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Activin A inhibits *Pax-6* expression and perturbs cell differentiation in the developing spinal cord *in vitro*

(dorsoventral patterning/floor plate/motor neuron/*Pax* gene regulation)

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ABSTRACT **We have developed an *in vitro* model of the isolated chicken neural plate. Here we demonstrate that even in the absence of notochord, the neural plate rapidly develops a typical dorsoventral patterning. This observation suggests that the ventral cell types are specified or at least predetermined prior to notochord formation and that permissive conditions are sufficient for differentiation of ventral structures. Treatment of the neural plate with activin A extinguishes *Pax-6* gene expression, whereas the dorsal markers *Pax-3* and *Pax-7* are still expressed. The absence of *Pax-6* transcripts can be correlated with an impeded differentiation of the motor neurons, whereas the floor plate seems to be enlarged. We propose that the region-specific expression of *Pax-6* in the spinal cord is under the control of activin-like molecules.**

Several members of the vertebrate paired box (*Pax*) gene family are expressed in restricted regions of the developing spinal cord (1). While *Pax-3* and *Pax-7* transcripts are restricted to the dorsal ventricular zone, *Pax-6* transcripts are present in more-ventral regions. The region-specific expression of these transcription factors suggests that they might play an important role as regulators of the dorsoventral patterning in that structure.

There is increasing evidence that cell differentiation in the ventral part of the spinal cord is controlled by signals emanating from the ventral midline cell groups: the notochord and the floor plate (2). While grafting a second notochord lateral to the neural plate induces the differentiation of an ectopic floor plate and of ectopic columns of motor neurons, the absence of notochord leads to the absence or delayed differentiation (3) of these ventral cell types. Interestingly, a strong reorganization of the *Pax* gene expression domains precedes the ectopic differentiation or the absence of differentiation of ventral cell types (4, 5). In that context, *Pax-3* and *Pax-6* are of particular interest because they constitute very early markers responding to the ventralizing effect of the ventral midline cells.

The aim of the present study is to identify the signaling molecules involved in the regulation of the region-specific expression of the *Pax* genes. For this purpose, we have established an *in vitro* model. The caudal neural plate of a stage 10 chicken embryo was isolated by microsurgery and cultured with or without the notochord in a collagen matrix. This *in vitro* model presents two main advantages: (*i*) to show the potential for differentiation of the caudal neural plate and (*ii*) to allow the effect of various factors on *Pax* gene expression and on cell differentiation to be tested. We have investigated the possible influence of activin A on the region-specific expression of the *Pax* genes in the spinal cord. Recent data suggest that this molecule, first involved in cell specification in mesoderm induction (6), might play an important role in neurogenesis (7). Further, in tailbud-stage *Xenopus* embryo, inhibin/activin mRNA was detected, among other places, in the notochord (8). Since a high level of transcripts coding for the activin type IIA receptor are present during the differentiation of chicken neuroepithelium (9), we used activin A as a tool to determine the influence of such molecules on *Pax* gene expression and on cell differentiation.

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MATERIALS AND METHODS

Microsurgery. A piece (300–400 μm) of the open neural plate positioned above Hensen's node (Fig. 1*A*) was excised from stage 9–10 chicken embryos (10) after the surrounding tissues were cleared off in 0.1% trypsin dissolved in Ca^{2+}- and Mg^{2+}-free Tyrode's solution. The dorsal limit of the dissection was at the junction between the neuroepithelium and the surface ectoderm. When present, the notochord was kept attached in its normal ventral position. In some cases the open neural plate was divided into presumptive floor plate, ventral plate, intermediate plate, and dorsal plate (11). The explanted tissue was then washed in F-199 culture medium (Sigma) supplemented with 5% fetal bovine serum, 2 mM L-glutamine, and 1% antibiotic solution (penicillin, 5000 units/ml; streptomycin, 5000 μg/ml; GIBCO).

Explant Cultures. The explanted tissues were placed in a tissue culture insert (diameter, 10 mm; Nunc) containing 40 μl of rat tail collagen mixture (3 mg/ml; rat tail collagen was from Boehringer Mannheim). Explants were cultured in the medium described above at 37°C in a 5% CO_2 atmosphere. At least two groups of five cultures were analyzed in each case except when mentioned in *Results*.

