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Determination of Neuroepithelial Cell Fate: Induction of the Oligodendrocyte Lineage by Ventral Midline Cells and Sonic Hedgehog

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Near the floor plate of the embryonic neural tube there is a group of neuroepithelial precursor cells that are specialized for production of the oligodendrocyte lineage. We performed experiments to test whether specification of these neuroepithelial oligodendrocyte precursors, like other ventral neural cell types, depends on signals from the notochord and/or floor plate. We analyzed heterozygous Danforth's short tail (*Sd*/+) mutant mice, which lack a notochord and floor plate in caudal regions of the neural tube, and found that oligodendrocyte precursors did not appear at the ventricular surface where there was no floor plate. Moreover, oligodendrocytes did not develop in explant cultures of *Sd*/+ spinal cord in the absence of a floor plate. When a second notochord was grafted into an ectopic position dorsolateral to the endogenous notochord of a chicken embryo, an additional floor plate was induced along with an ectopic focus of oligodendrocyte precursors at the ventricular surface. Oligodendrocytes developed in explants of intermediate neural tube only when they were cocultured with fragments of notochord or in the presence of purified Sonic hedgehog (Shh) protein. Thus, signals from the notochord/floor plate, possibly involving Shh, are necessary and sufficient to induce the development of ventrally derived oligodendroglia. These signals appear to act by specifying the future fate(s) of neuroepithelial cells at the ventricular surface rather than by influencing the proliferation or differentiation of prespecified progenitor cells in the parenchyma of the cord.

INTRODUCTION

The notochord and floor plate at the ventral midline of the neural tube are responsible, in part, for organizing the developing spinal cord. Short-range or contact-mediated signals from the notochord induce neuroepithelial cells at the ventral midline to form the floor plate (van Straaten *et al.*, 1985, 1988; Placzek *et al.*, 1990, 1993), which then acts as a secondary source of organizing signals for the spinal cord (Klar *et al.*, 1992; Serafini *et al.*, 1994; Kennedy *et al.*, 1994; Colamarino and Tessier-Lavigne, 1995; Guthrie and Pini, 1995; Hynes *et al.*, 1995; Tamada *et al.*, 1995). The notochord and floor plate can also act at a distance to induce the development of motor neurons (Yamada *et al.*, 1991, 1993; Tanabe *et al.*, 1995). A strong candidate for both the floor plate- and motor neuron-inducing activities of the notochord is the product of *Sonic hedgehog* (*Shh*), a vertebrate homologue of the *Drosophila* patterning gene *hedgehog* (*hh*) (for reviews see Smith, 1994; Perrimon, 1995; Johnson and Tabin, 1995). Recombinant Shh can act in a concentration-dependent manner on neural plate cells *in vitro*, inducing them to differentiate as either floor plate cells (higher con-

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centrations of Shh) or motor neurons (lower concentrations of Shh) (Roelink *et al.*, 1995; Martí *et al.*, 1995; Porter *et al.*, 1995). This concentration dependence might explain why contact between notochord and neuroepithelium is required for floor plate induction but not motor neuron induction and suggests that Shh might be a component of a long-range ''morphogenetic gradient'' that could induce several different neural cell types to differentiate at specified distances from the ventral midline.

Spinal cord oligodendrocytes are descended from a small number of specialized neuroepithelial precursor cells that reside at the ventricular surface in the ventral half of the developing cord (Warf *et al.*, 1991; Pringle and Richardson, 1993; Yu *et al.*, 1994; Ono *et al.*, 1995; Nishiyama *et al.*, 1996; reviewed by Richardson *et al.*, 1995; Miller, 1996). These neuroepithelial oligodendrocyte precursors subsequently give rise to proliferative, migratory oligodendrocyte progenitor cells that disperse throughout the spinal cord before differentiating into postmitotic oligodendrocytes (Warf *et al.*, 1991; Pringle and Richardson, 1993; Noll and Miller, 1993; Yu *et al.*, 1994; Ono *et al.*, 1995; Nishiyama *et al.*, 1995). Both the neuroepithelial precursors and the migratory progenitor cells can be visualized *in situ* with nucleic acid hybridization probes or antibodies against the platelet-derived growth factor α-receptor (PDGFRα) (Pringle and Richardson, 1993; Yu *et al.*, 1994; Ellison and de Vellis, 1994; Nishiyama *et al.*, 1995). In transverse sections of E13 mouse spinal cord (E14 in rat, E7 in chicken) the PDGFRα^+ precursors first appear as small, bilateral foci of neuroepithelial cells, 15% of the way from the floor plate toward the roof plate (Pringle and Richardson, 1993; Yu *et al.*, 1994) (see Figs. 1, 2, 5, and 6). The ventral location of the oligodendrocyte precursors raises the question of whether development of these cells, like that of other ventral cell types in the neural tube, depends on inducing signals from the notochord and/or floor plate. Here, we describe experiments that address this question. We investigated the development of oligodendrocyte precursors in the spinal cords of Danforth's short tail (*Sd*) mutant mice that lack a notochord and floor plate, and also in chicken embryos that possess a second, surgically introduced notochord. Our results demonstrated that development of the oligodendrocyte lineage depends on the presence of a notochord and/or floor plate and suggested that one or both of these structures might be the origin of a long-range morphogenetic gradient that influences the future fates of neuroepithelial precursor cells. In addition, we found that oligodendrocytes could be induced to develop in explants of chicken or quail intermediate neural plate, which does not normally give rise to oligodendrocytes, by coculturing with fragments of notochord or in the presence of purified recombinant Shh protein. The dose–response for induction of oligodendrocytes by Shh was the same as for motor neuron induction. These experiments indicate that a common signaling pathway involving Shh induces the development of ventrally derived oligodendrocytes and motor neurons.

MATERIALS AND METHODS

Preparation of Tissue Sections

Whole chicken or mouse embryos were sacrificed by decapitation and immersion-fixed in 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS) for 24 hr at 4°C and cryoprotected in 20% (w/v) sucrose in PBS for 24 hr at 4°C. Tissues were immersed in OCT embedding compound (BDH) and frozen on solid CO_2. Tissue was stored at −70°C until required for sectioning. Frozen sections were cut (10–15 μm nominal thickness) on a cryostat and collected on 3-aminopropyltriethoxysilane (APES)-coated glass microscope slides. Sections were air dried for 2 hr before being stored at −70°C.

In Situ Hybridization

Our *in situ* hybridization procedure has been described previously (Pringle *et al.*, 1992), except that the proteinase K digestion (20 μg/ml) was for 5–7 min at room temperature prior to hybridization. Our ^{35}S-labeled RNA probes were generated as previously described (Pringle *et al.*, 1992) by *in vitro* transcription from a 1.5-kb *Sac*I–*Pvu*II fragment encompassing most of the extracellular domain of rat PDGFRα cloned into PGEM1. The chicken PDGFRα probe was a 3.2-kb *Xho*I–*Bam*HI fragment of a full-length CDNA obtained from M. Mercola (Harvard Medical School, Boston), encompassing most of the 3′ untranslated region cloned into Bluescript SK. For autoradiography, the slides were coated with Ilford K5 nuclear emulsion, exposed for up to 3 months in the dark at 4°C, and developed in Kodak D-19. Some sections were lightly counterstained with hematoxylin (Gills No. 3, Sigma).

