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10. The elution of the two protein fractions with the ability to alter Ras GTPase activity from a PA affinity column seems to depend on the size of the column. In our previous experiments, when a small PA affinity column (2 ml) was used, only GAP activity was detected. However, in the present study, a large PA affinity column (5 ml) allows the separation of both GAP and GTPase inhibiting protein activities. The two proteins are thus apparently not separable by the smaller PA affinity column.
11. For filter binding assays, purified bacterially synthesized Ras was first loaded with [γ-^{32}P]GTP by incubation with 20 μl of incubation buffer containing 20 mM tris-HCl (pH 7.5), 1 mM dithiothreitol, and 2 μM [γ-^{32}P]GTP (10 Ci/mmol; Amersham) at 30°C for 10 min. GTPase activity was then assayed by incubating the [γ-^{32}P]GTP-bound Ras (0.1 μM) at 30°C for 60 min in 100 μl of reaction buffer [20 mM tris-HCl (pH 7.5), 1 mM dithiothreitol, 1 mM $MgCl_2$, 1 mM GTP]. The reaction mixture was then applied to nitrocellulose filters (0.45 μm, 25 mm; BA 85, Schleicher & Schuell), which were immediately washed with 10 ml of 20 mM tris-HCl (pH 7.5) containing 5 mM $MgCl_2$ and 50 mM NaCl. The amount of [γ-^{32}P]GTP remaining on the filters, which reflects the GTPase activity of Ras, was determined. The data described here obtained with renatured Ras protein, but similar results have been obtained with Ras prepared in soluble form, 70 to 80% of which binds nucleotide.
12. Nucleotide-free Ras (0.2 μM) was incubated for 20 min at 30°C with 1 μM [α-^{32}P]GTP (3000 Ci/mmol; Amersham) in 50 μl of tris-HCl (pH 7.5) containing 2 mM dithiothreitol. The GTPase reaction was initiated by addition of $MgCl_2$ with or without test samples in 100 μl of reaction buffer [final concentrations: 20 mM tris-HCl (pH 7.5), 5 mM $MgCl_2$, 0.15 M NaCl, 1 mM dithiothreitol]. After incubation at 30°C for 1 hour, Ras was immunoprecipitated by monoclonal antibody Y13-259 and protein A–Sepharose beads coated with rabbit antibody to rat immunoglobulin G. Bound nucleotides were released from the immunoprecipitate by boiling for 3 min, resolved on polyethyleneimine cellulose thin-layer chromatography plates (EM Science) in 1 mM potassium phosphate (pH 3.4), and visualized by autoradiography. Ras protein was prepared in a bacterial expression system.
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19. An affinity column (5 ml) containing immobilized 1-stearoyl, 2-arachonoyl PA was prepared by first chemically linking arachidic acid (20:0) (prepared as liposomes in acetate buffer) to agarose gel containing a six-atom hydrophilic spacer arm (AFF-gel 102; Bio-Rad). The covalently attached fatty acid is unavailable for association with GAP (*9*), but it was able to associate with and retain subsequently added liposomes, presumably by hydrophobic interactions. Liposomes of PA were passed over the column to generate the affinity column used. PA liposomes were prepared by dissolving PA (0.5 to 1.0 mg; Sigma) in chloroform, followed by drying under vacuum, in a glass tube (12 by 75 cm). The thin layer of lipid coating the glass was suspended in 1 ml of 0.1 M tris-HCl (pH 7.5) and sonicated in an ice bath for 30 s by inserting a titanium microtip (Fisher, Model 300 Sonic Dismembrator) into the bottom of the glass tube. Half-maximum power output was used for liposome-micelle preparation.
20. The cerebra from ten mice were homogenized in ice-cold hypotonic buffer [10 mM tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride]. The homogenate was centrifuged at 3000*g* for 10 min to remove unbroken cells, and resulting supernatant was then centrifuged at 100,000*g* for 30 min. The supernatant (5 mg of protein per milliliter), which contained GTPase inhibiting protein activity, was stored at −80°C. Before application to the PA affinity column, the preparation (30 ml) was incubated with 10 ml of DEAE-Sephacel (Pharmacia LKB) for at least 2 hours in tris-HCl (pH 7.5) containing 5 mM $MgCl_2$ and 1 mM EGTA. After washing the resin with 1 liter of the same buffer five times, proteins were eluted from the resin by the same buffer containing 0.3M NaCl.
21. We thank M. Roudebush, M. Golubic, and S. Dobrowolski for advice and technical assistance, and J. Leis and D. R. Lowy for helpful reviews of the work and manuscript. Supported in part by a grant (CA 48662) from the NIH and by the DeBartolo Fund.