Activin A Treatment. A recombinant human activin A (12) was diluted to the desired final concentration in culture medium containing 5% fetal bovine serum. The medium was replaced every 24 hr. To control for the specificity of the activin treatment, culture medium containing activin A at 150 ng/ml was incubated for 2 hr at 37°C with follistatin fixed on Affi-Gel beads (Bio-Rad) (a gift from Y. Eto, Central Research Laboratories, Ajinomoto, Japan), centrifuged for 10 min at 8500 × *g* and then used as culture medium. Activin effects were completely abolished in such conditions. The percent inhibition of *Pax-6* expression with increasing concentrations of activin was quantified with the NIH IMAGE 1.56 program (kindly provided by W. Rasband, National Institutes of Health). The domain expressing *Pax-6* was measured with this program in five independent explants.

Whole-Mount *in Situ* Hybridization. *Pax-3*, *Pax-6*, and *Pax-7* transcripts were analyzed with antisense RNA probes corresponding respectively to nt 468–1113 (13), 345–908 (14), and 86–720 (15) of the equivalent mouse cDNA. Prior to fixation, the cultures were incubated for 1–2 min with collagenase type V (Sigma) dissolved at 1 mg/ml in 50 mM tricine, pH 7.5/0.4 M NaCl/10 mM $CaCl_2$. The whole-mount *in situ* procedure was performed according to Wilkinson (16).

Whole-Mount Antibody Staining. After collagenase treatment as described above, immunostaining was monitored

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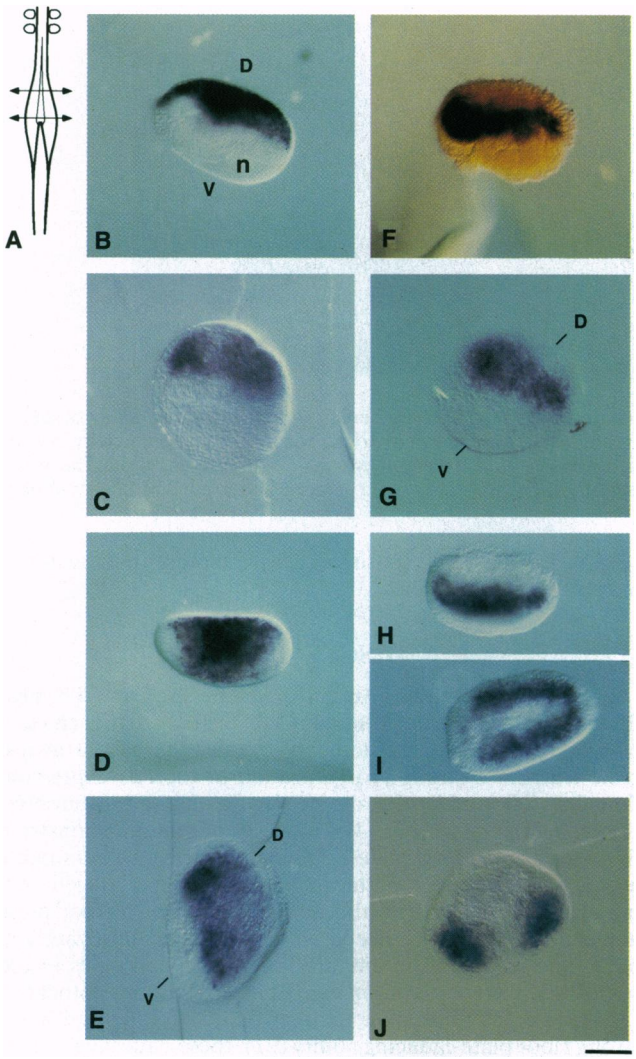

