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Antagonizing cAMP-dependent protein kinase A in the dorsal CNS activates a conserved Sonic hedgehog signaling pathway

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SUMMARY

Hedgehog (Hh) signaling plays a significant role in defining the polarity of a variety of tissue types along the anterior/posterior and dorsal/ventral axes in both vertebrate and invertebrate organisms. The pathway through which Hh transduces its signal is still obscure, however, recent data have implicated the cyclic AMP-dependent protein kinase A as a negative regulator of the Hh signal transduction pathway. One of the vertebrate *Hh* family members, *Sonic hedgehog* (*Shh*), can induce ventral neural cell types both in vivo and in vitro; high concentrations induce floor plate and lower concentrations motor neurons. To investigate whether PKA plays an active role in the suppression of ventral neural differentiation, we generated transgenic embryos expressing a dominant negative form of PKA (dnPKA) in primarily dorsal aspects of the mouse CNS. Similar to our earlier results with Shh, we observed the induction of floor plate and motor neuron markers in embryos expressing the dominant negative PKA transgene and the loss of dorsal gene expression at rostral levels. Thus suppression of PKA activity is sufficient to activate targets of the Shh signaling pathway in the vertebrate CNS suggesting that induction of ventral cell types occurs via the antagonistic action of Shh on PKA activity. Two mammalian target genes that are strongly expressed in ectopic dorsal locations in response to dnPKA are *Ptc* and *Gli*. As both of these are targets of *Drosophila* Hh signaling, our data point to an evolutionary conservation in both the mechanisms of signaling and the effectors of the signaling pathway.

Key words: sonic hedgehog, cAMP-dependent protein kinase A, CNS patterning, transgenic mouse

INTRODUCTION

The notochord has been known for some time to act as the source of an inductive signal(s) with the capacity to polarize both the neural plate and paraxial mesoderm (reviewed in Placzek, 1995). Recent grafting experiments in the chick have shown that a signal emanating from the notochord is capable of inducing medial neural plate cultures to become floor plate in a contact-dependent fashion (Placzek et al., 1993) while promoting the differentiation of motor neurons in a contact-independent fashion (Yamada et al., 1993). A notochord-derived signal has also been implicated in restricting dorsal gene expression in the neural tube (Goulding et al., 1993) as well as participating in the differentiation of sclerotome from paraxial mesoderm (Pourquie et al., 1993). Once established, the floor plate also serves as the source of a signal(s) with similar inductive properties as the notochord (reviewed in Placzek, 1995). Until recently, it was unclear as to whether a single or multiple signals were responsible for the aforementioned activities. However, current data support the view that a single signaling molecule, *Sonic hedgehog* (*Shh*), is a notochord/floor plate-derived inducer of differentiation.

Shh RNA and protein are localized to the notochord and floor plate when these tissues are known to exert their inductive influences (Echelard et al., 1993; Riddle et al., 1993; Krauss et al., 1993; Roelink et al., 1994; Chang et al., 1994; Martí et al., 1995a). Furthermore, the amino-terminal peptide of Shh can independently induce floor plate and a diverse set of ventrally defined neuronal cell types along the entire length of the rostrocaudal axis of the embryonic neural tube (Roelink et al., 1995; Martí et al., 1995b; Hynes et al., 1995; Ericson et al., 1995; Wang et al., 1995). Moreover, antibodies raised against amino-terminal Shh can block the notochord-derived induction of motor neurons (Martí et al., 1995b). Experiments have also shown that dorsal neuroepithelial cell fates can be repressed by culturing dorsal or intermediate neural explants in conditioned medium containing amino-terminal Shh (Roelink et al., 1995). Finally, infection of dorsal somitic mesoderm by a retrovirus expressing *Shh* or in vitro cultures of presomitic mesoderm incubated with amino-terminal Shh were shown to result in the ventralization of the somitic mesoderm as assessed by the

ectopic activation of the sclerotomal marker *Pax-1* (Johnson et al., 1994; Fan et al., 1994, 1995).

