled symbols). (A) Position of the labeled clusters along the dorsoventral axis of the neural tube. DiI- and LRD-injected cells initially located in the same domain of the neural plate have generated descendants located at the same relative position within the neural tube. Small clusters of cells located contralateral to the original label (plotted on negative axis) were found frequently in DiI- but not dextran-labeled spinal cords. (B) Dorsoventral dispersal of DiI- or LRD-labeled clusters. Using either dye, the extent of cluster expansion was found to be nonuniform and dependent on their position along the dorsoventral axis of the neural tube. (C) Whole embryo (stage 11; 13 somites), viewed dorsal side up, 12 h after application of DiI to the dorsal domain of the neural plate. Note the close proximity of the ipsilaterally and contralaterally labeled cells. DM, dorsal midline; s, somites; Bar, 100 μm.
difference in the size of the region initially labeled with each dye (8 ± 1 μm; n = 47 with LRD; 13 ± 1 μm; n = 49 with DIl; P < 0.001), final cluster size was significantly smaller in LRD- than DIl-labeled spinal cords (Fig. 4). Despite this, the overall pattern of cell behavior on the ipsilateral side of the neural tube was the same. After 24 h, clusters labeled with either dye occupied the same relative position within the neuroepithelium and showed the same nonuniform dorsoventral expansion pattern (Fig. 4). However, with the exception of ventral injections, which could possibly have initially labeled cells on both sides of the midline, in only 1 of 29 LRD-injected embryos (3%) was a small group of contralaterally labeled cells detected (Fig. 4A). By contrast, contralateral cells were observed in over 3
of 4 (76%; 19/25) Dil-labeled embryos examined at this time (Figs. 4A and 4B). Since LRD is cell autonomous, this demonstrates that some spinal cord progenitors can translocate to and integrate into the contralateral side of the neural tube. However, this must be a very rare event. Indeed, when the fate of large numbers of spinal cord progenitors was tracked using the chick–quail chimera technique, no cells were found which had migrated to the contralateral side of the cord (Catala et al., 1996). Thus, the frequency with which Dil-labeled cells were found on the side of the cord contralateral to the initial label strongly suggests that the labeling of these cells is an artifact and the result of secondary transcellular dye transfer between the tightly opposed walls of the neural tube. In support of this, in embryos examined prior to the onset of neural tube occlusion (stage 11; Desmond and Schoenwolf, 1985) or injected after the lumen of the spinal cord had reopened (stages 14–15; Schoenwolf and Desmond, 1986; see below), no Dil-labeled cells were ever found on the contralateral side of the cord.

**FIG. 6.** Summary fate map of the early neuroepithelium illustrating the formation of the neural tube from the neural plate and the associated changes in the Pax3 expression domain. The dorsoventral axis of the neural tube is not built by the simple equal expansion of the neural plate. During early spinal cord development (A, B), considerable mixing occurs between progenitors originating in the different domains of the neural plate. The cellular contribution of each domain to the expansion of the neuroepithelium also is unequal. (B, C) At later stages, the degree of progenitor cell mixing and dispersal is much more limited and more equal expansion of the ventricular epithelium occurs.

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**Progenitor Cell Movement and Pax3 Gene Expression**

Several genes show restricted patterns of expression along the dorsoventral axis of the neural tube. The relationship between progenitor cell dispersal and the expression pattern of one of these genes, Pax3, was investigated. Pax3, a homeobox-containing transcription factor, is initially expressed throughout the mediolateral axis of the caudal neural plate but, over the next 24 h, transcripts are gradually downregulated in the ventral neural tube by notochord-derived signals, limiting its expression to progenitors in the dorsal half of the neuroepithelium (Liem et al., 1995; Bang et al., 1997; Fig. 7). Thus, Dil-labeled progenitors located in all five domains of the neural plate initially expressed Pax3 (Figs. 6 and 7A). However, 24–48 h later, transcription had not simply been uniformly switched off in the descendants of cells initially labeled in the ventral half of the neural tube (ventral/ventrolateral domains) or maintained in all cells originating more dorsally. Due to the widespread dispersal and mixing of progenitors which occur during this period, fluorescent clusters with cells in both Pax3-expressing and nonexpressing regions were found frequently (Figs. 3 and 6). Final, rather than initial position in the ventricular neuroepithelium appears to be the primary determinant of whether an individual progenitor continues to express Pax3.