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Mesodermal Control of Neural Cell Identity: Floor Plate Induction by the Notochord

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The floor plate is a specialized group of midline neuroepithelial cells that appears to regulate cell differentiation and axonal growth in the developing vertebrate nervous system. A floor plate–specific chemoattractant was used as a marker to examine the role of the notochord in avian floor plate development. Expression of this chemoattractant in lateral cells of the neural plate and neural tube was induced by an ectopic notochord, and midline neural tube cells did not express the chemoattractant after removal of the notochord early in development. These results provide evidence that a local signal from the notochord induces the functional properties of the floor plate.

THE DEVELOPMENT OF THE VERTEbrate nervous system begins with the induction of the neural plate from undifferentiated ectoderm in response to signals that derive from adjacent mesoderm (*1, 2*). Within the neural plate, the first cells to differentiate are located at its midline (*3*) and give rise to the floor plate, a distinct structure at the ventral midline of the neural tube (*4, 5*). The floor plate appears to be the source of a signal that regulates the pattern of cell differentiation along the dorsoventral axis of the neural tube (*6, 7*). The floor plate also contributes to axonal patterning by releasing a diffusible factor that may attract commissural axons to the ventral midline of the spinal cord (*8*) and by guiding these axons after they cross the midline (*9*). The specialized functions of the floor plate have led us to examine the interactions that control its differentiation.

Prospective floor plate cells are located immediately above the notochord, and notochord grafts placed next to the neural tube cause wedging of the adjacent neural epithelium similar to that observed during the early development of the floor plate (*10*). These results have led to the suggestion that floor plate differentiation is induced by the notochord (*10, 11*). However, comparable changes in neuroepithelial cell shape occur in many regions of the developing nervous system (*12*); thus it is not possible unambiguously to identify the floor plate by its morphology. The floor plate–specific chemoattractant (*8*) provides a marker with which to detect the floor plate and its development in response to the notochord. Here, we report that this chemoattractant is induced in lateral cells of the chick neural plate and neural tube in vivo and in vitro and that the chemoattractant does not appear in cells at the ventral midline of the neural tube in the absence of the notochord. A local signal from the notochord therefore appears to act on overlying neural plate cells to induce floor plate differentiation.

To study the induction of the floor plate in the chick embryo, we initially examined whether chick floor plate causes outgrowth and orientation of commissural axons from explants of E11 rat dorsal spinal cord (*13, 14*). Production of the chemoattractant was quantified by counting the number of axon bundles that emerged from the rat dorsal explants. Floor plate explants from stage 6

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to 24 chicks (*15*) were as active as rat floor plate (*8*) and always evoked outgrowth and orientation of commissural axons. Dorsal spinal cord explants cultured with lateral regions of stage 6 to 24 chick neural epithelium showed only the low amount of outgrowth normally observed when they were cultured alone (see below).