Mouse Spinal Cord Explant Cultures

Spinal cords of E13 *Sd*/+ mice were dissected away from surrounding tissue, split along the dorsal midline, and flattened with the ventricular surface uppermost. The floorplate, where present, could be easily recognized at the ventral midline of such preparations. In the *Sd*/+ embryos used for these experiments, the region lacking a floor plate was about 1 mm long at the caudal end; unlike the embryos described in Fig. 1, there was no adjacent normal spinal cord further posterior to this abnormal region. Transverse segments, approximately 0.5 mm in the anteroposterior dimension, were cut from the caudal region of the cord using a tungsten needle. First, the spinal cord was cut transversely into anterior (normal, with floor plate) and posterior (abnormal, no floor plate) sections at the visible boundary between normal and abnormal cord. The tail section was then removed and discarded. The remaining two sections were further divided into equal segments, the two most posterior segments without floor plate, and the more anterior segments with floor plate. Spinal cords from wild-type littermates were aligned with the *Sd*/+ cords, and cuts made in equivalent positions. Each spinal cord segment was attached to the surface of a tissue culture dish with 1 μl of chicken plasma, clotted *in situ* by adding an equal volume of 1 mg/ml thrombin (Sigma). The explants were cultured at 35.5°C in 5% CO_2 on a rocking platform (12 cycles/min), so that the explant was exposed above the surface of the culture medium (4 ml in a 6-cm-diameter dish) during about half of each cycle. Half of the culture medium (Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum) was exchanged every other day. Explants were cultured for 19 days, until the equivalent of P12, and oligodendrocytes visualized by

immunohistochemistry with an antiserum against myelin basic protein (MBP). This procedure labeled only oligodendrocytes that lay at or close to the surface of the explants.

Preparation of Donor Notochords

White Leghorn chicken eggs were incubated at 38°C for approximately 48 hr (stage 12–13) (Hamburger and Hamilton, 1951). Embryos were dissected from the eggs and placed in dispase (1 mg/ml; Boehringer Mannheim, FGR) in Dulbecco's modified Eagle's medium (Gibco-BRL). The notochords were isolated from surrounding tissue using flame-sharpened tungsten needles, washed in DMEM, and stored in DMEM on ice before use. Notochords were used for transplantation within 3 hr of isolation.

Notochord Grafts

White Leghorn chicken embryos were incubated at 38°C for approximately 36 hr (stage 9) (Hamburger and Hamilton, 1951). The eggs were washed with 70% ethanol, 1 ml of albumin was removed, a window was cut in the shell over the embryo, and India ink (Pelikan drawing ink number 17 black, Hanover, FGR) diluted 1:30 in Howard's Ringer solution was injected under the blastoderm to visualize the embryo. The vitelline membrane was deflected with a flame-sharpened tungsten needle. An incision was made in the unsegmented mesoderm region, lateral to the neural tube over a distance of approximately two to three somites. A donor notochord was transferred in 5 μl of DMEM and placed close to the incision. The notochord was oriented parallel to the incision and inserted into the cut with a tungsten needle. Following surgery the eggs were sealed with Tesa Band (Bieirdorf, FGR) and incubated at 38°C until they reached stage 32–33 (7.5 to 8 days). At the ages and survival periods that we employed in this study, we were unsuccessful in obtaining viable embryos that had an ectopic notochord positioned more dorsally than that shown in Fig. 6C.

Avian Neural Tube Explant Cultures

Quail or chicken neural tube tissue was dissected from Hamburger–Hamilton stage 9–10 (E1.5–2) embryos as described by Yamada *et al.* (1993). The neural tube with notochord attached was dissected away from the rest of the embryo under MEM–Hepes containing 1 mg/ml dispase. With the neural tube lying on its side, two longitudinal vertical cuts were made with a flame-sharpened tungsten needle to subdivide the tube into ventral, intermediate, and dorsal thirds, which were subsequently cut into smaller fragments approximately 100 μm long. The notochord was also isolated for some experiments.

Neural plate explants were cultured in three-dimensional collagen gels as previously described (Guthrie and Lumsden, 1994), in Bottenstein and Sato (1979) medium lacking transferrin and containing concanavalin A, 0.5% FCS, 1% (v/v) chick embryo extract and antibiotics. The embryo extract was made from E11 chick embryos as described by Stemple and Anderson (1992). In some experiments intermediate regions of neural tube were placed in contact with notochord such that the neural tube ''saddled'' the notochord. Continual contact between the notochord and intermediate neural tube explant was maintained within the collagen gel. In other experiments, intermediate neural tube was cultured alone in collagen gels in culture medium containing different concentrations of purified Shh. The Shh was recombinant amino-terminal fragment purified from *Escherichia coli* (Roelink *et al.,* 1995), and was added once only at the beginning of the culture period. In calculating the concentration of Shh, allowance was made for the volume of the collagen gel (100 μl) to which the medium (900 μl) was added. Cultures were assayed for expression of floor plate and motor neuron markers after 36–48 hr of culture and for oligodendrocytes after 12 days.

Immunolabeling of Explants

Cultured explants were washed in PBS, fixed with 4% (w/v) paraformaldehyde in PBS for 2 hr, washed for at least 2 hr in PBS containing 0.1% (v/v) Triton X-100, and then washed in PBS as before. After incubation at 4°C overnight in the primary antibody (see below) they were washed in PBS and incubated in fluorescein-conjugated goat anti-rabbit IgG or rhodamine-conjugated rabbit anti-mouse IgG for 2 hr at room temperature. Anti-MBP rabbit serum (a gift from D. Colman, Mount Sinai School of Medicine, NY) was diluted 1:1000 in PBS. Monoclonal anti-SMP (Dulac *et al.,* 1988) was used as undiluted cell culture supernatant. Monoclonal antibody 4D5 (which recognizes Lim homeodomain transcription factors Isl-1 and Isl-2) (Tsuchida *et al.,* 1994) was used as a 1:100 diluted tissue culture supernatant. Monoclonal antibody FP1, which recognizes chicken floor plate cells (Yamada *et al.,* 1991), was used as a 1:300 dilution of ascites fluid. All primary antibody solutions contained 0.1% (v/v) Triton X-100. After final washes in PBS, the explants were mounted under glass coverslips in Citifluor anti-fade reagent (City University, London) and examined in the fluorescence microscope.

Immunolabeling of Tissue Sections

Cryosections on glass slides were incubated overnight at 4°C in FP1 ascites diluted 1:300 in PBS containing 1% (v/v) Triton X-100 and 10% (v/v) goat serum. After washing in PBS, slides were incubated at room temperature in biotinylated anti-mouse Ig followed by streptavidin-conjugated peroxidase complex (ABC kit, Vector Laboratories). The staining was developed with a diaminobenzidine (DAB) peroxidase substrate kit (Vector Laboratories). The slides were washed again in PBS, dehydrated, cleared in xylene, and mounted in XAM (BDH).

Computer Imaging

Corresponding bright- and dark-field photographic images were converted to digital images using a video camera and frame grabber connected to a Macintosh computer. The monochrome images were assigned false colors (bright field, blue; dark field, yellow) and superimposed using Adobe Photoshop software. The composite images were photographed using a Sapphire slide recorder.