Between stage 14 and stage 20 stable expression of Pax3 is maintained, with a sharp boundary, at the same relative position along the dorsoventral axis of the neural tube, delimiting the ventral extent of the expression domain (Figs. 7D–7H). This coincides with the period over which progenitor cell dispersal and mixing become much more restricted (Figs. 5 and 6). However, by comparing the position, size, and shape of the Dil clusters with the gene expression domain we found no evidence that the Pax3 boundary constitutes a barrier to cell movement. In several cases, progenitors marked at stage 14 were found, 24 h later, to have generated clusters with cells in both Pax3-expressing and nonexpressing regions (Fig. 5C). Moreover, the edges of each individual cluster were found at random positions within the neuroepithelium and not preferentially at specific dorsoventral locations as would be expected if cell movement was inhibited at gene expression boundaries (Fig. 5C). Finally, the shape formed by the labeled cell population (e.g., Figs. 5B and 8B–8F) is not consistent with their having abutted any boundary to cell movement. If this was the case, then D-shaped clusters should have been produced (see Fraser et al., 1990).

**Spatial Origin of Distinct Neuronal Phenotypes**

The early chick spinal cord contains a simple set of neuronal cell types which can be phenotypically classified according to the position of their cell body and direction of growth of their axon. By stage 22, five broad classes can be identified: motor neurons and four different types of c-cells (interneurons), the axons of which initially run circumfer-
ential along the dorsoventral axis of the neural tube before turning to ascend or descend in the ipsilateral or contralateral ventrolongitudinal fasciculus (Oppenheim et al., 1988; Yaginuma et al., 1990; Fig. 8A). The position of origin of each of these different neuronal cell types was determined. A small slit was made in the roof plate of the cervical spinal cord and 5–10 progenitor cells in the ventricular zone of stage 14–15 embryos were labeled by direct iontophoretic application of DiI. Following this, the embryos were reincubated for a further 36 h until they reached stages 21–23. During this time, brightly labeled neurons in association with discreet clusters of progenitor cells were produced (Figs. 8B–8F). Confocal microscopy was used to distinguish between these cell types, which lie at different depths of the neuroepithelium (see above), and the phenotype of the neurons in each cluster was determined. Motor neurons were identified by their location close to the floor plate and dorsally growing axons, which extend for only a short distance in the neuroepithelium before exiting via the ventral roots (Fig. 8F). The cell bodies of c-cells were found at all dorsoventral levels, their axons initially growing ventrally toward the floor plate before turning into the appropriate longitudinal tract (Figs. 8A–8E). Within the ventrolongitudinal fasciculus they either extended, often tightly fasciculated together, adjacent to the floor plate (Fig. 8E) or, after a short distance, deviated from this position to grow more dorsally within the neural tube (Figs. 8B and 8C). Each cluster contained between one and three different types of neurons.

Following their differentiation, spinal cord neurons can migrate considerable distances away from their place of birth (e.g., Leber and Sanes, 1995). Thus, since during this period little cell movement occurs within the ventricular zone (Figs. 5C and 6), the position of the undifferentiated progenitors in each cluster was determined and used as a marker of the site of origin of the related neurons. Distinct sets of neuronal cell types were found associated with progenitors located at different dorsoventral positions of the neural tube (Fig. 9). Along the dorsoventral axis there was a gradual switch in the phenotype of the neurons contained in each cluster, each cell type being generated in distinct but overlapping regions of the neuroepithelium. At progressively more dorsal positions, clusters were found which contained motor neurons, followed in order by c-cells with ipsilaterally projecting axons (idc-cells), c-cells with contralaterally projecting axons (cac-cells), and, finally, c-cells with contralaterally projecting axons (cdc-cells; Fig. 9). Motor neurons and interneurons with ipsilaterally projecting axons were born exclusively in the ventral half of the neuroepithelium, outside the Pax3 domain. By contrast, interneurons with contralaterally projecting axons were generated more dorsally, with the vast majority of the undifferentiated progenitors remaining in cdc-cell-containing clusters located within the domain of Pax3 expression (Fig. 9).

