Ectopic neural expression of a floor plate marker in frog embryos injected with the midline transcription factor Pintallavis

(F-spondin/hepatocyte nuclear factor 3/fork head/amphibian development/*Xenopus*)

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ABSTRACT The floor plate, a cell group that develops at the midline of the neural plate in response to inductive signals from the notochord, has been implicated in the control of dorsoventral neural pattern. The frog Pintallavis gene, encoding a member of the HNF-3/fork head transcription factor family, is expressed in the notochord and in midline neural plate cells that give rise to the floor plate. To examine whether Pintallavis might be involved in regulating the differentiation of the floor plate, we ectopically expressed Pintallavis by injection of synthetic mRNA into two-cell frog embryos. Injection of Pintallavis mRNA resulted in the ectopic expression of F-spondin, a gene encoding a floor plate-specific adhesion molecule, at the dorsal midline of the neural tube. The expression of Pintallavis in midline cells may therefore contribute to the establishment of the floor plate fate.

The patterning of the nervous system in vertebrate embryos is established by signals that specify the fate of cells at distinct positions within the neural plate. Patterning of the neural plate is initiated during gastrulation as the dorsal mesoderm involutes under the prospective neural ectoderm (1). Regionalization of the neural plate along the anteroposterior and mediolateral (later dorsoventral, D-V) axes appears to involve both planar and vertical inductive signaling pathways. Signals that spread through the plane of the ectoderm may derive from the organizer (2–4) and from midline cells of the notoplate (3). Vertical signals derive from the prechordal plate and notochord and appear to cooperate with planar signals to establish the complete axial pattern (3, 5–7).

An early event in the elaboration of the D-V pattern of the neural tube is the induction of floor plate differentiation at the midline of the neural plate in response to vertical signals from the notochord (5, 8, 9). Notochord and floor plate cells then provide signals that induce the differentiation of many ventral neuronal types such as motor neurons (5). Signals from the floor plate also have later roles in the guidance of commissural axons (10–13). An understanding of the mechanisms involved in early patterning along the D-V axis of the neural tube requires a definition of the steps involved in floor plate induction. Identification of transcription factors that control the signaling properties of the notochord and the change in fate of midline neural plate cells should provide insights into this inductive interaction.

In frog embryos, a gene encoding a member of the hepatocyte nuclear factor 3 (HNF-3)/fork head family of transcription factors, Pintallavis (14) (XFKH1, ref. 15; and XFD1/1', ref. 16), is expressed by the notochord and by cells at the midline of the neural plate. Pintallavis expression by midline neural plate cells appears to depend on signals from the underlying notochord (14, 15), suggesting that it may

represent an early response to floor plate induction. A previous analysis of Pintallavis function involved the injection of synthetic Pintallavis mRNA into developing frog embryos. Embryos injected with Pintallavis mRNA exhibited defects in neural development along both the anteroposterior and D-V axes of the neural tube (14). Since Pintallavis expression in the neural plate is normally restricted to midline cells, it is possible that this gene is involved in floor plate differentiation. Ectopic expression of Pintallavis could therefore lead to the acquisition of floor plate properties by neural cells in other regions.

To address the possibility that deregulation of Pintallavis induces the expression of floor plate-specific traits, we have ectopically expressed Pintallavis in frog embryos and monitored changes in floor plate differentiation. As a marker of floor plate differentiation, we have assayed changes in the pattern of expression of F-spondin (17), a gene encoding a floor plate-specific neural adhesion molecule.*

MATERIALS AND METHODS

Library Screens. A tadpole-stage (stage 30) *Xenopus laevis* cDNA library was screened with the entire coding region of the rat F-spondin gene (17). Hybridization was performed at 60°C in 10% polyethylene glycol/7% SDS/220 mM NaCl/15 mM sodium phosphate, pH 7.4/1.5 mM EDTA containing denatured herring sperm DNA at 100 μg/ml. Six overlapping cDNA clones were isolated and the largest insert (4 kb) was sequenced on both strands.