To test the inductive properties of the notochord in vivo, we grafted a 500- to 600-μm-long piece of stage 10 chick notochord to a site immediately adjacent to one side of the neural tube of stage 10 host embryos (*16*) (Fig. 1, A and B) and allowed the embryos to develop to stages 19 to 23. Those in which the supernumerary notochord was located next to the spinal cord and approximately midway between the roof plate and floor plate were analyzed further. A 700- to 900-μm region of the neural tube adjacent to the grafted notochord, the equivalent region of the spinal cord from the contralateral side, the floor plate of the host embryo, and the grafted notochord were isolated and tested separately for their ability to promote commissural axon outgrowth (Figs. 1 and 2). Explants of spinal cord from the region adjacent to the grafted notochord evoked axon outgrowth in most (83%) experiments and were almost as active as the host floor plate (Fig. 1, C and D, and Fig. 2, B and D). Neither the neural epithelium from the contralateral unoperated side of the spinal cord nor the grafted notochord had any chemotropic activity (Fig. 1, C and D, and Fig. 2C). Similarly sized grafts of embryonic chick gut or human hair also did not induce chemotropic activity in adjacent neural epithelium. Thus the notochord induced expression of chemotropic activity in regions of the chick neural tube that were normally devoid of this activity.

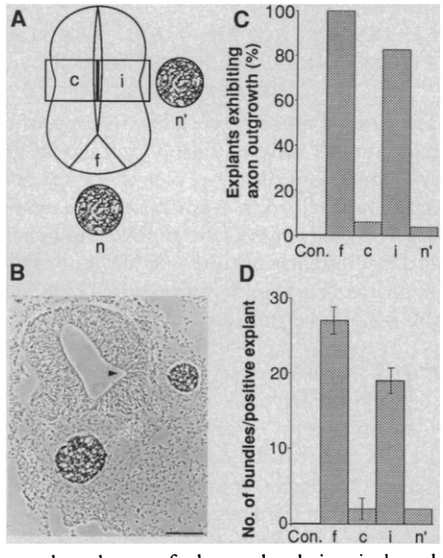

Fig. 1. The notochord can induce chemotropic activity in lateral neural epithelium in vivo. (**A**) Regions of chick spinal cord assayed for chemotropic activity, subsequent to grafting a supernumerary notochord. (**B**) Phase-contrast micrograph of a transverse section of chick spinal cord 40 hours after grafting of a supernumerary notochord. The section has been labeled with monoclonal antibody (MAb) Not-1, which recognizes an epitope expressed selectively by chick notochord. Both host and donor notochords are labeled. The location of the induced floor plate is indicated (arrowhead). Bar represents 100 μm. (**C** and **D**) Quantitative analysis of outgrowth from dorsal spinal cord explants. (C) Percentage of dorsal explants showing axon outgrowth, when cultured alone (Con.), or when cocultured with the three different regions of the spinal cord or with the grafted notochord. (D) Average number of axon bundles from those dorsal explants that showed outgrowth. Standard error bars are shown. The number of explants for each value in (C) is as follows: Con. 9; f, 36; c, 36; i, 36; n′, 23. Abbreviations: f, host floor plate; c, spinal cord contralateral to grafted notochord; i, spinal cord ipsilateral to grafted notochord; n, host notochord; n′, grafted notochord.