From the described activities of Shh, a number of compelling questions remain to be addressed including the identification of the effectors mediating the transmission of Shh signaling. Shh is initially synthesized as a 45×10^3 M_r precursor protein, which enters the secretory pathway and undergoes signal peptide cleavage in addition to an autoproteolytic cleavage event yielding a 19×10^3 M_r (amino-terminus) peptide, which remains tightly associated with the cell surface, and a 26×10^3 M_r (carboxy-terminus) peptide, which freely dissociates from the cell (Lee et al., 1994; Bumcrot et al., 1995; Porter et al., 1995). All known signaling activities ascribed to Shh, or its *Drosophila* homologue hh reside in the amino-terminal peptide (reviewed in Ingham, 1995). Interestingly, there appears to be a concentration-dependent mode of action to amino-Shh inductions, with a significantly lower concentration requirement for the induction of motor neurons compared to floor plate (Roelink et al., 1995). Analysis of the crystal structure of the amino-terminal Shh peptide has revealed structural similarity with known zinc hydrolases raising the speculation that long-range Shh signaling (sclerotome and motor neuron induction) is achieved through the release of amino-terminal Shh from the cell surface via an intermolecular proteolytic activity (Tanaka Hall et al., 1995).

Some insight into the manner by which hh transduces its signal has come from experiments performed in *Drosophila* which implicate the cAMP-dependent protein kinase A (PKA) signal transduction pathway. Clones of cells lacking PKA activity in the anterior margins of wing or leg disks and anterior to the morphogenetic furrow in the eye disc induce ectopic wing duplications or ectopic furrows (Jiang and Struhl, 1995; Li et al., 1995; Strutt et al., 1995; Lepage et al., 1995; Pan and Rubin, 1995). These findings mimic the effects of ectopically expressing *hh* in anterior regions of imaginal disks (reviewed in Perrimon, 1995; Kalderon, 1995; Ingham, 1995). Initial findings in vertebrates have also implicated the PKA pathway in regulating Shh signaling. Using pharmacological agents which increase cAMP levels Fan et al. (1995) and Hynes et al. (1995) were both able to block the Shh-mediated induction of sclerotome and midbrain-derived dopaminergic neurons, respectively. In addition, expression of dominant negative and constitutively active forms of PKA in the zebrafish embryo lead to the transcriptional activation or inhibition of several targets of the Shh signaling pathway, respectively (Hammerschmidt et al., 1996; Concordet et al., 1996).

To further our understanding of the role that PKA plays in Shh signaling in the vertebrate CNS, we generated transgenic mouse embryos expressing a dominant negative form of PKA in primarily dorsal aspects of the CNS utilizing the *Wnt-1* enhancer. The cAMP-dependent PKA holoenzyme is composed of two regulatory and two catalytic subunits. The enzyme is inactive in the tetrameric state and only becomes active upon the binding of four cAMP molecules by the regulatory subunit resulting in the release of the active catalytic subunits, which then allows for their nuclear translocation and subsequent phosphorylation of specific target genes. The dominant-negative mutation utilized in our experiments maps to the cAMP-binding domain of the regulatory subunit and prevents dissociation of the holoenzyme upon cAMP binding (Clegg et al., 1987).

We report here that ectopic expression of the dominant-negative form of *PKA* mimics the effects of ectopic expression of *Shh* (Echelard et al., 1993; Goodrich et al., 1996). These include the dorsal activation of floor plate markers, *HNF-3β* and *Shh*, motor neuron induction and induction of *Ptc* and *Gli*, two vertebrate homologues of *Drosophila* genes involved in the regulation and transduction of the hh signaling pathway. Taken together, these results suggest that PKA is a negative regulator of the Shh signaling pathway in the vertebrate CNS. Furthermore, some of the downstream targets of hh signaling in *Drosophila* are conserved targets in the mammalian CNS.