Embryos. Pigmented and homozygous albino *X. laevis* embryos were obtained, reared, and staged by standard procedures (18). Exogastrulae were obtained by incubation in high salt on Petri dishes containing a layer of 2% agarose (3).

RNA Injections. Sense Pintallavis RNA was synthesized *in vitro* (19) and 2–5 ng was injected into one cell of two-cell embryos. Injected embryos were allowed to develop to the tadpole stage (stages 34–38) and assayed for F-spondin expression by whole-mount *in situ* hybridization. As a control, synthetic RNA encoding a truncated Pintallavis cDNA, which eliminates the DNA-binding domain (14), was also injected into developing embryos at the two-cell stage.

In Situ **Hybridization.** Whole-mount *in situ* hybridization (20) was performed using a digoxygenin-labeled antisense RNA probe consisting of the full F-spondin or Pintallavis (14) sequence. The probe was not hydrolyzed and the embryos were not prehybridized. The hybridization signal was detected with alkaline phosphatase-coupled anti-digoxygenin antibodies and reaction with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate, resulting in a blue

Abbreviations: D-V, dorsoventral; RLDx, rhodamine-lysine-dextran.
*The frog F-spondin sequence reported in this paper has been deposited in the GenBank data base (accession no. L09123).

precipitate. Embryos were viewed and photographed with a Zeiss Axiophot microscope.

Lineage Tracing. Rhodamine-lysine-dextran (RLDx) at 25 mg/ml in water (Molecular Probes) was injected into one cell at the two-cell stage. Injected embryos were fixed with formaldehyde, embedded in Paraplast, and sectioned on a microtome.

RESULTS

To provide a molecular marker with which to assess floor plate identity in frog embryos, the *X. laevis* homologue of the rat F-spondin gene (17) was cloned. The rat and frog F-spondin proteins exhibit 84% sequence identity and a similar domain organization (Fig. 1). Examination of the pattern of F-spondin expression by whole-mount *in situ* hybridization showed that in the hindbrain and spinal cord, F-spondin mRNA was restricted to a three- to five-cell-wide strip at the ventral midline corresponding to the floor plate (Fig. 2 *A* and *C–F*). F-spondin mRNA was first detected in single midline cells at the early tailbud stage (stages 20–22; Fig. 2*G*), and expression later expanded to occupy the entire ventral midline of the neural tube (Fig. 2 *A* and *C–F*). Beginning at approximately stage 30, low levels of F-spondin mRNA were also detected laterally in the midbrain and in the hypochord, a rod-like structure that underlies the notochord (Fig. 2 *C* and *D*).

Floor plate differentiation from neural plate cells appears to require contact-dependent signals from the underlying notochord (5, 8, 9). To determine whether F-spondin expression by neural plate cells requires the notochord, we examined its expression in exogastrula embryos, in which the neural ectoderm and notochord remain segregated (1, 3). F-spondin mRNA was absent from the neural ectoderm of exogastrula embryos (Fig. 2*B*), suggesting that F-spondin transcription in the neural tube depends on induction by the notochord. Thus the expression of F-spondin can be used to define floor plate differentiation in frog embryos.

To test whether ectopic expression of Pintallavis can induce markers of floor plate differentiation at ectopic sites within the neural tube, we examined the distribution of F-spondin transcripts in tadpoles injected at the two-cell stage with synthetic Pintallavis mRNA. Consistent with previous results (14), injection of Pintallavis mRNA resulted in neural defects which arose, at least in part, from its expression in mesoderm. Sixty-five to 70% of injected embryos displayed abnormal neural axis formation at tailbud stages, most notably a loss of anterior neural structures and an expansion of posterior neural tissue (Fig. 3 *C, E, H,* and *I*). Injection of similar amounts of other, unrelated transcription factors did not result in the same phenotype (14), indicating that the effects of Pintallavis are specific. Fifteen percent (22/145) of injected embryos exhibited ectopic F-spondin expression. None of 218 control embryos displayed ectopic F-spondin expression (Table 1). Ectopic F-spondin mRNA was found only in the hindbrain, where it was detected in cells adjacent to the ventral (Fig. 3 *C, E,* and *G*) and dorsal (Fig. 3 *A, B, D,* and *F*) midlines at a similar frequency. The effect of Pintallavis mRNA injection was reproducible and specific. Injection of equal amounts mRNA encoding a mutant form of Pintallavis lacking the putative DNA-binding domain did not result in ectopic F-spondin expression (*n* = 83 embryos; Table 1).