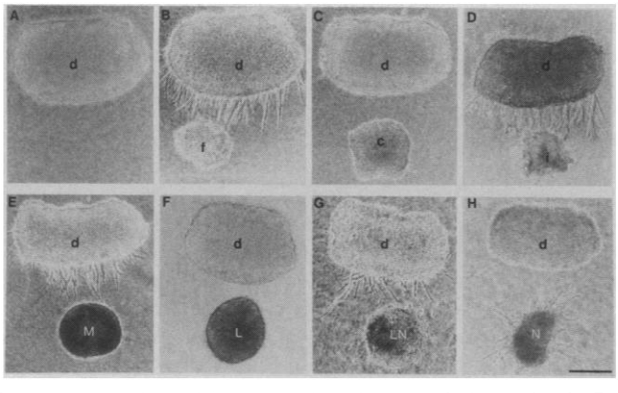

Fig. 2. Phase-contrast micrographs showing examples of outgrowth from dorsal explants cultured for 40 to 44 hours in collagen matrix. (**A**) No axons emerge from a rat dorsal spinal cord explant cultured alone. (**B**) Extensive outgrowth of commissural axons is observed from the ventralmost edge of a dorsal explant cultured in the presence of stage 22 chick floor plate. (**C**) No axon outgrowth occurs from a dorsal explant presented with stage 22 neural epithelium isolated from a region of spinal cord contralateral to a grafted notochord. (**D**) Profuse axon outgrowth is evoked by stage 22 neural epithelium isolated from a region of spinal cord immediately adjacent to a grafted notochord. (**E**) The ventral midline of stage 6 neural plate evokes outgrowth from a dorsal explant. (**F**) Stage 6 lateral neural plate does not evoke outgrowth. (**G**) Axon outgrowth is evoked when stage 6 lateral neural plate is cultured in direct contact with stage 7 notochord. (**H**) No axon outgrowth is observed in a dorsal explant in response to a stage 7 notochord alone. Bar represents 180 μm. Abbreviations: d, dorsal spinal cord explant; M, midline neural plate; L, lateral neural plate; N, notochord; LN, notochord plus lateral neural plate; f, host floor plate; c, spinal cord contralateral to grafted notochord; i, spinal cord ipsilateral to grafted notochord.

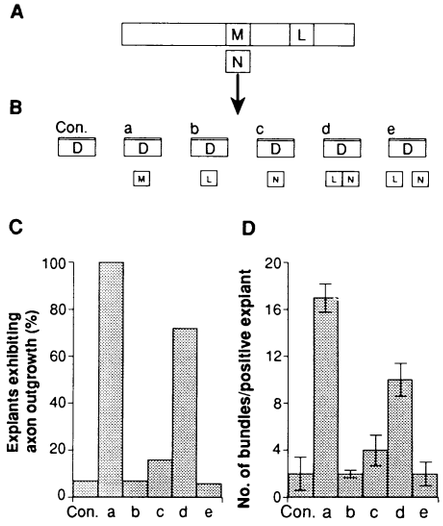

Fig. 3. Induction of chemotropic activity in the lateral neural plate in vitro. (**A**) Schematic cross section through stages 6 to 7 chick embryo neural plate and underlying notochord. Regions assayed for chemotropic activity are boxed. (**B**) Dorsal spinal cord explants (D) were cultured for 40 to 44 hours, either alone (Con.), with midline neural plate (a), with lateral neural plate alone (b), with notochord alone (c), and with lateral neural plate and notochord in contact (d) or apart (e). (**C** and **D**) The percentage of dorsal explants showing axon outgrowth (C) and the average number of axon bundles from those dorsal explants that showed outgrowth (D), when cultured alone (Con.) or in each experimental paradigm (a to e). Standard error bars are shown. The number of explants for each column in (C) is as follows: Con, 67; a, 40; b, 59; c, 44; d, 39; e, 31. Abbreviations: D, dorsal spinal cord explant. M, midline neural plate; L, lateral neural plate; N, notochord.

Induction of the chemoattractant was markedly reduced or abolished in older embryos (Table 1). The neural epithelium may therefore be competent to respond to inductive signals that derive from the notochord only up to about stage 11. However, we cannot exclude the possibility that older embryos express factors that destroy or inhibit the action of the notochord-derived inductive signal. The period of competence coincides with that over which wedging of neuroepithelial cells can be observed after notochord grafts (*11*).

It is possible that in vivo other tissues act in combination with the notochord to induce chemotropic activity. To test whether the notochord alone is sufficient for induction, we examined the interactions between isolated stage 7 chick notochord and lateral

Table 1. Induction of chemotropic activity depends on the age of the host neural tube. Quantification of axon outgrowth was performed as described (*14*) after grafting of stage 10 notochord adjacent to the neural tube of different aged host embryos. Bundle numbers are mean ± SEM where appropriate. Explants were obtained from 11, 5, and 12 grafted embryos for stage 10, 11, and 12 to 14, respectively. Ipsi, neural tissue ipsilateral to the grafted notochord; Contra, neural tissue contralateral to the grafted notochord.