RESULTS

Specification of Oligodendrocyte Precursors Requires the Notochord/Floor Plate

The Danforth's short tail (*Sd*) mutation causes early degeneration of the notochord and, in homozygotes, results in perinatal lethality (Dunn *et al.,* 1940; Theiler, 1959). Het-

erozygous *Sd*/+ mice are often viable but the notochord is discontinuous in caudal regions of the embryo, leading to loss of a variable portion of the distal tail and discontinuous induction of floor plate in the most caudal regions of the remaining neural tube. Thus, *Sd*/+ embryos have regions of apparently normal caudal spinal cord interspersed with dorsalized regions that have no floor plate (for a recent description see Dietrich *et al.*, 1993).

We serially sectioned *Sd*/+ heterozygotes aged E13 (three animals from two litters) from the tip of the vestigial tail to the midthoracic region. Embryos were aged by morphological criteria (Theiler, 1959). The presence or absence of the floor plate at any particular level could be scored easily by the morphology of the spinal cord at the ventral midline (Fig. 1); this was confirmed by assaying the sections *in situ* for the enzyme acetylcholinesterase, which is normally expressed in floor plate cells and in motor neurons (Karnovsky and Roots, 1964) (Fig. 2). In some litters that we examined, the most caudal spinal cord did possess a floor plate but this disappeared, and then reappeared again further rostrally. A subset of the sections was hybridized *in situ* with a probe to PDGFRα to visualize oligodendrocyte precursors. Bilateral foci of PDGFRα^+ cells were present at the ventricular surface only in those regions of spinal cord that possessed a floor plate (Figs. 1 and 2). Thus, the development of oligodendrocyte precursor cells at the ventricular surface correlates with, and is presumably dependent on, the presence of the notochord and/or floor plate.

Surprisingly, there were PDGFRα^+ cells distributed evenly throughout the spinal cords of E19 *Sd*/+ mice, even in caudal regions which would have lacked a floor plate at E13. At an intermediate age, E15, there were still regions of spinal cord that lacked both a floor plate and PDGFRα^+ cells (Fig. 3a). There were also regions that lacked a floor plate but nevertheless contained dispersed PDGFRα^+ cells (Fig. 3b). However, we never found these cells at the ventricular surface, even when the section contained only a few PDGFRα^+ cells, suggesting that they were not generated locally but had invaded those regions without a floor plate by migrating longitudinally from neighbouring regions where spinal cord development was wholly normal (Fig. 3c). Oligodendrocyte progenitors are known to migrate large distances during normal development (Small *et al.*, 1987; Levison *et al.*, 1993; Leber and Sanes, 1995; Richardson *et al.*, 1995).

Oligodendrocytes Do Not Develop in Vitro in the Absence of a Floor Plate

To ask whether mature oligodendrocytes can be generated locally in regions of spinal cord that lack a floor plate, while avoiding the complication of long-range longitudinal migration of oligodendrocyte progenitors, we cultured thick (0.5 mm) transverse slices of spinal cord cut from the caudal regions of E13 *Sd*/+ mice and their wild-type littermates. After culturing for 19 days, to the equivalent of Postnatal Day 12 (P12), we labeled the explants with an antibody against myelin basic protein (MBP) to visualize differentiated oligodendrocytes (Fig. 4). MBP-positive oligodendrocytes developed at all rostro-caudal levels of wild-type spinal cord and in segments of *Sd*/+ cord that had an associated floor plate. However, oligodendrocytes did not develop, or developed in much reduced numbers relative to wild-type, in sections of *Sd*/+ cord that lacked a floor plate at the time the sections were cut (Fig. 4). This result was reproduced in explants from three *Sd*/+ and four wild-type embryos from two separate litters. The same result was obtained when monoclonal anti-galactocerebroside (GC), rather than anti-MBP, was used to visualize oligodendrocytes (not shown). Thus, neither oligodendrocyte precursors nor mature oligodendrocytes develop normally in the absence of a floor plate.

Specification of Oligodendrocyte Precursors by a Grafted, Ectopic Notochord

To discover whether the notochord/floor plate complex is sufficient to induce neuroepithelial cells to adopt an oligodendroglial fate, we performed notochord grafting experiments in chick embryos. Before doing this, we wished to determine whether oligodendrocyte precursors in the ventricular zone of the embryonic chick spinal cord express PDGFRα, as they do in rodent spinal cord. We subjected transverse sections of normal embryonic chick spinal cord at various stages of development to *in situ* hybridization with a probe against chicken PDGFRα. Bilateral foci of PDGFRα^+ cells appeared in the ventral ventricular zone, and these cells subsequently increased in number and appeared to migrate away from the ventricular surface, just as in the rodent. The PDGFRα^+ cells were first apparent at the ventricular surface in E7.5 chick embryos (stage 32; Hamburger and Hamilton, 1951) (Fig. 5), but the strength of the PDGFRα signal was weaker at this stage than at the equivalent developmental stage in mouse or rat, requiring more than a month of autoradiographic exposure compared to 1–2 weeks in the rodent to provide a similar signal-to-noise ratio. The chicken PDGFRα signal was significantly up-regulated soon after the cells had left the ventricular surface. These data, together with the recent study by Ono *et al.* (1995), who used monoclonal antibody O4 (Sommer and Schachner, 1981) to label oligodendrocyte precursors in chick spinal cord, demonstrate that early oligodendrocyte development in the chick is similar to that in mouse or rat.

We grafted segments of notochord from donor chick embryos into recipient embryos at the open neural plate stage of development (stage 9, E1.5). We fixed these embryos on E7.5–E8 (stage 32–34), sectioned through the region of the graft, and visually assessed the success of the operation. Selected embryos were subjected to *in situ* hybridization to visualize PDGFRα^+ oligodendrocyte precursors. In most of the embryos (56/61) the grafted notochord was missing, or had fused with, the endogenous notochord. The position and appearance of the floor plate and the foci of PDGFRα^+ precursors appeared normal or near-normal in such embryos