The failure to detect F-spondin mRNA along the entire D-V axis of the neural tube could, in principle, result from the differential segregation of injected mRNA within the neural tube. To determine whether there was segregation of injected molecules within the neural tube, we performed a lineage analysis by coinjecting Pintallavis mRNA with RLDx. We used RLDx, a stable macromolecule, since the analysis of Pintallavis mRNA stability (see below) showed that the level of injected transcripts at the time of neural tube closure was too low to detect. In >50% of injected embryos (*n* = 30), RLDx was found uniformly in the injected half of the neural tube and the underlying mesoderm (Fig. 2*J*). Although the


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F-spondin    NH2 [                          ▨▨▨▨▨▨▨▨▨   ] COOH
                                             1  2  3  4  5  6

Frog         MGLIFOPLFWQYVATSYALMVLGFLDETVEKAIKSEGYCSRILRAQGGTRKEGYNEFSMR    60
Rat          MRLSPAPLRLSRGPALLALALPLAAA.A.S...LD.VA............-...R...T...L.

             VEGDPEFYKPGNTYRVTILAVSPAYFRGFTLIALKEGKEGEKEEDHAGSFQIIDEEDTQF   120
             .....D.....SS....LS.AP.S............NR..D.......T.......E...

             MSNCPVAVTESTPRRRTRIQVFWTAPSIGTGCVILKASIVQKKIIYFQDEGSLTKRMCEL   180
             .................I..PT...............R............KL..Q

             DLTLEGGNEKTIPDCCACGTAKYRLTFYGNWSEKAHPKDYPRRANHWSAIIGGSHSGEYV   240
             .P..D.VTDRP.L..................T.....................KN..

             LWEYGQASDGVKQVAELGSPVKMEEEIRQKGDEVLTVIKAKAQWPAWQPLNVRAAPSAEF   300
             .....G..E..................QS.............S...V.........

             SVDRSRHLMSFLAMMGPSPDWNVGLTSEDLCTKECGWVQKVVQDLIPWDAGTDSGVTYES   360
             ....T.......T........SA......................