FIG. 1. *Pax-3* and *Pax-6* transcripts detected by whole-mount *in situ* hybridization in 1-day-old neural plate culture. Schematic representation (*A*) shows the level of explantation of the open neural plate on a stage 10 chicken embryo. *Pax-3* (*B–E*) and *Pax-6* (*G–J*) expression domains were observed in the presence (*B, C, F,* and *G*) or absence (*D, E,* and *H–J*) of notochord. Double *in situ* detection (*F*) shows the respective expression domain of *Pax-3* (in red) and *Pax-6* (in blue) in the presence of notochord. *B, D, F,* and *H* represent lateral views and *I* represents a ventral view of the whole explants. *C, E, G,* and *J* correspond to representative cross-sections through such cultures. In each photograph the dorsal side of the explant is at the top, except where indicated otherwise. n, Notochord; D, dorsal; V, ventral. [Bar = 100 μm (*B, D, H,* and *I*), 90 μm (*F*), or 50 μm (*C, E, G,* and *J*).]

according to Wright *et al.* (17). The absence of notochordal cell contaminations on the neural plate cultured alone was verified with the monoclonal antibody Not1 obtained from the Developmental Studies Hybridoma Bank (data not shown). The polyclonal antibody to Isl-1 (working dilution, 1:100) was a gift from S. Thor (18). The monoclonal antibody JC7/SC1 (hereafter JC7; working dilution, 1:500) was a gift from J. Covault (19). The appropriate secondary antibodies were purchased from Organon Teknika–Cappel.

Vibratome Sectioning. The samples were embedded in a gelatin/albumin mixture and sectioned at 30 μm with a vibrating microtome.

RESULTS

Expression of *Pax* Genes in the Caudal Neural Plate Grown 24 hr in Culture. To determine whether the neural tube would respond to regulatory signals in the same way *in vitro* as *in vivo*, small pieces of the open neural plate of stage 10 embryos were explanted and cultured in a collagen matrix with or without the underlying notochord (Fig. 1*A*). After 24 hr *in vitro* these cultures were analyzed by whole-mount *in situ* hybridization with antisense RNA probes recognizing *Pax-3, Pax-6,* or *Pax-7* transcripts.

In the presence of notochord, *Pax-3* expression was restricted to the most dorsal part of the neural tube (Fig. 1 *B* and *C*), whereas *Pax-6* was expressed in the dorsolateral part of this tissue (Fig. 1 *F* and *G*) partially overlapping with the *Pax-3*-expressing region but extending more ventrally. As expected, no staining was observed in the most ventral part of the neural tube. In the absence of notochord, the ventral limit of *Pax-3* expression extended more ventrally but still excluded the midline cells (Fig. 1 *D* and *E*). Surprisingly, *Pax-6*, which is either absent or expressed at a very low level in the most ventral part of the neural tube after deletion of the notochord *in vivo*, was still strongly expressed in these cultures as two bands on both side of the ventral midline (Fig. 1 *H–J*). Nevertheless, like *Pax-3*, the expression domain of *Pax-6* also showed a clear ventral shift (Fig. 1*H*). The pattern of *Pax-7* expression was comparable to the one observed for *Pax-3* (data not shown).

In the absence of notochord, the strong *Pax-6* expression can be dependent on the presence of the cells at the ventral midline. To test this hypothesis, the open neural plate was separated into ventral, intermediate, and dorsal plate and each piece was cultured separately. Despite the absence of ventral midline cells, *Pax-6* expression was detected in ventral plates (see Fig. 4*B*) but not in lateral or dorsal plates (data not shown).

Differentiation of Ventral Cell Types in the Open Neural Plate Grown *in Vitro*. The possible differentiation of ventral cell types in our culture model was analyzed by double immunostaining. We used a polyclonal antibody against Isl-1 (islet 1), which is the earliest marker for motor neurons (18), and the monoclonal antibody JC7, which recognizes a cell surface adhesion molecule present both on the motor neurons and on the floor plate (19). Apart from these ventral cell types, both antibodies label cells located in the dorsal root ganglion and in the dorsal part of the neural tube.

When the open neural plate was cultured for 3 days in the presence of notochord, two symmetric groups of Isl-1-positive nuclei were clearly visible in the lateral plates of the neural tube (Fig. 2*A*). Some of these cells were also JC7-immunopositive. In these cultures, an average of 39 ± 8 Isl-1-positive nuclei per section (n = 8 cultures, 5 sections counted per culture) were found positioned in these two columns of putative motor neurons. A strong JC7 immunostaining was also observed in a triangular structure at the level of the ventral midline. These JC7-positive but Isl-1-negative cells constituted the floor plate (Fig. 2*A*).