MATERIALS AND METHODS

Preparation of construct and generation of transgenic embryos

The *Wexp3-dnPKA* construct was prepared by blunt end ligation of a 1.3 kb *Xba*I fragment containing the *PKA* dominant negative regulatory subunit coding sequence (Clegg at al., 1987) into the *Eco*RV site of the *Wexp3* expression construct. The only modification in *Wexp3* from the previously published *Wexp2* expression construct is the insertion of an 825 bp portion (*Eco*RV to *Sac*I) of the *lacZ* gene immediately 3′ of the *Eco*RV cloning site, which provides a tag for the transgene-derived RNA (Fig. 1).

For microinjection, the *Wexp3-dnPKA* construct was linearized with *Sal*I, gel purified by electroelution and applied to a 'Wizard DNA Clean Up System' column (Promega). The DNA was diluted to 2 ng/μl in 10 mM Tris, 1 mM EDTA and injected into the male pronuclei of (C57BL/6J×CBA/J) F_1 (Jackson Labs) zygotes as described by Hogan et al. (1994). Pseudopregnant Swiss Webster (Taconic) females were used as recipients of the injected embryos. G_o mice were collected at 10.5, 12.5 and 14.5 dpc, and photographed using an Olympus SZH stereo microscope and Kodak EPY 64T color slide film, and further processed as described below.

Embryos carrying the *Wexp3-dnPKA* transgene were identified by PCR analysis of Proteinase K (Boehringer-Mannheim)-treated yolk sacs as previously described (Echelard et al., 1993). PCR genotyping was performed using an upstream primer specific for the *PKA* regulatory subunit cDNA (primer no. 1420) and a downstream primer

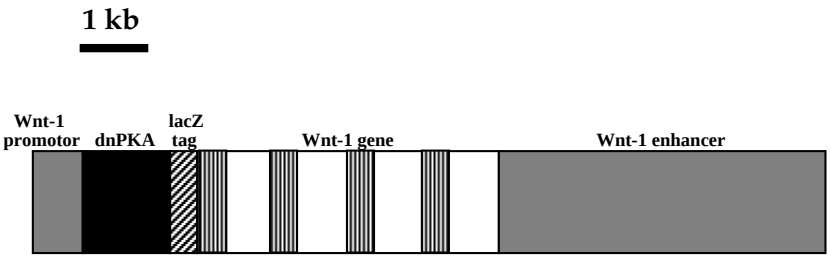

Fig. 1. Organization of the *Wexp3-dnPKA* transgene. Solid gray bar at the 5′ end of the construct represents the *Wnt-1* promoter. Solid gray bar at the 3′ end of the construct represents the *Wnt-1* enhancer. Black box indicates the dominant-negative regulatory subunit cDNA. Diagonally hatched box represents 825 base pairs of untranslated *lacZ* tag. Longitudinally striped boxes and white boxes represent the *Wnt-1* gene which harbors a disrupted translational start site.

specific for the *lacZ* tag sequence (primer no. 137) yielding a PCR-positive product of 228 bp under the following conditions: 1 minute at 94°C, 1 minute at 60°C, 1 minute at 72°C for 30 cycles followed by a final extension at 72°C for 10 minutes.

In situ hybridization, immunostaining and histological analysis

In situ hybridizations on sections were performed with [^{35}S]UTP-labeled RNA probes as previously described (Wilkinson, 1992). The following riboprobes used in the present experiments were described elsewhere: *Shh* (Echelard et al., 1993); *HNF-3β* (Sasaki and Hogan., 1993); *Ptc* (Goodrich et al., 1996); *Gli* (Hui et al., 1994); *cRet* (Pachnis et al., 1993); *Pax-3* (Goulding et al., 1991); *lacZ* (Eco RV/Eco ICR1) subclone, linearized with Eco R1 and transcribed with T7 polymerase). HNF-3β whole-mount antibody staining was performed as in Martí et al. (1995a).