             PNKPTIPQDKIRPLTSLDHPQSPSMTRGGPIIPIARVVIERIARKGEQCNIIPDNVDDIV   420
             ........E..............FYDP..S.TQV..............V........
                                                        ┌1─────
             ADLVTEEKDEDDTPETCIYSNWSPWSACSSATCDKGKRMRQRMLKAQLDLSVPCPDTQDF   480
             ...AP........................S..E.....................
             ┌────────────2────────
             EPCMGPGCSDDEASTCMMSEWITWSPCSASCGMGIEVERERYVKQFPEDGSLCKVPTEETE   540
             Q.........EDG...T............V.....MRS.............V.ML.....
             ┌─│ │──────3──────
             KCIVNEECEPSSCIVTEWAEWEECSATCRMGMKKRHRMIKMTPADGSMCKADTTEVEKCM   600
             ..T...S....L....G..DD.....G.........V..S.........E.SQA....
             ┌─│ │──────4──────
             MPECHTIPCVLSPWSEWSDCSVTCGKGTRTRQRMLKSPSELGDCNEELELKQVEKCMLPE   660
             .........L..............M.........LA.......D..--.A.......
             ─│ │───────5──────
             CPISCELTEWSYWSECNKSCGKGHMIRTRMITMEPQFGGAVCPETVQRKKCRLRKCQKSS   720
             ...D...S...Q..................T.Q........P...........A...LR.P
                                 ┌────6────
             GNERRHLKDAREKRRSEKIKEDSDGEQYPVCKMKPWTAWTECTKFCGGGIQERFMTVKKR   780
             SIQKLRWRE...S....QLR.E.....F.G.R.R.....S....L.........Y.....
             ──────────│
             FKSSQFTSCKDKKEIRACNVHPC                                        803
             ......................
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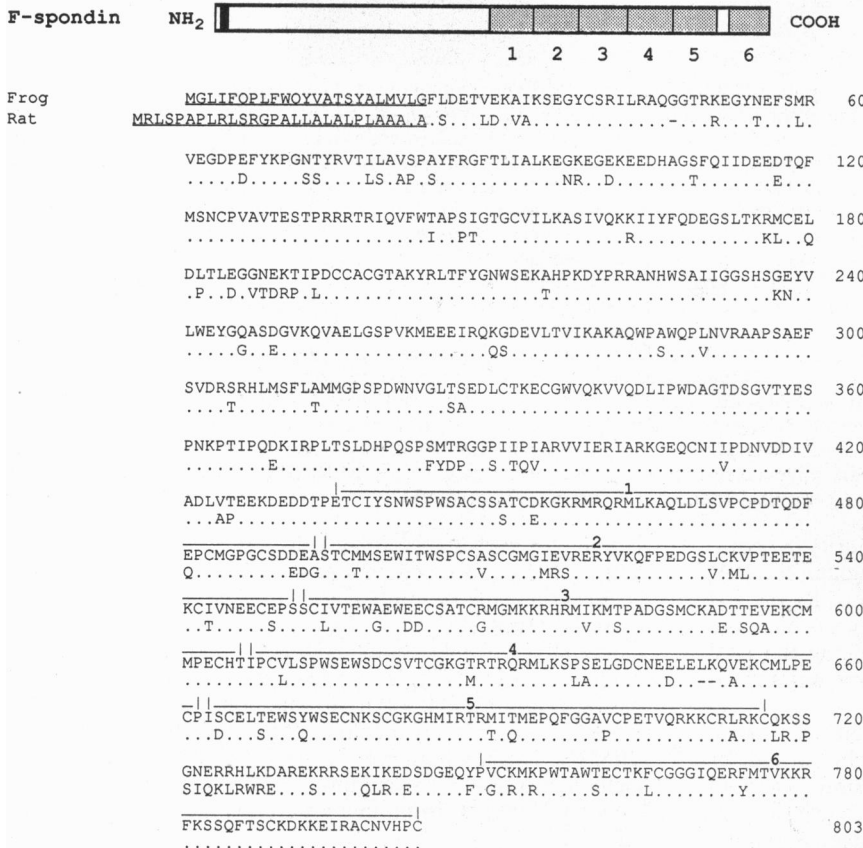
FIG. 1. Comparison of the frog and rat F-spondin proteins. (*Upper*) Schematic representation of the domain structure of F-spondin protein. The black box represents the signal sequence. The gray boxes represent the six thrombospondin type I repeats (TSRs). (*Lower*) Amino acid alignment of the frog and rat F-spondin proteins. Underlined NH2-terminal sequences correspond to the putative signal sequences. Bars above the sequence delineate the TSRs, numbered 1–6 as in the schematic. The overall similarity of the frog and rat F-spondin proteins is 84%. The NH2-terminal domain of the proteins, which does not contain the TSRs, exhibits 86% identity, indicating that this domain may have a role in the structure or function of the protein. Apart from the signal sequence, the only region of extensive sequence divergence is the highly charged segment between TSRs 5 and 6. Numbers at right refer to the frog F-spondin sequence.
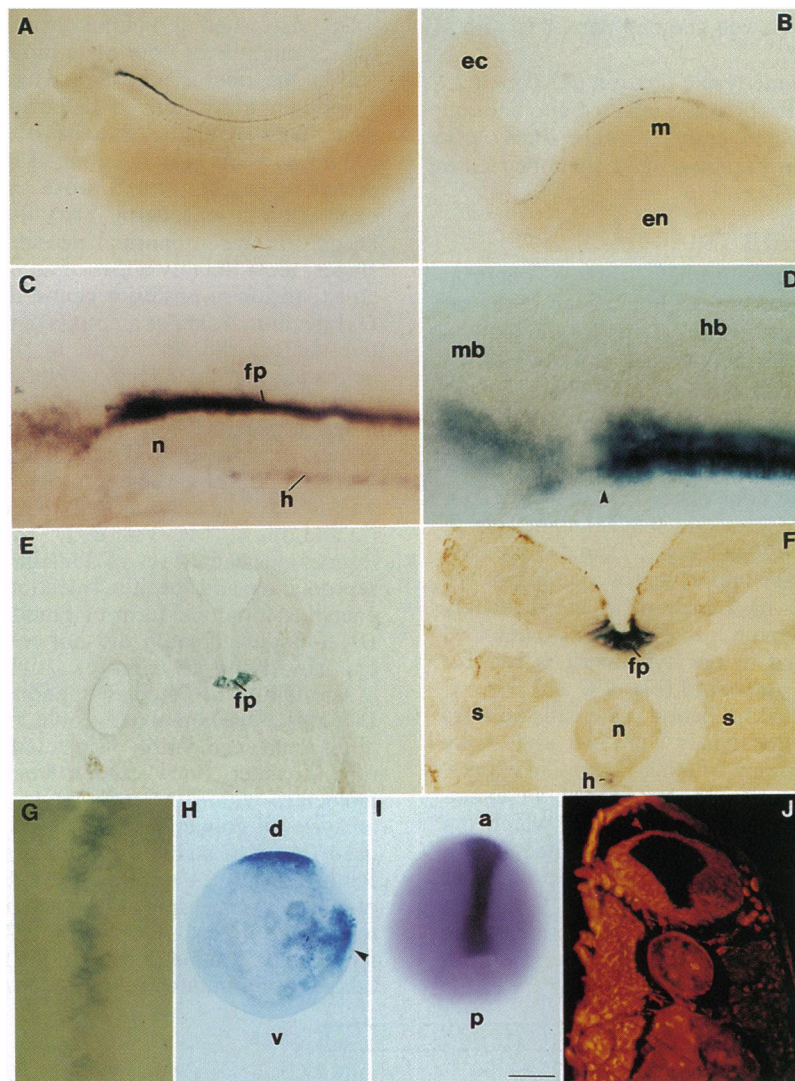