**DISCUSSION**

Limitations to the Use of DiI/DiA to follow Cell Fate

As a consequence of their lipophilic properties, carbocyanine dyes such as DiI and DiA have been widely used to study cell movements and cell fate (e.g., Serbedzija et al., 1988; Yaginuma et al., 1990; Fig. 8A). The position of origin of each of these different neuronal cell types was determined. A small slit was made in the roof plate of the cervical spinal cord and 5–10 progenitor cells in the ventricular zone of stage 14–15 embryos were labeled by direct iontophoretic application of DiI. Following this, the embryos were reincubated for a further 36 h until they reached stages 21–23. During this time, brightly labeled neurons in association with discreet clusters of progenitor cells were produced (Figs. 8B–8F). Confocal microscopy was used to distinguish between these cell types, which lie at different depths of the neuroepithelium (see above), and the phenotype of the neurons in each cluster was determined. Motor neurons were identified by their location close to the floor plate and dorsally growing axons, which extend for only a short distance in the neuroepithelium before exiting via the ventral roots (Fig. 8F). The cell bodies of c-cells were found at all dorsoventral levels, their axons initially growing ventrally toward the floor plate before turning into the appropriate longitudinal tract (Figs. 8A–8E). Within the ventrolongitudinal fasciculus they either extended, often tightly fasciculated together, adjacent to the floor plate (Fig. 8E) or, after a short distance, deviated from this position to grow more dorsally within the neural tube (Figs. 8B and 8C). Each cluster contained between one and three different types of neurons.

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1989; Selleck and Stern, 1991; Fishel et al., 1993; Birgbauer and Fraser, 1994; Selleck and Bronner-Fraser, 1995; Vargesson et al., 1997). These dyes can be applied to small groups of neighboring cells and, since they are insoluble in water, under most conditions are only transferred during cell division. Detailed and accurate fate maps of developing structures therefore can be produced. However, in situations where very close cell–cell contact takes place, transcellular spread of these dyes can occasionally occur (see Honig and Hume, 1986, for review). We have found that this may be a potential problem when DiI or DiA is used to follow cell fate in the development of the early spinal cord. We believe that the occlusion of the lumen of the caudal neural tube which occurs during early stages of chick development (stages 11–14; Desmond and Schoenwolf, 1985; Schoenwolf and Desmond, 1986) allows dye transfer between progenitors located in the tightly opposed ventricular surfaces (Fig. 4C). As a result, two discrete clusters of fluorescent cells at similar dorsoventral positions on either side of the ventral midline are produced (e.g., Fig. 1D). This appears to be the most likely explanation for the high percentage (61%) of embryos containing fluorescent cells on both sides of the spinal cord. However, since we found one case with bilateral clusters when using the intracellular, cell-autonomous dye LRD, the transposition of progenitors from one side of the neural tube to the other may be a normal occurrence for a small number of spinal cord cells. The generation of bilaterally symmetrical progenitor cell clusters has previously been unequivocally demonstrated in the avian hypothalamus (Arnold-Aldea and Cepko, 1996) and secondary neural tube (Catala et al., 1996), but using the chick–quail chimera system Catala et al. (1996) did not detect any bilateral clusters in more anterior regions of the spinal cord. In the developing zebrafish CNS (Kimmel et al., 1994) bilateral clusters in brain and spinal cord are generated from the oriented division of cells that lie at the midline of the prospective neural keel. Despite the fact that we believe that the high percentage of bilateral clusters is artifactual in our studies of chick spinal cord, the main findings of our study remain valid. No obvious differences were found in the overall patterns of unilateral dispersal and mixing of progenitors marked using either DiI or LRD (Figs. 4A and 4B). Moreover, in embryos labeled with DiI after the spinal canal had reopened, no secondary labeling was apparent (Figs. 5 and 8). Consequently, the data presented here on unilateral progenitor cell dispersal and fate are reliable.