In the absence of notochord, again two symmetric groups of Isl-1/JC7-positive cells were observed in 3-day-old cultures (Fig. 2*B*). The Isl-1-positive nuclei, localized more ventrally than in the presence of notochord, were also less numerous (15 ± 4 positive nuclei per section, n = 7 cultures, 5 sections per culture). The presence at the ventral midline of cells that were JC7-immunopositive but Isl-1-negative suggested that a floor plate-like structure developed in the neural tube even in the absence of notochord (Fig. 2*B*).

Activin A Abolishes *Pax-6* Expression in a Dose-Dependent Manner. To determine whether activin A could regulate *Pax* gene expression in the neural plate, various concentrations of this factor, 5–600 ng/ml, were added to the culture medium. In choosing this range of concentrations, we took into account that 5 ng/ml (0.2 nM) corresponds to 1 unit (12) and that an activin A concentration of 200 ng/ml is necessary to increase goosecoid mRNA accumulation in mouse embryos (20). The

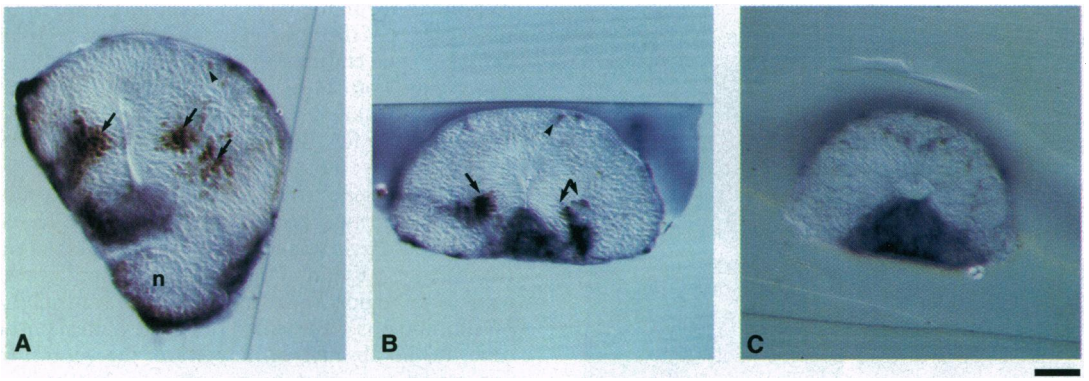

FIG. 2. Cell differentiation in neural plate cultures. (*A* and *B*) Vibratome sections showing the characteristic double immunostaining observed in 3-day-old cultures in the presence (*A*) or absence (*B*) of notochord. Isl-1 immunoreactivity is shown in brown and JC7 immunoreactivity in blue. Note the presence of a few positive cells in the dorsal part of the neural tube (arrowhead). (*C*) Effect of activin A on cell differentiation. The motor neuron columns are no longer visible, but some scarce positive cells are present in the neural tube. A very strong and expanded JC7 staining is observed at the ventral midline. n, Notochord. (Bar = 50 μm.)

neural plates explanted without notochord were cultured in the presence of activin A for 24 hr, fixed, and analyzed for *Pax-6* expression.

For doses lower than 50 ng/ml, no significant effect on *Pax-6* expression was observed. At 50 ng/ml, *Pax-6* expression was clearly reduced, with only 50% of the staining left (Fig. 3*B*). The characteristic expression pattern of *Pax-6* (i.e., two bands of positive cells in both side of the ventral midline) was still discernible, suggesting that the progressive disappearance of the transcripts was not accompanied by a ventral shift (Fig. 3*B*). At 100 ng/ml, the effect was more pronounced, with an ≈75% decrease in the staining (Fig. 3*C*); above 150 ng/ml, *Pax-6* expression was completely abolished (Fig. 3*D*).

To define whether the effect of activin A on *Pax-6* expression was mediated by the cells at the ventral midline, the ventral plate was separated from the ventral midline and from the intermediate plate and cultured in the presence of activin A. *Pax-6* expression was abolished in ventral plate cultured in the presence of activin (Fig. 4), showing that the effect can be independent of the presence of the ventral midline cells.