FIG. 2.   Localization of F-spondin mRNA in frog embryos. (*A*) Whole-mount *in situ* hybridization of a *X. laevis* tadpole, stage 34–36, showing F-spondin RNA in the floor plate at the ventral midline of the neural tube and in cells of the hypochord. Note the higher F-spondin expression in hindbrain compared with spinal cord. (*B*) Whole-mount *in situ* hybridization of an exogastrulated embryo at a stage equivalent to that in *A*, showing that F-spondin RNA is not present in the ectoderm (ec). In contrast, F-spondin RNA is detected in cells of the hypochord found adjacent to the notochord (n). en, Endoderm; me, mesoderm. (*C* and *D*) High magnification of midbrain–hindbrain region of tadpole (stage 36) labeled in whole-mount, showing F-spondin RNA in the floor plate (fp) of the hindbrain and in the hypochord (h), located under the notochord (n). There is also F-spondin RNA in the lateral region of the midbrain. In contrast to the expression in rat embryos (17), in frogs F-spondin is not expressed by the most anterior floor plate cells located in the midbrain and there is a clear boundary of expression in the floor plate at the boundary of the midbrain (mb) and the hindbrain (hb) (arrowhead in *D*). F-spondin is expressed at low levels in the posterior part of the notochord and in branchial arches (data not shown). (*E* and *F*) Cross sections of the hindbrain of tadpoles (stage 36) after whole-mount *in situ* hybridization. F-spondin RNA is restricted to the floor plate (fp) at the ventral midline. In *F*, F-spondin RNA is detected in the hypochord (h), an embryonic endodermal structure located ventrally to the notochord (n). s, Somites. (*G*) Dorsal view of the anterior hindbrain of a tailbud-stage (stage 22) embryo after whole-mount *in situ* labeling, showing early expression of F-spondin by small groups of cells at the ventral midline of the neural tube. (*H* and *I*) Whole-mount *in situ* hybridization of late blastula (stage 9) (*H*) and late gastula (stage 12.5) (*I*) embryos. (*H*) Vegetal view showing normal expression of Pintallavis in the organizer region, located dorsally, and the injected RNA (arrowhead). d, Dorsal; v, ventral. (*I*) Dorsal view of an embryo injected with Pintallavis RNA, showing normal expression of Pintallavis in the midline. a, Anterior; p, posterior. (*J*) Cross section of a tadpole (stage 36) injected with RLDx into one cell at the two-cell stage, showing unilateral restriction of the injected tracer. Note the labeling of decussated axons in the ventral funiculus of the unlabeled half. [Bar = 600 μm (*A* and *B*), 150 μm (*C*), 30 μm (*D*), 40 μm (*E* and *J*), 20 μm (*E* and *G*), or 200 μm (*H* and *I*).]