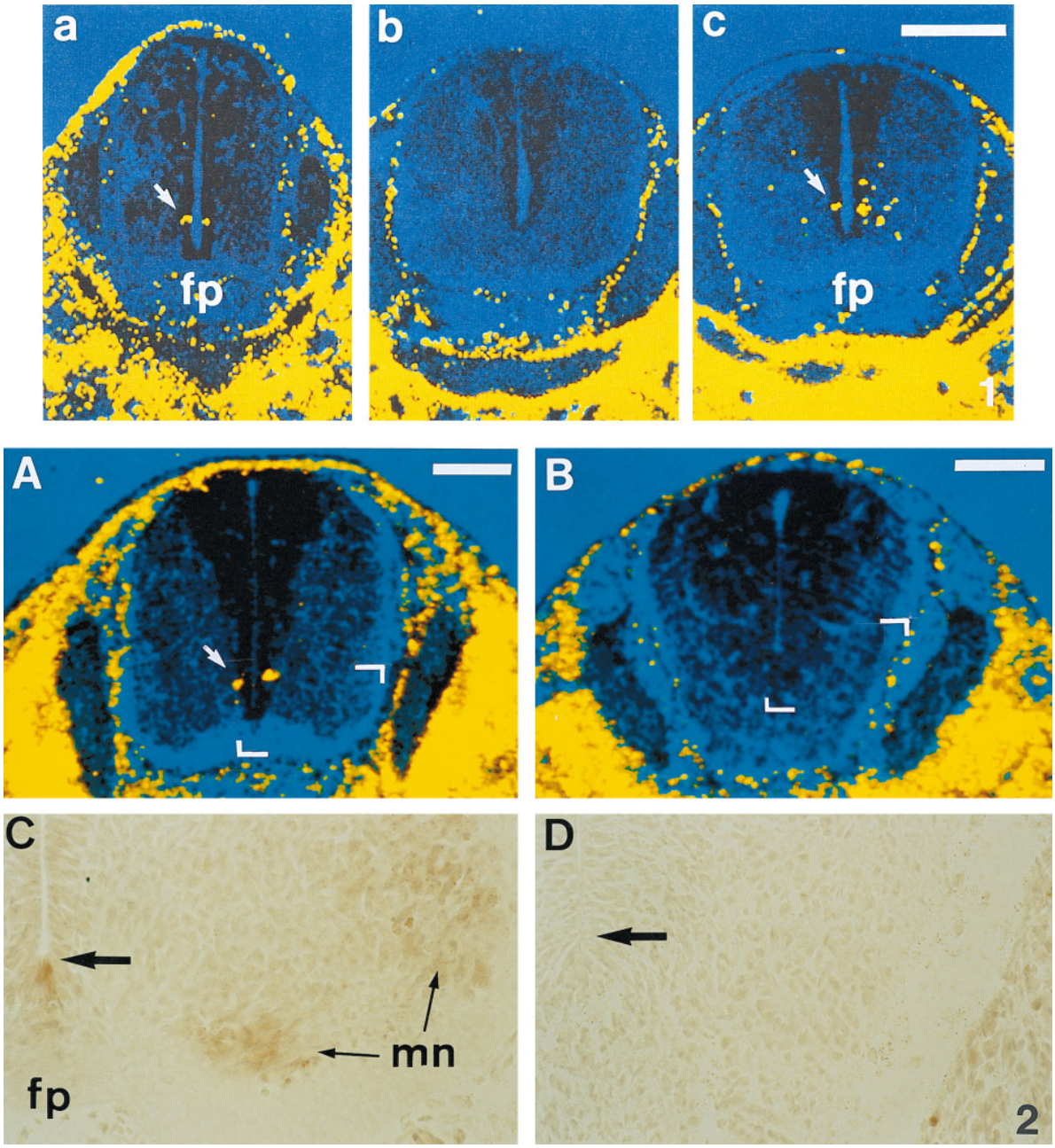

FIG. 1. The appearance of oligodendrocyte precursors at the ventricular surface of Danforth's short-tail mouse mutant depends on the presence of a notochord/floor plate. Transverse cryosections (10 μm) were cut through the spinal cords of heterozygous E13 *Sd*/+ embryos, from the tip of the vestigial tail to the upper thoracic region. In three embryos that we examined, the most caudal sections did possess a floor plate (fp; a), but anterior to this was a region approximately 600 μm in length that lacked a floor plate (b). More anterior still, the floor plate reappeared (c). The sections depicted in the figure are evenly spaced by approximately 1 mm; section (a) came from the caudal spinal cord, 2 mm from the tip of the tail. After hybridization to the PDGFRα probe, the sections were autoradiographed, photographed under dark-field illumination in a dissecting microscope, stained with hematoxylin, and rephotographed under bright-field illumination. The bright- and dark-field images were converted to digital format using a video camera and frame grabber attached to a Macintosh computer, assigned false colors (bright-field, blue; dark-field, yellow), and superimposed using Adobe Photoshop software. PDGFRα is expressed in many tissues outside of the CNS (a–c). In regions of spinal cord that were associated with a floor plate (a, c) there were bilateral foci of PDGFRα^+ cells in the ventral ventricular zone (arrows) as described previously in the rat (Pringle and Richardson, 1993; Yu *et al.*, 1994). Where there was no floor plate, there were no PDGFRα^+ cells in that region of the spinal cord (b). Scale bar, 200 μm.

FIG. 2. Acetylcholinestase assay for floor plate cells and motor neurons in E13 *Sd*/+ heterozygous mice. The presence or absence of a floor plate, judged by the morphology of the ventral midline region, was confirmed in some sections by an immunohistochemical assay for acetylcholinesterase (Karnovsky and Roots, 1964). A and B depict sections from the caudal region of a *Sd*/+ heterozygote hybridized with the PDGFRα probe to show the presence (A, arrow) or absence (B) of PDGFRα^+ cells in the ventricular zone (within the boxed areas). C and D show consecutive sections to those of A and B, respectively, stained for acetylcholinesterase activity. The presence of PDGFRα^+ oligodendrocyte precursor cells at the ventricular surface correlates with the presence of the floor plate (fp) and motor neuron pools (mn). The ventral-most tip of the central canal is indicated (large arrows in C and D). Scale bars, 200 μm.