Results	Stage 10		Stage 11		Stage 12–14	
	Ipsi	Contra	Ipsi	Contra	Ipsi	Contra
Explants exhibiting axon outgrowth (%)	76	7	38	0	7	8
No. of bundles/positive explant	20 ± 2.1	2	22.5	0	2.7 ± 1.5	1
No. of cultured explants	25	28	8	8	44	24

regions of stages 6 and 7 neural plate in vitro (Fig. 3). Explants of the lateral region of the neural plate alone did not exhibit chemotropic activity (Figs. 2F and 3). When lateral neural plate and notochord were placed in direct contact and about 300 μm away from a segment of E11 rat dorsal spinal cord, significant axon outgrowth was observed in 72% of experiments (Figs. 2G and 3). No significant axon outgrowth was observed when the notochord (*17*) and lateral neural plate were placed 50 to 100 μm apart (Fig. 3), which rules out the possibility that the uninduced neural plate and notochord release different factors that act in combination. Notochord alone, therefore, can induce chemotropic activity in the neural plate (*18*). Proximity or direct contact between cells of the notochord and neural plate may be necessary for induction of the floor plate. In support of this, during normal development only those neural plate cells contacted by the notochord exhibit the shape changes (*19*) and surface antigens (*6*) characteristic of early floor plate differentiation.

We next determined whether the notochord is required for the normal development of the floor plate by examining the effect of notochord removal on the expression of chemotropic activity by cells at the ventral midline of the neural tube. The ventral midline of the caudal neural tube of stages 10 to 12 embryos does not exhibit chemotropic activity (*20*), suggesting that floor plate development in the caudal neural tube is significantly delayed relative to the rostral neural tube (*21*). We therefore examined whether removal of the notochord from the caudal region of stage 10 to 12 embryos (*22*) prevented the appearance of the chemoattractant. The ventral midline of the neural tube was isolated from the notochordless region and from regions just rostral and caudal and tested independently for chemotropic activity. The ventral midline at levels lacking a notochord did not evoke axon outgrowth, whereas the rostral and caudal regions of ventral midline evoked extensive outgrowth (Fig. 4).

Our results show that the notochord is required for the expression of chemotropic activity by ventral midline cells and suggest that in grafting experiments, the notochord alone is responsible for the appearance of this activity in lateral neuroepithelial cells. Because there is little cell death within the chick neural tube before stage 15 (*23*), expression of chemotropic activity in neural cells presumably results from induction rather than from the rescue of a population of precursor cells that die in the absence of the notochord. Notochord grafts also induce floor plate surface antigens (*6*) and cell shape changes (*10, 19*) in lateral neuroepithelial cells. Thus many, perhaps all, of the phenotypic properties of the floor plate are induced by the notochord (*24*).

In vertebrate embryos, the floor plate and notochord share an early lineage (*25, 26*) and exhibit several molecular and functional properties in common (*5–7*). On this basis we previously raised the possibility that the specialized lineage of the floor plate may account for its functional properties (*5*). However, our present results show that cells of the lateral neural tube that do not have any lineage relationship with the notochord can acquire floor plate properties. Moreover, fate mapping studies in the chick hindbrain have shown that cells that originate in the lateral neural plate can populate the floor plate (*27*). These two observations suggest that the common cell ancestry that exists between midline neural plate cells and the notochord does not contribute to the known functions of the floor plate.

More generally, these results suggest that mesodermally derived inductive signals have sequential and progressively more refined roles in neural differentiation. During gastrulation, these signals are responsible for the initial induction of the neural plate and for its early regionalization along the anteroposterior axis (*1, 2*). Our results show that mesoderm also controls the identity of specific cells within the neural plate. Induction of the floor plate by the notochord appears to be a critical step in many subsequent aspects of cell differentiation and axon guidance in the developing nervous system.