remaining embryos exhibited mosaicism, a majority of neural cells were labeled, and these were found throughout the D-V axis of the neural tube. Thus, the distribution of RLDx and that of other injected molecules (21–23) suggests that the restriction of ectopic F-spondin expression along the D-V axis of the neural tube does not result from differential segregation of Pintallavis mRNA.

One possible explanation for the detection of ectopic F-spondin expression in only a fraction of embryos and for the restriction of ectopic expression to the hindbrain is a rapid decay of injected RNAs after the midblastula transition (24). To examine the stability of injected message, synthetic

Pintallavis mRNA was injected at the two-cell stage and detected later in development by whole-mount *in situ* hybridization. Injected RNA was present at high levels at blastula stages (stages 6–7; Fig. 2*H*; n = 15) but at levels only marginally above the threshold for detection in late gastrula–neurula embryos (Fig. 2*I*; n = 15). Thus, by the time of neural plate formation and floor plate induction, the level of injected RNA was much lower than that of the endogenous transcript (Fig. 2*I*). This finding supports the idea that the relatively low incidence of embryos exhibiting ectopic F-spondin expression could result from the progressive decay of injected mRNA.
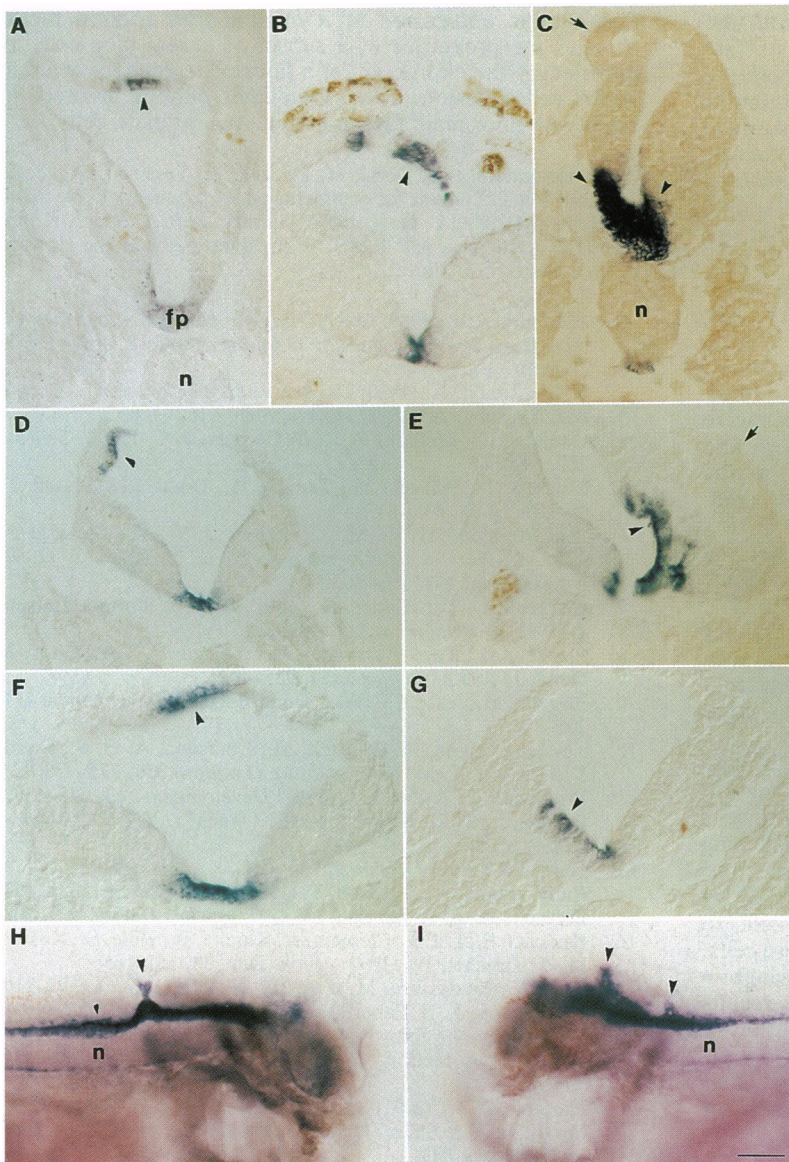
FIG. 3. Localization of F-spondin mRNA in Pintallavis mRNA-injected tadpoles. (*A–G*) Cross sections of the hindbrain of tadpole stage embryos injected with Pintallavis RNA at the two-cell stage, showing the localization of F-spondin RNA by whole-mount *in situ* hybridization. F-spondin RNA is expressed in floor plate (fp) cells at the ventral midline of the neural tube. Arrowheads depict the sites of ectopic F-spondin RNA expression. Embryos shown in *A*, *B*, *D*, and *F* show ectopic F-spondin RNA in cells adjacent to the dorsal midline, which form part of the roof plate. In *B* and *D* the dorsal cells were displaced slightly during the labeling procedure. The embryo shown in *B* was pigmented. Embryos shown in *C*, *E*, and *G* show an expanded area of F-spondin expression adjacent to the floor plate. *C* shows F-spondin RNA expression in hypochord cells under the notochord (n). As previously