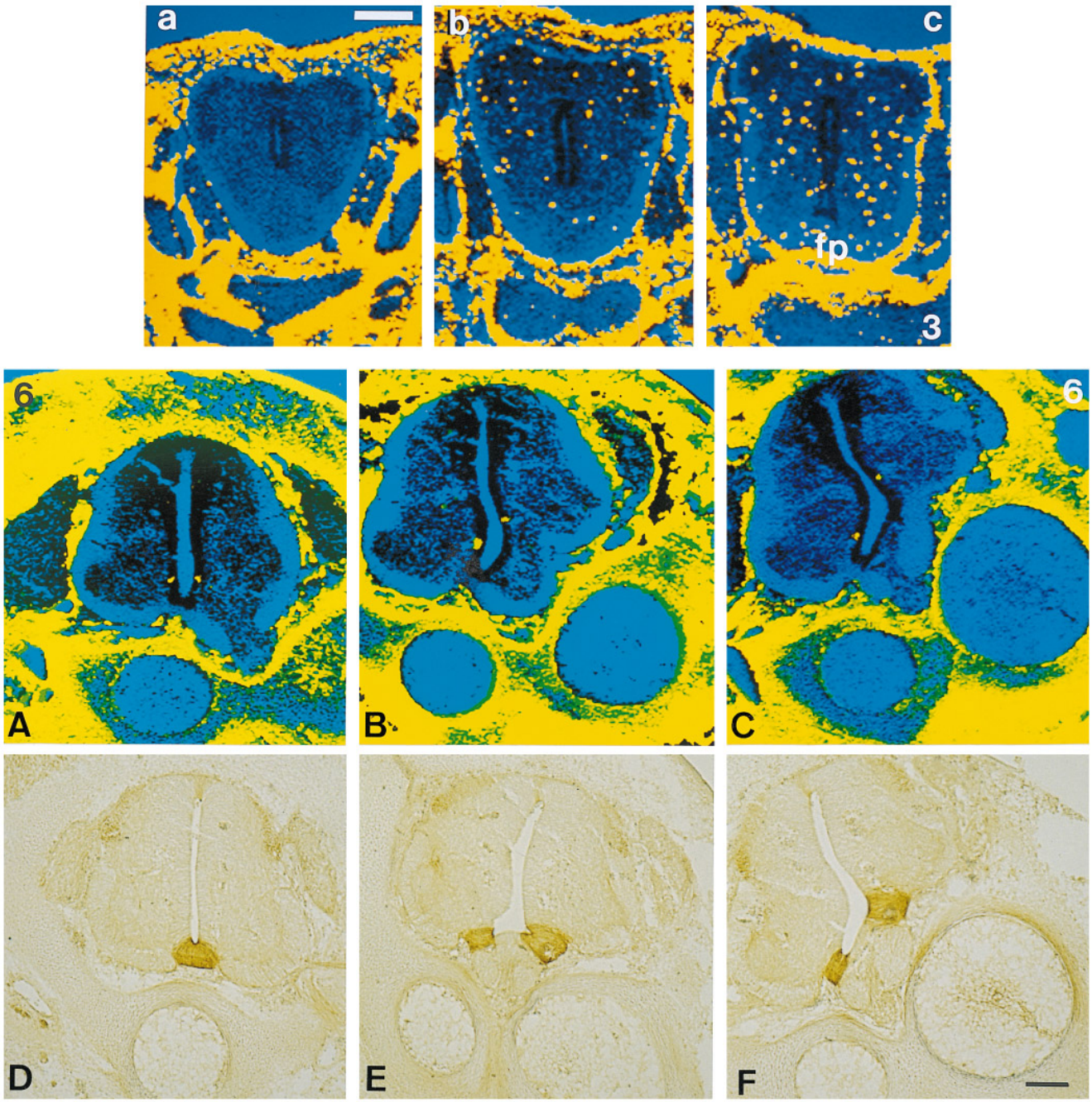

FIG. 3. Oligodendrocyte progenitor cells invade floor plate-less regions of *Sd*/+ mouse spinal cord by longitudinal migration from neighboring regions of normal spinal cord. Sections were taken from the caudal region of an E15 *Sd*/+ embryo and hybridized *in situ* with the PDGFRα probe (see the legend to Fig. 1). The sections of (a) and (b) lack a morphologically recognizable floor plate, whereas the more anterior section of (c) possesses a floor plate (fp). Note that the abnormal morphology of the spinal cord in (a) and (b) results from the loss of motor neurons and other ventral cell types due to the lack of a notochord/floor plate. The distance between (a) and (b) is approximately 150 μm; the distance between (b) and (c) is 300 μm. PDGFRα^+ cells appear in (b) despite the absence of a floor plate, presumably because of longitudinal migration of these PDGFRα^+ cells from the more anterior, normal region of spinal cord. Since the region lacking a floor plate (and PDGFRα^+ cells) in E13 *Sd*/+ mice is around 600 μm (see Fig. 1), and PDGFRα^+ cells are found at all levels of the neuraxis at E19 (see text), this suggests that oligodendrocyte progenitors can migrate at least 300 μm during embryonic development. Scale bar, 200 μm.

FIG. 6. An ectopic, grafted notochord induces an additional floor plate and an ectopic focus of oligodendrocyte precursors in the ventricular zone of the E7.5 (stage 32) chick spinal cord. Transverse sections through the spinal cord of a chick embryo with an ectopic, grafted notochord were subjected to *in situ* hybridization with a probe for chicken PDGFRα (A–C) or immunhistochemistry with antibody FP-1 (D–F). All sections were taken from one manipulated animal, progressing from the edge of the graft (A, D) into the graft in a rostral-to-caudal direction. The total length of the graft in this animal was approximately 400 μm. The corresponding upper and lower micrographs are of close, but not necessarily consecutive, sections. Where the grafted notochord is displaced from the natural notochord, it has induced an additional, ectopic floor plate (E, F) and an associated focus of PDGFRα^+ cells in the ventricular zone (B, C). Note that no PDGFRα^+ cells appear in the region between the ectopic and natural floor plates. The larger size of the grafted notochord relative to the endogeneous notochord presumably reflects the slightly older age of the donor compared to that of the host (E2 versus E1.5). Scale bars, 200 μm.

(data not shown). In the remaining manipulated embryos (5 animals), the grafted notochord was displaced from, and dorsolateral to, the endogenous notochord. Judging by morphology and immunolabeling with monoclonal antibody FP1 (Yamada *et al.*, 1991), an additional floor plate was induced adjacent to the ectopic notochord in each of these embryos (Fig. 6). One of these embryos was developmentally too immature and no PDGFRα^+ cells were found anywhere in the ventral half of the cord. Another was developmentally too advanced and many PDGFRα^+ cells were distributed throughout the cross section of the cord. In the remaining three manipulated embryos there was a new focus of PDGFRα^+ cells at the ventricular surface, the same distance away from the induced floor plate as the original foci were from the endogenous floor plate (Fig. 6). However, a new focus of PDGFRα^+ cells was induced only to one side of the ectopic floor plate, and simultaneously one of the original pair of foci was repressed, so that no PDGFRα^+ cells appeared in the neuroepithelium between the two floor plates (Fig. 6). This phenomenon was observed consistently in all the sections that we analyzed from these grafts (approximately 10 sections total). The reason for this observation was *not* because there was insufficient space between the two floor plates: on the contrary, there were more than enough intervening neuroepithelial cells to accommodate two additional nonoverlapping foci of PDGFRα^+ cells at the expected distances from the floor plates. The distance between the lateral margins of the floor plate and the foci of PDGFRα^+ cells is normally 75 μm whereas, as shown in Fig. 6C for example, the distance between the margins of the natural and ectopic floor plates is approximately 200 μm. Therefore, we tentatively interpret this observation as evidence for the existence of a diffusible ''morphogen'' originating from the floor plate and/or notochord (see Discussion).

Induction of Oligodendrocytes by Notochord and Purified Sonic Hedgehog Protein in Avian Neural Plate Explants

To extend the *in vivo* grafting experiments described above and to confirm that the notochord can induce oligodendrocyte development in naive neural tube tissue, we explanted fragments of E1.5–2 (stage 9–10) quail or chicken ventral, intermediate, or dorsal neural plate and cultured these in collagen gels either alone or in contact with pieces of notochord from the same embryos. After culturing the explants for 12 days, we fixed and immunolabeled them as whole-mount preparations with anti-SMP and/or anti-MBP to visualize oligodendrocytes. Most of our experiments were conducted using quail explants because the time required for appearance of oligodendrocytes *in vitro* was less than with chicken explants and, in addition, we had two independent antibodies (anti-MBP and anti-SMP) with which to score quail oligodendrocytes. However, most of the quail experiments described below were also confirmed qualitatively for chicken explants.

Two independent preliminary experiments were performed to test the inherent capacity of stage 9–10 ventral, intermediate, or dorsal quail neural tube tissue to generate oligodendrocytes *in vitro*. In the absence of notochord, large numbers of (MBP$^+$, SMP$^+$) oligodendrocytes developed in most ventral neural tube explants (13/14), but not in either intermediate (0/7) or dorsal (0/9) explants. However, intermediate neural tube was able to generate hundreds of oligodendrocytes when cultured in direct contact with notochord (17/17 explants; Fig. 7). These results are consistent with those published by Trousse *et al.* (1995), who performed similar experiments with neural tube explants from E4 (stage 23–24) chick embryos.