Expression of the Dorsal Markers *Pax-3* and *Pax-7* Is Not Abolished by Activin A. To determine whether activin A would similarly influence the expression of the dorsal *Pax* genes, the neural plates were cultured for 24 hr in presence of activin A (150 ng/ml) and the expression of *Pax-3* and *Pax-7* was analyzed. Both *Pax-3* and *Pax-7* were expressed in the activin-treated cultures (Fig. 5).

Effect of Activin A Treatment on Cell Differentiation. To determine whether activin would affect cell differentiation *in vitro*, double immunostaining experiments were performed as described above on 3-day-old cultures treated with activin A (150 ng/ml). While the presence at the ventral midline of JC7-positive but Isl-1-negative cells suggested that a floor plate-like structure still differentiated in the presence of activin A, the two clusters of Isl-1/JC7-positive cells, clearly visible on both sides of the floor plate in the control cultures, were generally not identifiable in the activin-treated cultures (Fig. 2*C*). Some scarce positive cells were present in the dorsal part of the neural tube.

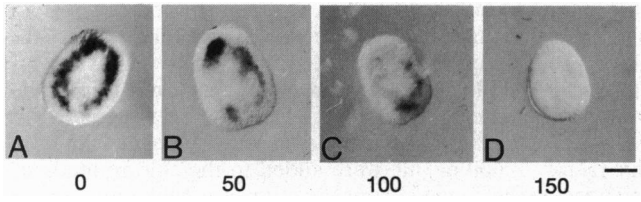

FIG. 3. *Pax-6* expression in neural plates (ventral view) cultured for 24 hr without activin A (*A*) or with activin A at 50 ng/ml (*B*), 100 ng/ml (*C*), or 150 ng/ml (*D*). (Bar = 100 μm.)

DISCUSSION

Permissive Conditions Are Sufficient for the Caudal Neural Plate to Differentiate Ventral Cell Types. In the chicken embryo, deletion of the notochord under the caudal neural plate leads to a strong reorganization of the *Pax* expression domains, followed by the absence or the delayed differentiation of ventral structures (4). However, our data demonstrate that the caudal neural plate isolated in culture displays rapidly a typical dorsoventral patterning according to *Pax* gene expression domains. Later, the differentiation of a floor plate and of motor neurons proceeds in such cultures. Other authors have reported the differentiation of ventral cell types in the spinal cord differentiating *in vitro* or *in vivo* without notochord (3, 11). Therefore, despite the fact that the notochord has a strong floor plate-inducing ability (21), these data suggest that the initial inductive interaction takes place before notochord organization. This suggestion supports the model proposed by Artinger and Bronner-Fraser (3), in which either the floor plate is induced early by the chordamesoderm of Hensen's node or limited interactions between predisposed ventral neural midline cells and the notochord immediately after neurulation are sufficient for floor plate determination. An alternative is that the prospective ventral neuroepithelial cells are predetermined to differentiate a floor plate according to their lineage. The observation that prospective median (ventral) and prospective lateral neuroepithelial cells exhibit behavior differences prior to notochord inductive interaction supports such a hypothesis (22).

Nevertheless, permissive conditions are necessary to get the expression of these ventral traits. Indeed, in the chicken embryo 2 days after notochord ablation, the neural tube presents a dorsalized fate as indicated by the pattern of *Pax* gene

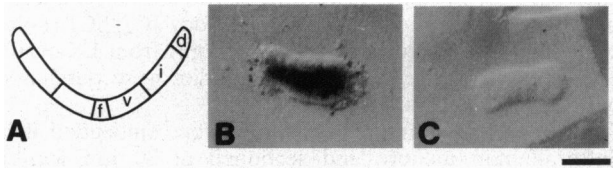

FIG. 4. Activin abolishes *Pax-6* expression even in the absence of midline cells. (*A*) Schematic representation showing the limits of dissection for the presumptive floor plate (f), ventral plate (v), intermediate plate (i), and dorsal plate (d). (*B* and *C*) One-day-old ventral plates (v) cultured in the absence (*B*) or presence (*C*) of activin. (Bar = 100 μm.)