shown (8), injection of Pintallavis RNA leads to an increase in the amount of posterior neural tissue in affected embryos. *C* and *E* show enlarged neural tubes (arrows) and (in *C*) a secondary neurocoel. (*H* and *I*) Lateral view of tadpoles (stage 36) injected with Pintallavis RNA after whole-mount *in situ* localization of F-spondin RNA. Arrowheads point to the sites of ectopic F-spondin expression. The embryo in *H* faces right and that in *I* faces left. In all cases, dorsal side is up. [Bar = 10 μm (*A–G*) or 200 μm (*H* and *I*).]

In addition, the incidence of ectopic F-spondin expression in injected embryos ($\approx$15%) was lower than that of overt neural defects ($\approx$65%) (14). Our previous studies have provided evidence that many of the neural defects obtained after injection of Pintallavis mRNA can result from early actions of Pintallavis on mesoderm at the time of neural induction (14). In contrast, the induction of F-spondin expression is likely to result from later actions of Pintallavis during neural plate formation, providing a possible explanation for the lower incidence of affected embryos. The restriction of ectopic F-spondin to the hindbrain may also result from the progressive decay of injected mRNA. Floor plate differenti-

Table 1. Summary of Pintallavis RNA injection results

Injected RNA	No. of embryos		
	Total	Normal	Ectopic
Pintallavis	145	123	22
Mutant	83	83	0
Uninjected	135	135	0

The results of three independent experiments are summarized. Numbers refer to embryos displaying normal or ectopic F-spondin RNA patterns as assayed by whole-mount *in situ* labeling followed by serial sectioning. The mutant Pintallavis RNA derives from a template carrying a frameshift mutation and encodes a truncated protein lacking the putative DNA-binding domain (14).

ation in the hindbrain occurs several hours before that in the spinal cord (Fig. 2*G* and data not shown; see ref. 8). Thus, Pintallavis mRNA may be present at levels sufficient to induce F-spondin expression only in the hindbrain, and there only in a fraction of injected embryos. Despite these technical limitations, detection of the ectopic neural expression of a floor plate marker *in vivo* provides evidence that Pintallavis is involved in the control of floor plate differentiation.