General histological analysis of wild-type and *Wexp3-dnPKA* transgenic embryos was performed on 4% paraformaldehyde- or Bouin's-fixed embryos that were paraffin-embedded, sectioned and stained with haematoxylin and eosin.

RESULTS

To ascertain whether inhibition of cAMP-dependent PKA activation in areas of the dorsal CNS would mimic the inductive properties of Shh, we generated transgenic embryos that ectopically expressed a dominant-negative form of *PKA* under the transcriptional control of the *Wnt-1* enhancer. The *Wnt-1* enhancer, previously defined as a 5.5 kb stretch of DNA 3′ of the *Wnt-1* locus, directs proper temporal and spatial expression of the *Wnt-1* gene and has previously been utilized for the misexpression of transgenes, including *Shh*, to the *Wnt-1* expression domain (Echelard et al., 1993, 1994). The earliest expression of *Wnt-1* is detected in the presumptive midbrain just prior to somite formation (Wilkinson et al., 1989). As the neural folds close to form the neural tube, *Wnt-1* becomes localized to a ring of expression just anterior of the mid/hindbrain junction as well as the ventral midbrain and dorsal aspects of the diencephalon, midbrain, myelencephalon and spinal cord (Wilkinson et al., 1989; Parr et al., 1993). Expression of various forms of *Shh* under the control of this regulatory element ventralizes the dorsal brain and spinal cord (Echelard et al., 1993; B. St-Jacques, D. Rowitch and A. P. M., unpublished data).

One copy of the mutated *PKA* regulatory subunit cDNA was cloned into the *Wnt-1* misexpression construct (*Wexp3-dnPKA*) depicted in Fig. 1. The linearized construct was injected into fertilized eggs and generation zero (G_0) embryos were collected at either 10.5, 12.5 or 14.5 dpc (Table 1). A progressive CNS phenotype with varying degrees of severity was detected at all stages in 19% of the embryos carrying the *Wexp3-dnPKA* transgene. Only those transgenic animals displaying an obvious and consistent morphological phenotype were analyzed in detail.

Morphological aberrations in embryos expressing the *Wexp3-dnPKA* transgene

Of 18 transgenic embryos collected at 10.5 dpc, three displayed morphologically detectable phenotypes. All three embryos showed an unusual cranial morphology including the disruption of the mid/hindbrain isthmus, a flattened and thickened rather than rounded shaped midbrain and, in one embryo, an uncharacteristic bulging of the neural tube in the region of the diencephalon (Fig. 2B). The spinal cord at this stage showed no obvious malformations in any of the 10.5 dpc transgenic embryos.

Table 1. Summary of *Wexp3-dnPKA* transgenics

Embryonic age (days post coitum)	Total number of embryos collected	Number of transgenic embryos collected	Number of embryos displaying a detectable phenotype
10.5 dpc	44	18	2
12.5 dpc	64	17	3
14.5 dpc	37	17	5
Total	145	52 (35.8%)*	10 (19.2%)†

*Percent of total embryos collected that are transgenic.
†Percent of transgenic embryos displaying a phenotype.

At 12.5 dpc three of 17 *Wexp3-dnPKA* transgenic embryos were identified with a striking CNS phenotype consisting of an unusual ruffling of the dorsal tissue in both brain and spinal cord regions. In the most severe case, the ruffling was present along the entire length of the spinal cord. Histological analysis of two of these embryos revealed that, while the ventral aspect of the CNS appeared normal in morphology, dorsal regions of the CNS were highly disrupted from the diencephalon to the spinal cord (Fig. 3). An apparent overproliferation within the dorsal neuroepithelium resulted in the expansion of dorsal ventricular zone cells overlying the caudal most aspect of the third ventricle (dorso/caudal diencephalon) (Fig. 3C) and the entire midbrain (Fig. 3D-F). As a consequence of the overproliferation, the most rostral aspect of the midbrain vesicle has overgrown and come to lie atop the diencephalic vesicle (Fig. 3C). At the level of the mid/hindbrain junction, no cerebellar anlage was observed (Fig. 3E,F). Transverse sections at the level of the spinal cord revealed a striking overproliferation in the dorsal half of the spinal cord resulting in the branching of the central canal and overlying ventricular zone into two or more folds (Fig. 3G). A similar proliferative effect has been reported in the spinal cord of transgenic embryos ectopically expressing *Shh* under the control of the *Wnt-1* enhancer (Echelard et al., 1993). Interestingly, the presence of dorsal root ganglia suggests that, despite the dorsal expression of the *dnPKA* transgene, neural crest cells were formed (Fig. 3G).