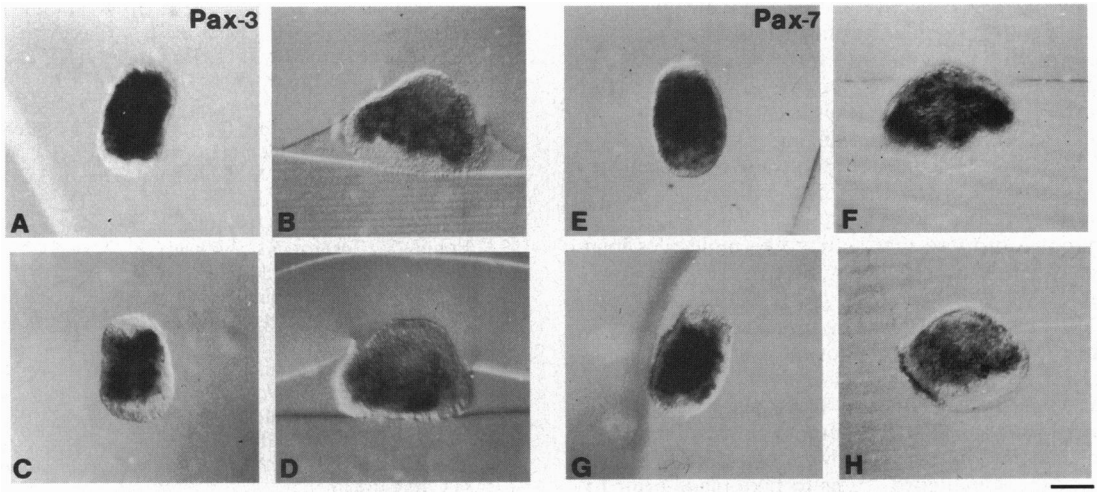

FIG. 5. *Pax-3* and *Pax-7* expression in neural plate explants treated with activin A. Whole mount *in situ* staining shows *Pax-3* (*A–D*) or *Pax-7* (*E–H*) expression in 1-day-old control cultures (*A, B, E,* and *F*) or in 1-day-old activin-treated (150 ng/ml) cultures (*C, D, G,* and *H*). *A, C, E,* and *G* are ventral views of whole neural tubes, and *B, D, F,* and *H* are the corresponding cross-sections. In *B, D, F,* and *H* the dorsal side of the section is at the top, and the ventral side is at the bottom. [Bar = 100 μm (*A, C, E,* and *G*) or 50 μm (*B, D, F,* and *H*).]

expression as well as by the absence of ventral cell-type differentiation (4, 23). Since the neural plate never undergoes a complete dorsalization *in vitro*, this may reflect either the presence of permissive ventralizing factor(s) in these cultures (perhaps due to the presence of 5% fetal bovine serum) or the absence of inhibitors of the ventral differentiation. *In vivo*, such an inhibitory effect could be due to the surface ectoderm and/or the paraxial mesoderm, both of which are in close contact with the developing neural tube.

Activin-Like Molecules and Dorsoventral Polarity of the Neural Tube. *Pax-6* expression in the spinal cord is initially detected in a broad band of cells, with the exception of prospective roof plate and floor plate cells, and then is progressively repressed in both the dorsal and the ventral region, resulting in the region-specific expression of *Pax-6* in the mid-lateral region of the spinal cord (4). Are activins expressed at the right time and place to regulate *Pax-6* expression in the spinal cord? After neural tube closure, inhibin/activin βB mRNA is detected in cells located in the dorsal part of the neural tube and in the notochord (27). Therefore, activins may be involved in both the dorsal and ventral downregulation events which lead to *Pax-6* expression in the mid-lateral region of the spinal cord (see model in Fig. 6). The grafting of a supernumerary notochord beneath the closing neural plate rapidly represses *Pax-6* expression in cells adjacent to the graft (4), an observation compatible with the hypothesis that activins may play an important role in the regulation of *Pax-6* expression in the ventral part of the spinal cord.