DISCUSSION

Expression of the transcription factor Pintallavis is transient and restricted to midline cells of all three germ layers during gastrulation and neurulation (14–16). Within the neural plate, Pintallavis is expressed by midline cells that give rise to the floor plate of the neural tube. Here we show that deregulation of Pintallavis by injection of synthetic mRNA into developing embryos leads to the ectopic expression of F-spondin, a gene encoding a floor plate-specific adhesion molecule, in the neural tube of injected tadpoles.

Ectopic F-spondin expression was detected only in cells adjacent to the floor plate and in the roof plate. Cells that give rise to all D-V regions of the hindbrain and spinal cord, however, appear to be competent to differentiate into floor plate in response to inductive signals from the notochord (5, 8, 9). Thus, the restriction in expression of F-spondin RNA

along the D-V axis of the neural tube, together with the widespread distribution of injected mRNA, suggests that recipient cells differ in their competence to respond to Pintallavis. One possible explanation for the restriction of F-spondin expression is the existence of positive cofactors in the ventral and dorsal midline regions or of inhibitory factors in other areas. For example, homeobox genes such as *Ghox-2.9* (the chicken *HoxB1* gene; ref. 25) show widespread expression along the D-V axis of the hindbrain but are excluded from the floor plate and roof plate.

The present results, however, do not resolve whether ectopic Pintallavis functions in the same cells that eventually express F-spondin ectopically. It remains possible, for example, that ectopic Pintallavis confers, in a selective manner, floor plate-inducing properties to lateral plate mesoderm, which underlies the lateral edges of the neural plate that give rise to the roof plate. Indeed, cells of the notochord and lateral plate mesoderm may share properties, such as the expression of the twist gene (26), that could facilitate Pintallavis function.

The primary domain of ectopic F-spondin expression occurs adjacent to the dorsal midline in the roof plate. Since floor plate progenitors never populate the roof plate (27–29), the expression of F-spondin by roof plate cells provides strong evidence that the fate of neural plate cells has been changed. These results also raise the possibility that roof plate and floor plate precursor cells share underlying properties.

The domain of ectopic F-spondin expression that is adjacent to the ventral midline could also result from a change in fate of cells that are located next to the floor plate. These cells may normally be exposed to levels of a floor plate-inducing signal from the notochord that are insufficient to induce floor plate differentiation. The reinforcement of this signal by ectopic Pintallavis expression could cause these cells to differentiate as floor plate. The ventral position of these cells, however, makes it difficult to exclude that the change in ventral expression of F-spondin in injected embryos results from the enhanced proliferation or abnormal migration of floor plate progenitors.

Additional markers will be required to determine whether roof plate cells that express F-spondin display a full floor plate phenotype. Based on the results of notochord grafts in chicken embryos (5, 30), one functional consequence of a dorsally located floor plate is the suppression of dorsal cell markers and the induction of ventral cell types in adjacent regions of the neural tube. In our previous analysis of the ectopic expression of Pintallavis mRNA (14) we observed an inhibition of the differentiation of Rohon–Beard neurons, a dorsal neural cell type. In the light of the present findings, it is possible that the suppression of Rohon–Beard differentiation reflects the expression of floor plate-like properties in dorsal midline cells.

In normal development, the expression of Pintallavis and that of F-spondin in midline neural plate cells overlap for only a short period, suggesting that Pintallavis may be involved in activating but not in maintaining the transcription of F-spondin. Genes encoding cell adhesion molecules can be activated directly *in vitro* by transcription factors implicated in the specification of cell identity (31, 32). Our *in vivo* results show

that ectopic expression of a midline transcription factor changes the properties of a subset of neural tube cells as revealed by the expression of a floor plate gene involved in neural cell adhesion. Thus, Pintallavis may be involved in specifying floor plate fate in midline neural plate cells.

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