Sonic hedgehog, a vertebrate homologue of the *Drosophila* patterning gene product hedgehog, is known to be able to induce the development of ventral cell types including floor plate cells, motor neurons, and dopaminergic neurons in neural tube explants. It seemed possible that Shh, which is expressed in the notochord and floor plate during early neurogenesis, might be at least partly responsible for the oligodendrocyte-inducing activity of the notochord identified by our experiments and those of Trousse *et al.* (1995). Therefore, we cultured intermediate neural tube explants in the presence of different concentrations of the autoproteolytic amino-terminal fragment of Shh (Roelink *et al.*, 1995). The results of these experiments are shown in Table 1 and Fig. 7. Whereas floor plate cells were induced by only the highest concentration of Shh tested (7×10^{-9} *M*), both motor neurons and oligodendrocytes were induced by Shh over a range of concentrations from 7×10^{-9} to 7×10^{-10} *M* (Table 1 and Fig. 7). For both motor neurons and oligodendrocytes, there appeared to be a sharp decline in the inducing activity of Shh at lower concentrations than this (Table 1).

DISCUSSION

The experiments reported here show that the influence of the notochord/floor plate extends to the oligodendrocyte lineage in addition to ventral neurons such as motor neurons (Yamada *et al.*, 1991, 1993) and midbrain dopaminergic neurons (Hynes *et al.*, 1995). This suggests that the processes governing development of glial cells and neurons are fundamentally similar. The fact that we can detect the very first PDGFRα^+ oligodendrocyte progenitor cells at a precisely defined site at the ventricular surface of the E13 mouse spinal cord (E7.5 chick spinal cord) demonstrates that neuroepithelial precursor cells are not all equivalent at this age and indicates that oligodendrocyte progenitors are prespecified as such before they move away from the central canal. The same might be true for other glial and neuronal lineages; indeed, it has been suggested (Wenger, 1950; Yu *et al.*, 1994) that the entire ventricular zone might consist of a mosaic of predetermined precursor cells, each dedicated to the production of a distinct subset of differentiated neural cell types. The data presented here show that loss of the notochord/floor plate in *Sd* mice results in a

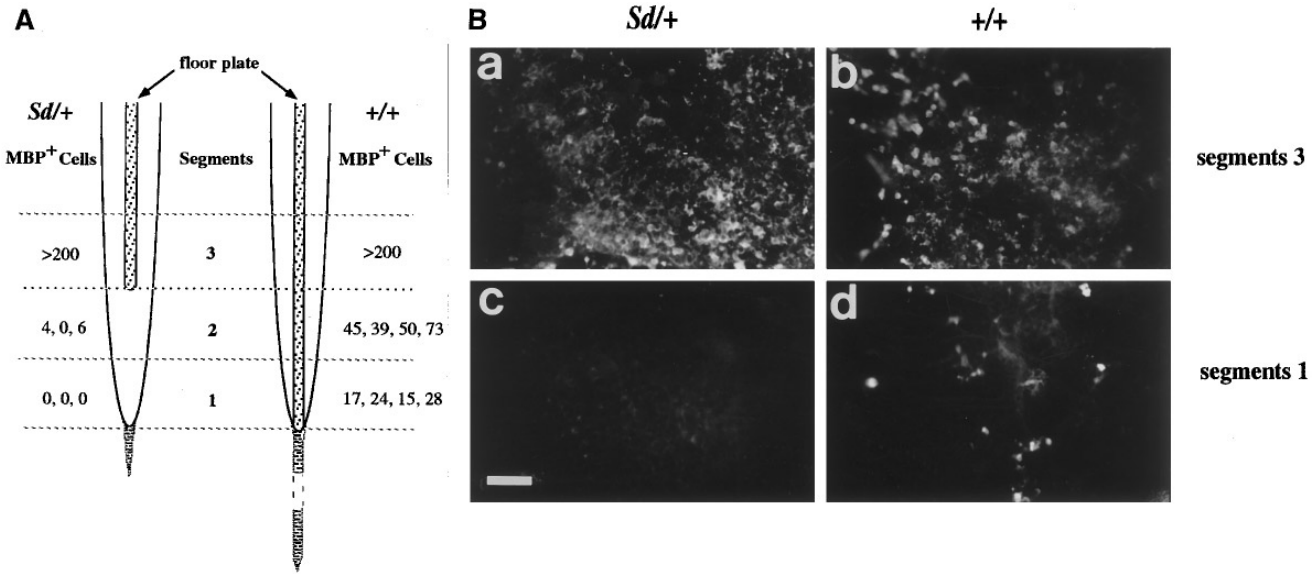

FIG. 4. Oligodendrocytes do not develop in explant cultures of *Sd*/+ mouse spinal cord in the absence of a floor plate. Spinal cords of E13 *Sd*/+ mice were dissected away from surrounding tissue, split along the dorsal midline, and flattened with the ventricular surface uppermost. The floor plate, where present, could be easily recognized at the ventral midline of such preparations (illustrated in A). In the *Sd*/+ embryos used for these experiments, the region lacking a floor plate was about 1 mm long at the caudal end; unlike the embryos described in Fig. 1, there was no normal spinal cord further posterior to this abnormal region. Transverse sections (0.5 mm thick) of caudal spinal cord with or without a floor plate (corresponding to the indicated segments 1–3 in A) were cultured for 19 days (until the equivalent of Postnatal Day 12), and then oligodendrocytes were visualized with an anti-MBP rabbit serum (see Materials and Methods). Three *Sd*/+ and four wild-type embryos from two litters were analyzed. There were large numbers (>200) of MBP^+ oligodendrocytes in anterior, floor plate-containing explants from both wild-type and *Sd* mice (A, segments 3; B, panels a and b). In the two most posterior wild-type segments the number of oligodendrocytes decreased significantly (A, segments 1 and 2; B, panel d). However, in the equivalent *Sd*/+ segments (which lacked a floor plate) there was a precipitous drop in oligodendrocyte numbers to zero in all of the most posterior explants (A, segment 1; B, panel c) and less than 10% of wild-type in the penultimate segments (A, segment 2). We could detect no obvious visible differences between *Sd*/+ and wild-type explants.

failure of oligodendrocyte founder cells to develop in the ventricular zone. This suggests that signals from the ventral midline might act, in general, by specifying the future fates of neuroepithelial precursors rather than by influencing proliferation of prespecified progenitor cells, or the differentiation pathways that they adopt.