Besides inhibiting *Pax-6* expression, activin also affects cell differentiation in our *in vitro* model (Fig. 6). In the ventral part of the neural tube, the two motor neuron columns are either absent or underdeveloped after treatment with activin. Three main scenarios can be proposed to explain the absence of motor neuron differentiation. (*i*) Activin A may mimic the effect of dorsalizing factors such as Dsl-1 (24). Dsl-1, which is present in the dorsal region of the spinal cord, has been shown to inhibit the differentiation of the motor neurons *in vitro*. However, activin A seems to stimulate floor plate differentiation, an observation which is difficult to explain as a dorsalin-like effect. (*ii*) The absence of motor neuron differentiation observed in activin-treated explants may be directly related to the prior extinction of *Pax-6* expression. (*iii*) High concentrations of activin A may favor the differentiation of other neural cell types at the expense of motor neuron differentiation (Fig. 6). Activins have been shown to act as morphogens in mesoderm induction (6) and could play a similar role in spinal cord development. Several studies indicate that distinct classes of neural cells in the ventral neural tube are induced by the ventral midline cell groups, the floor plate, and notochord (23, 25). Cells in the proximity of a source of activin would be

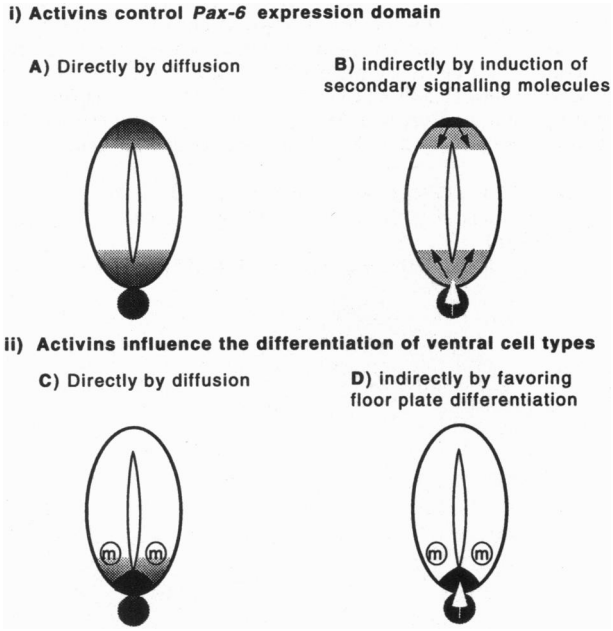

FIG. 6. Diagrams summarizing the possible functions of activin-like molecules in the regulation of *Pax-6* expression and cell differentiation. (*i*) Establishment of the region-specific expression of *Pax-6*. The two putative sources of activins are cells in the dorsal part of the neural tube and the notochord. (*A*) Activins may diffuse from these sources and repress *Pax-6* expression, leading to its specific expression domain (in white on the diagram). (*B*) Despite the fact that activin A can abolish *Pax-6* expression *in vitro* in the absence of ventral midline cells (see *Results*), we cannot exclude the possibility that, in the ventral and/or dorsal zone of the neural tube, activins induce secondary signaling molecules involved in *Pax-6* regulation. (*ii*) Activins may influence ventral cell differentiation. With (*C*) or without (*D*) activin diffusion throughout the neural tube, the cells exposed to the highest concentrations of activins in the ventral part of the neural tube are those positioned in the proximity of the notochord, such as the floor plate precursors. High concentrations of activins may favor their differentiation, whereas low concentrations or no activin may be a permissive condition for cells positioned further away from the notochord, such as the motor neurons.

submitted to the highest concentrations of that factor. High concentrations of activins would thus favor the differentiation of these cells whereas low concentrations of activin or the absence of activin could be the permissive condition for differentiation of cells which are farther away from the activin source. An interesting observation in agreement with this hypothesis is that high concentrations of activin A seem to favor the differentiation of the floor plate whereas differentiation of motor neurons may be repressed.

In conclusion, we propose that activin-like molecules may play an important role as regulators of the region-specific expression of *Pax-6* and therefore in the control of the dorsoventral polarity in the developing spinal cord. Further, it has recently been suggested that the formation of the nervous system might be the result of the derepression of a default state (26). The data presented here lead us to suggest that the differentiation of ventral cell types could proceed by following the same scenario. The initial inductive event leading to the ventralization of the spinal cord seems to take place prior to notochord formation. Therefore, ventral cell types are already specified in the open neural plate and permissive conditions would be sufficient for their differentiation. In the absence of notochord, the ectopic presence of inhibitor(s) could repress the differentiation of these precursor cells. The presence of the notochord would create permissive conditions for their differentiation. Their differentiation would then be the result of the derepression of a default state.

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