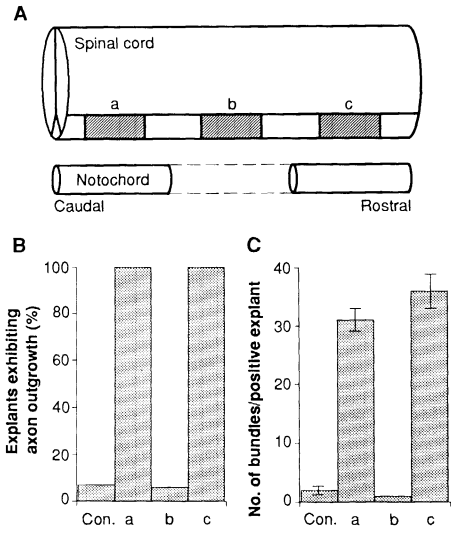

Fig. 4. The notochord is required for the expression of chemotropic activity in the developing neural tube. (**A**) Stages 19 to 23 chick spinal cord showing the regions of the ventral midline assayed for chemotropic activity (hatched) following the removal of a segment of notochord 40 to 48 hours previously. (**B**) Percentage of dorsal explants showing axon outgrowth, when cultured alone (Con.) or when cocultured with the regions from (A) as indicated beneath each column. (**C**) Average number of axon bundles in those dorsal explants that showed any axon outgrowth, when cultured alone (Con.) or with each region of the ventral midline of the neural tube. Standard error bars are shown. Number of explants for each value in (C) is as follows: Con, 27; a, 18; b, 17; and c, 18.

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13. Dissections and chemotropic assay were performed as follows. Tissues from Hamburger-Hamilton (*15*) stage 6 to 24 chick embryos and E11 rat embryos were dissected after treatment with Dispase (Boehringer Mannheim; 1 mg/ml in L15 air) at room temperature for 10 to 15 min (stages 6 to 10 chick) and 30 min (stages 19 to 24 chick and E11 rat embryos). After isolation, 50- to 200-μm-long pieces of chick tissues were embedded, together with two-segment pieces of the dorsal spinal cords of rat embryos (24 to 27 stage somites), in a collagen gel (*8*). Explants were cultured for 40 to 44 hours in serum-free supplemented Ham's F12 (Gibco) or with 5% heat-inactivated horse serum added, at 37°C in a 5% CO_2 environment.
14. In the presence of floor plate explants or induced neural epithelium, the vast majority of axons grew in fascicles that emerged perpendicular to the ventralmost edge of the dorsal explant and were directed toward the chick target tissue. Although there was some variability in bundle thickness, each bundle was scored as a single unit. Short single axons that were not target-directed were excluded from the analysis, because they were observed only rarely and with similar frequency whether dorsal explants were cultured alone or with targets. Commissural axons were identified by antibodies to TAG-1 (*8*). All axons that emerged in response to floor plate or induced neural epithelium expressed TAG-1. The ability of the chick floor plate or induced lateral tissue to orient commissural axons was determined by placing them adjacent to one end of a long dorsal explant (*8*). Floor plate and induced lateral neural epithelium, but not noninduced lateral neural epithelium, were able to reorient the growth of commissural axons.
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16. Notochords were removed from stage 10 chick donor embryos. The vitelline membrane of host chicks above the area to be operated was removed, an incision was made through ectoderm and mesoderm, and the endoderm was left intact. In stage 10 hosts, grafts were positioned adjacent to the closing neural tube at nonsegmented levels. In stages 11 to 14 hosts, grafts were positioned between the spinal cord and prospective somites 10 to 18. Eggshells were sealed and embryos allowed to develop a further 40 to 48 hours at 38°C. The approximate location of the grafted notochord was indicated by displacement of somites from the spinal cord. The grafted notochord often elongated in ovo. The grafted notochord and appropriate regions of the spinal cord were dissected and tested for expression of the chemoattractant.
17. Stage 7 notochord alone occasionally induced axon outgrowth (Fig. 3) (16% of explants, $n = 44$; 4 ± 1 bundles per positive explants, SEM; $P < 0.16$ when compared with dorsal explant cultured alone; Student's t test), although the amount is not sufficient to account for the chemotropic activity detected in neural plate-notochord cocultures. Stage 10 notochord, which can induce chemotropic activity in neural tissue in vitro (*20*), never evoked axon outgrowth ($n = 23$). The low amount of activity detected in stage 7 notochord may have been due to production of chemoattractant by the notochord or to leaching of chemoattractant previously released by the floor plate and bound to the notochord before its removal from the embryo.
18. The dorsal spinal cord explant may provide a cofactor that acts in concert with the notochord-derived signal to induce chemotropic activity in the lateral neural plate. However, we cannot test this directly because the assay of axon outgrowth requires the presence of spinal cord tissue.
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20. Stages 10 to 12 caudal ventral midline cultured alone with a dorsal explant did not evoke axon outgrowth [6% of explants, 2 bundles per positive explant (mean), $n = 16$]. In contrast, stages 10 to 12 caudal ventral midline cultured with stage 10 notochord opposite a dorsal explant evoked extensive outgrowth (88% of explants, 11 ± 3.7 bundles per positive explant SEM; $n = 8$). Axon outgrowth was also observed when stage 10 lateral neural tube was cultured together with stage 10 notochord (55% of explants; 7 ± 1.4 bundles per positive explant SEM; $n = 20$). This suggests that induction of chemotropic activity in the lateral neural epithelium is not dependent on the presence of a host floor plate within the neural tube.
21. Chemotropic activity can be detected at stages 6 to 7 in the midline of the neural plate at the axial level of the second somite. Ventral midline neural tube tissue from stages 10 to 12 that does not exhibit chemotropic activity was taken from the level of unsegmented mesoderm that will give rise to somites 11 to 20.
22. Notochord removal was as follows. Embryos were exposed and treated with in 0.15% trypsin. After incisions were made through ectoderm and mesoderm adjacent to the spinal cord, the notochord was freed from overlying neural tube and underlying endoderm. The notochord was removed, the spinal cord was placed back in its original position, and embryos were allowed to develop for a further 40 to 48 hours at 38°C. In sham operations, the notochord and spinal cord were displaced and then repositioned.
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Correction of a Defect in Mammalian GPI Anchor Biosynthesis by a Transfected Yeast Gene