The results of our notochord grafting and coculture experiments demonstrate that signals from the notochord and/or floor plate are sufficient to respecify the fate of neuroepithelial cells as oligodendrocyte precursors. This might be achieved *in vivo* by a ''domino effect,'' in which notochord-derived signals initiate a cascade of short-range signaling events along the plane of the ventricular surface, with each precursor cell signaling in turn to its nearest neighbor in a ventral-to-dorsal direction. However, our finding that the appearance of $PDGFR\alpha^+$ oligodendrocyte precursors in the *in vivo* grafting experiments was repressed in the region between the ectopic and natural floor plates (Fig. 6) is difficult to explain by a ''domino-effect'' model. It is more easily explained by postulating that the notochord or the floor plate acts as the source of a diffusible molecule (''morphogen'') that can act in a concentration-dependent fashion to influence cell fate over a distance of several cell diameters. According to this model, oligodendrocyte precursors might fail to be specified between the ectopic and natural floor plates because the putative morphogen might accumulate there to an inappropriately high concentration. A similar observation was made previously by Yamada *et al.* (1991), who found that motor neurons sometimes did not appear between the endogeneous and ectopic floor plates in analogous grafting experiments. These authors also interpreted their data in terms of gradients of diffusible morphogens. A morphogenetic gradient model is also favored over a domino effect model by the recent finding that notochord explants can induce neural plate cells to generate motor neurons at a distance *in vitro*, without the need for intervening floor plate (Tanabe *et al.*, 1995). A morphogenetic gradient model is also supported by the fact that the soluble N-terminal autoproteolytic fragment of Shh, whose homologue is known to act as a morphogen during *Drosophila* development (Smith, 1994; Porter *et al.*, 1995), can induce neural plate cells to generate either floor plate cells or motor neurons *in vitro*, according to the concentration of Shh in the culture medium (Roelink *et al.*, 1995; Martí *et al.*, 1995;

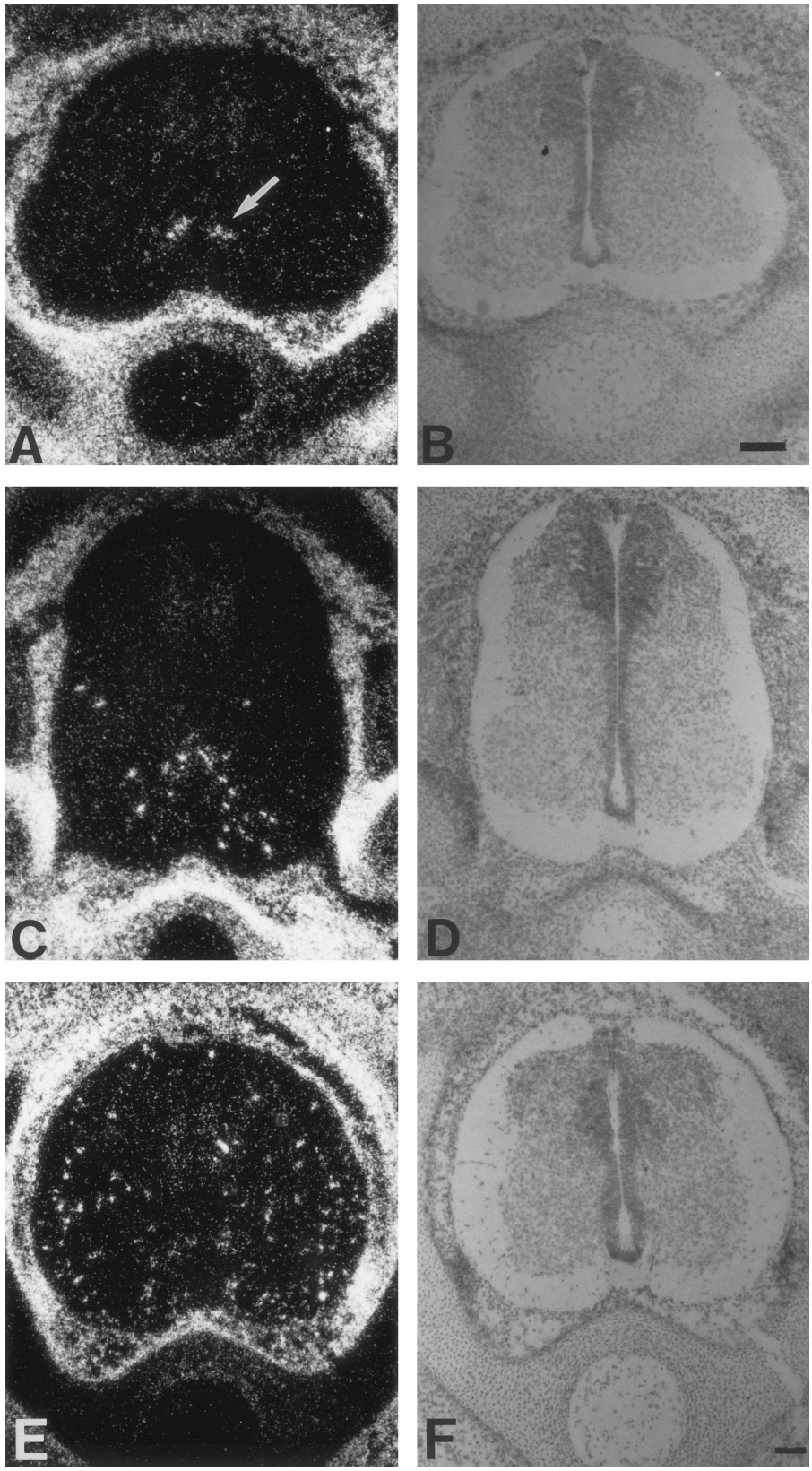

FIG. 5. PDGFRα^+ oligodendrocyte precursors first appear in the ventral ventricular zone of the E7.5 (stage 32) chick spinal cord. Transverse sections through the lumbar (A, B) and thoracic (C, D) spinal cord of a normal E7.5 embryo and the lumbar cord of an E9 (stage 35) embryo (E, F) were subjected to *in situ* hybridization with a probe for chicken PDGFRα (see legend to Fig. 1) and photographed under dark-field (A, C, and E) and bright-field (B, D, and F) optics. There are bilateral foci of PDGFRα^+ cells in the ventral ventricular zone of the lumbar cord at E7.5 (arrow in A; also see Fig. 6), very similar to that of the E13 mouse (Fig. 1). In the thoracic cord (developmentally more advanced) these PDGFRα^+ cells have increased in number and started to migrate away from the ventricular surface (C). By E9 (stage 35, lumbar region); the PDGFRα^+ cells are distributed throughout the cross section of the cord (E). Scale bars, 200 μm.