**Progenitor Cell Dispersal**

During the first 24 h of chick spinal cord development, considerable dorsoventral dispersal and mixing occur within the ventricular epithelium. As we have previously shown for the early chick hindbrain (Clarke et al., 1998), the amount of progenitor dispersal is not uniform but related to the initial position of the progenitors along the mediolateral axis of the neural plate. Thus, not all regions of the neural plate make an equal cellular contribution to the development of the neural tube. Later in development, progenitor cell dispersal becomes much more restricted (see also Stern et al., 1991; Leber and Sanes, 1995) and more uniform expansion of the ventricular epithelium also occurs (Figs. 5 and 6).

Several different factors (rates of proliferation, death, cell adhesion differences) may contribute to the differential dispersal of cells along the dorsoventral axis of the forming neural tube. Our cell-labeling techniques have not allowed us to determine relative rates of proliferation, but cells in the floor plate have a longer cell cycle (Smith and Schoenwolf, 1987) and do not mix with cells from other regions of the neuroepithelium (Alvarez and Schoenwolf, 1991), thus restricting their degree of dorsoventral dispersal. The dorsal and dorsoventral regions of the neural plate contribute cells to the roof plate, neural crest, and surface ectoderm (Selleck and Bronner-Fraser, 1995; Fig. 3), cell types not included in our measurements. Cell death during closure of the neural tube (e.g., Well et al., 1997) may also decrease the relative expansion of these regions. Finally, there may be constraints on the movement of progenitors where the greatest bending of the neural plate occurs, i.e., the dorsal and ventral midlines.

**Progenitor Cell Dispersal and Pax3 Gene Expression**

One of the earliest events in the development of the dorsoventral pattern of the spinal cord is the restriction of Pax3 expression to the dorsal half of the neural tube.

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**FIG. 8.** (A) Schematic diagram of a flat-mounted stage 22 spinal cord showing the principal neuronal cell types present at this time and the relative positions they occupy along the dorsoventral axis of the neural tube. For clarity each cell type is shown only on one side. mn, motor neuron; iac, ipsilateral ascending cell; cac, contralateral ascending cell; cdc, contralateral descending cell; idc, ipsilateral descending cell; FP, floor plate. (B–F) Examples of neuron-containing clusters analyzed, in flat-mounted stage 22–23 spinal cords, 36 h after labeling at stage 14. Two confocal z-series projections were made of each specimen, one close to the pial surface to reveal the neurons (pseudo-colored green) and one close to the ventricular surface to reveal the progenitors (pseudo-colored red), and the two images were superimposed using Adobe Photoshop software. (B) Large cluster containing both iac-cells and cac-cells. Arrow points to two cells which appear to be migrating dorsally away from the main cluster. (C) Example of an idc-cell- and iac-cell-containing cluster. (D) Cluster in which only one neuron has been generated (cac-cell). (E) Cluster, located in the dorsal half of the neural tube, in which only cdc-cells have been generated. (F) Motor neuron-containing cluster. Dotted lines indicate the edges of the floor plate and roof plate. Bar, 100 μm.

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(Goulding et al., 1993; Liem et al., 1995; Fig. 7). By stage 14, a ventral boundary of expression is established approximately midway between the dorsal and ventral midlines. However, although by in situ analysis Pax3 shows homogeneous labeling of all progenitors dorsal to this boundary, we found no evidence for any restrictions to cell mixing (Figs. 5 and 6). Progenitors appear able to disperse from Pax3-expressing to nonexpressing regions of the ventricular epithelium and vice versa. Maintenance of the sharp expression pattern of Pax3 must involve a rapid and complete switch in the expression of Pax3 within cells crossing the boundary.