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Glycosylphosphatidylinositol (GPI) serves as a membrane anchor for a large number of eukaryotic proteins. A genetic approach was used to investigate the biosynthesis of GPI anchor precursors in mammalian cells. T cell hybridoma mutants that cannot synthesize dolichol-phosphate-mannose (Dol-P-Man) also do not express on their surface GPI-anchored proteins such as Thy-1 and Ly-6A. These mutants cannot form mannose-containing GPI precursors. Transfection with the yeast Dol-P-Man synthase gene rescues the synthesis of both Dol-P-Man and mannose-containing GPI precursors, as well as the surface expression of Thy-1 and Ly-6A, suggesting that Dol-P-Man is the donor of at least one mannose residue in the GPI core.

A NUMBER OF EUKARYOTIC PROteins, such as Thy-1, Ly-6A, and the variant surface glycoprotein (VSG) of the protozoan *Trypanosoma brucei*, are attached to the cell membrane by a GPI anchor (*1, 2*). The GPI anchors of *T. brucei* VSG and rat brain Thy-1 have a remarkably conserved core structure (*3, 4*), which suggests that a common biosynthetic pathway may have been conserved throughout eukaryotic evolution. For VSG, the first step in GPI anchor biosynthesis is the transfer of *N*-acetylglucosamine (GlcNAc) from uridine 5′-diphosphate (UDP)–*N*-acetylglucosamine to phosphatidylinositol, which is followed by N-deacetylation to form glucosaminylphosphatidylinositol (*5, 6*). Subsequently, three mannose residues are added from either guanosine 5′-diphosphate (GDP)–mannose or Dol-P-Man, after which phosphoethanolamine is added. The completed GPI core is then transferred en