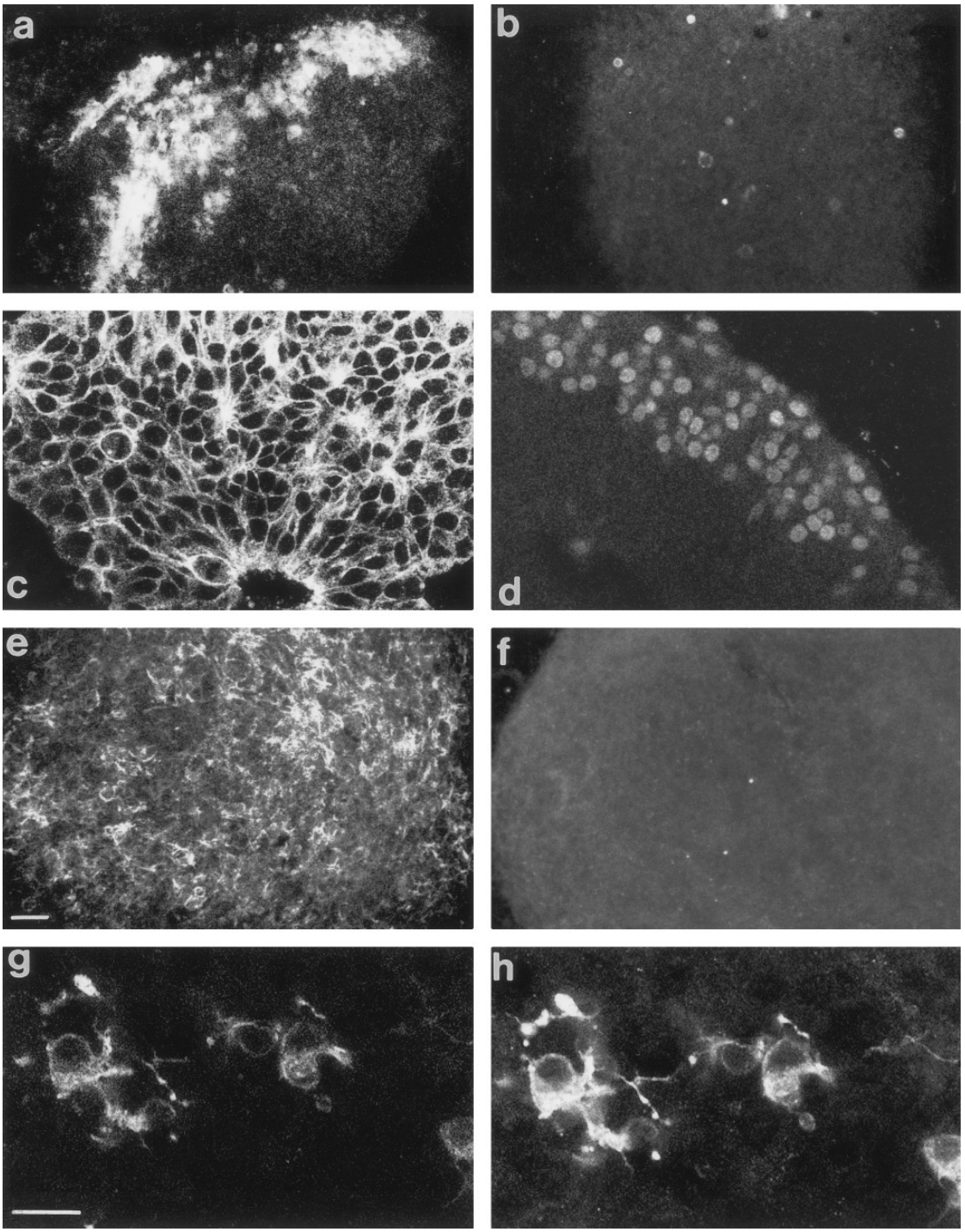

FIG. 7. Induction of floor plate cells, motor neurons, and oligodendrocytes in intermediate neural tube explants by notochord and purified Shh. Intermediate neural tube explants from E1.5–2 quails were cultured in collagen gels on their own or in contact with notochord tissue or in the presence of different concentrations of Shh (see Materials and methods). (a) Oligodendrocytes visualized with anti-SMP in an explant cultured for 12 days in contact with notochord tissue. Most of the oligodendrocytes are localized to a region overlying the original position of the notochord. (b) Control explant cultured in the absence of notochord for 12 days, labeled with anti-SMP. (c) Induced floor plate cells visualized with monoclonal FP1, 48 hr after the addition of Shh (7×10^{-9} *M*) to a cultured explant. (d) Motor neurons labeled with monoclonal 4D5, 48 hr after adding Shh (2×10^{-9} *M*) to a cultured explant. (e) Oligodendrocytes visualized with anti-SMP 12 days after adding Shh (2×10^{-9} *M*) to an explant culture. (f) Control explant, labeled with anti-SMP after 12 days *in vitro*. (g, h) Higher magnification views of part of a explant cultured for 12 days in the presence of Shh, showing oligodendrocytes double-labeled with anti-MBP (left) and anti-SMP (right). All images are confocal micrographs. Scale bars, 25 μm.

TABLE 1
Induction of Oligodendrocytes, Motor Neurons, and Floor Plate Cells in Neural Tube Explants by Sonic Hedgehog Protein

[Shh]	7×10^{-9}	2×10^{-9}	7×10^{-10}	2×10^{-10}	7×10^{-11}	Control
Oligodendrocytes	19/21	25/26	14/17	0/16	0/22	1/24
Motor neurons	12/12	16/18	10/13	0/6	0/6	0/13
Floor plate cells	10/10	0/6	ND	ND	ND	0/13

Note. Intermediate neural tube explants from stage 9–10 quail embryos were cultured in the presence of the indicated molar concentrations of Shh (amino terminal fragment, Roelink *et al.*, 1995). Motor neurons were labeled after 36–48 hr in culture with monoclonal 4D5 (anti-LIM domain proteins), floor plate cells were labeled after 36–48 hr with monoclonal FP1, and oligodendrocytes were labeled after 12 days with anti-MBP and/or anti-SMP (see Materials and Methods). Tabulated are the numbers of explants that contained cells of the indicated class and the total number of explants examined. Each data point is compiled from at least three independent experiments. The controls were parallel explants cultured in the absence of Shh. The one control experiment that scored positive contained fewer than 10 oligodendrocytes whereas explants cultured with notochord or in the presence of Sonic hedgehog always contained at least 50 oligodendrocytes, and usually more. The precise number of oligodendrocytes could not be determined accurately because the cell bodies were generally not clearly distinguishable from the background of immunopositive cell processes. ND, not done.

Porter *et al.*, 1995). Our finding that soluble Shh can also induce the development of oligodendrocytes in cultured neural tube explants extends the range of ventral cell types known to be specified by Shh, and further increases the likelihood that this molecule plays an important patterning role for the ventral neural tube *in vivo.*

Retroviral lineage analysis in the embryonic chick spinal cord suggests that motor neurons and oligodendrocytes share a common precursor cell at some stage of their ancestry (Leber *et al.*, 1990; Leber and Sanes, 1995). Our observation that motor neurons and oligodendrocytes are induced in neural tube explants by the same range of concentrations of Shh suggests that we might be witnessing the specification of a common motor neuron–oligodendrocyte precursor. However, motor neurons and oligodendrocyte lineage cells are generated at different times during development. In the rat spinal cord, for example, motor neurons are produced between E11 and E13 (Nornes and Das, 1974; Altman and Bayer, 1984), whereas PDGFRα^+ oligodendrocyte precursors do not appear in the ventricular zone until E14 (Pringle and Richardson, 1993; Yu *et al.*, 1994). This suggests that the fates of neuroepithelial precursors might change with time, either because of a cell-intrinsic mechanism that switches cell fate with successive cell divisions, or because of a changing instructive environment in the cord, or a combination of both. For example, the primary patterning signals from the notochord/floor plate might exert their effects early, conceivably in the open neural plate, and set in motion a sequence of cell-autonomous events that progressively alter the patterns of gene expression in neuroepithelial cells and their subsequent phenotypic fates. Alternatively, fate switching of neuroepithelial precursors might be determined by feedback signals from postmitotic motor neurons or other types of differentiated cells in the cord. Perhaps the fates of neuroepithelial precursors are subject to two complementary systems of control: a time-variant component derived from the evolving cellular composition of the cord superimposed on a fixed spatial coordinate system established earlier from the ventral midline.

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