Movement of progenitors across gene expression boundaries has been reported previously in the developing hindbrain (Birgbauer and Fraser, 1994). Along its anterio-posterior axis the hindbrain is transiently subdivided into eight segmental units termed rhombomeres, each of which shows a distinct pattern of gene expression (reviewed by Lumsden and Krumlauf, 1996). Although cell mixing is inhibited at rhombomere boundaries (Fraser et al., 1990), this restriction is not absolute and some cells can occasionally move between adjacent rhombomeres (Birgbauer and Fraser, 1994). Again, since cells expressing ectopic genes are rare, these boundary crossers must either be eliminated or undergo a rapid change in their expression and fate. Thus, dynamic regulation of gene transcription may play a role in maintaining the sharp patterns of gene expression along both the anterio-posterior and dorsoventral axes of the developing nervous system.

**Spatial Origin of Neuronal Phenotypes**

Most neurons in the chick spinal cord are born between stages 14 and 32 (Langman and Haden, 1970; Hollyday and Hamburger, 1977; McConnel and Sechrist, 1980; Oppenheim et al., 1988; Yaginuma et al., 1990). By labeling small...
groups of neighboring progenitors at the onset of this period we have been able to map the domains of the neural tube in which the early classes of neurons are produced. Motor neurons are born in the most ventral part of the neural tube, followed by interneurons with ipsilaterally and, more dorsally, contralaterally projecting axons. Neurons whose axons extend cranially are also generated in disparate regions of the neuroepithelium from those whose axons grow caudally (Fig. 9). Thus, since substantial dispersal and mixing of progenitors occur within the ventricular epithelium (see above; Fig. 6), this provides direct evidence that the spatial origin of a neuron determines its morphological phenotype. There is some overlap between the regions in which distinct cell types are generated and this most likely arises because some clusters contained progenitors within more than one environmental domain. In support of this, clusters were often found which contained two or more morphologically distinct neuronal cell types.

These results provide insights into the fate of area x, the region of the neuroepithelium between the floor plate and motor neuron pools which, it has been suggested, differentiates into a third, as yet unidentified, cell type (Yamada et al., 1991, 1993; Roelink et al., 1995). One candidate for this is early-born neurons, located adjacent to the floor plate, whose axons pioneer a primitive longitudinal pathway (PL-cells; Yaginuma et al., 1990). By stage 22, we were unable to unambiguously distinguish these cells from c-cells with ipsilaterally projecting axons and therefore have considered these as one class. Despite this, our data indicate an alternative fate for area x. Clusters with progenitors in area x always were found to contain motor neurons (Fig. 9). Occasionally these clusters also contained interneurons, but always in association with motor neurons. This strongly suggests that motor neurons are generated from progenitors located in area x and that, following their differentiation, they rapidly migrate to lie at a more dorsal position within the neural tube. Dorsal migration of motor neurons has been described previously (Leber and Sanes, 1995). Additionally, in our analysis, the motor neurons in each cluster often were located at a more dorsal position than the undifferentiated progenitors suggesting they had moved away from their more ventral site of origin.

The region of the neural tube in which interneurons with contralateral descending axons (cdc-cells) are born maps to the dorsal half of the neuroepithelium and encompasses the domain of Pax3 expression, suggesting a potential role for this transcription factor in the specification of this neuronal cell type. Other early patterning genes are also expressed in distinct dorsoventral domains of the neuroepithelium (e.g., Takahashi et al., 1992; Basler et al., 1993; Goulding et al., 1993; Hollyday et al., 1995; Liem et al., 1995, 1997), one of which, Pax6, has been shown to be required for the generation of specific classes of ventral interneurons (Burrill et al., 1997; Ericson et al., 1997). This strongly suggests that at all dorsoventral levels of the neural tube the early patterns of gene expression within the ventricular neuroepithelium may play a critical role in determining neuronal identity.

Although by their direction of axonal growth cdc-cells are the only class of interneuron generated within the dorsal part of the neural tube, using molecular markers three distinct cell types, D1, D2, and D3 neurons, have been identified within this region (Liem et al., 1997). Based on their expression of PAX2, EN1 and EVX1 more ventrally located interneurons also can be divided into distinct molecular subtypes (Burrill et al., 1997). Thus, an important next step to understand more fully the relationship between the activity of the early patterning genes and neuronal identity will be to combine these approaches and determine the relationship between the spatial origin of each distinct cell type and both its morphological and molecular phenotypes.

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