The phenotype of the *Wexp3-dnPKA* transgenic embryos at 14.5 dpc was generally consistent with the phenotype observed at 12.5 dpc. Of the seventeen 14.5 dpc transgenic embryos, five showed morphological abnormalities with varying degrees of severity. Three showed fluid-filled cysts arising from the dorsal midbrain in addition to a disrupted mid/hindbrain isthmus (data not shown). Two other transgenic embryos showed more severe phenotypes including a similar, albeit, substantially larger overgrowth of tissue overlying the midbrain compared to that observed at 12.5 dpc (data not shown). Transverse sections through the spinal cord of one of these embryos showed a highly disorganized dorsal half of the neural tube. The lumen did not appear to extend dorsally. In fact, at the level of the sulcus limitans, it split in two with each half progressing dorsolaterally rather than dorsally. The ventral half of the spinal cord was normal in appearance.

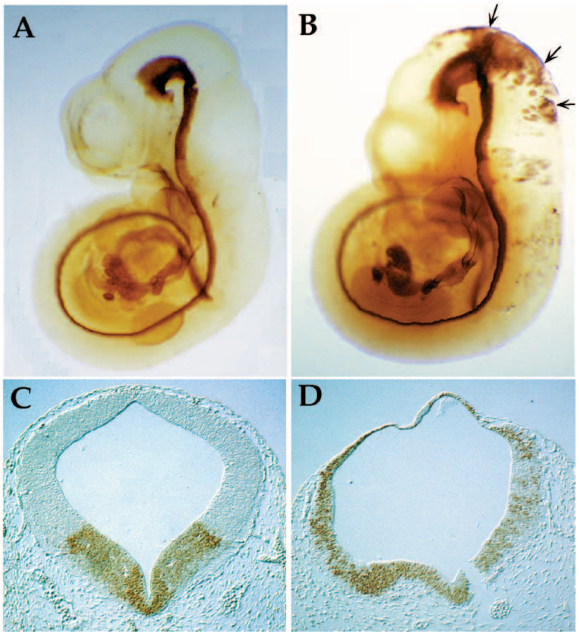

Fig. 2. HNF3β immunostaining of 10.5 dpc wildtype and *Wexp3-dnPKA* transgenic embryos. Whole-mount immunostaining of a wild-type (A) and transgenic (B) embryo showing the normal and ectopic distribution of HNF3β (arrows). (C) Section through the caudal midbrain of a wild-type embryo showing the ventral restriction of HNF3β expression. (D) Section through the mid-hindbrain junction of a transgenic embryo showing ectopic expression of HNF3β in lateral and dorsal neural tissue.

Ectopic activation of floor plate and motor neuron markers

A substantial number of experiments have recently documented the ability of Shh to mediate the induction of floor plate and distinct ventral neurons. The type of neurons formed is dependent on the anterior-posterior level of the responding neural tissue (Echelard at al., 1993; Krauss et al., 1993; Roelink et al., 1994, 1995; Ericson et al., 1995; Martí et al., 1995b; Hynes et al., 1995; Wang et al., 1995). To determine whether the *Wexp3-dnPKA* transgene was also capable of ectopically activating similar ventral fates, whole-mount immunostaining and *in situ* hybridizations were performed with a variety of floor plate- and neuron-specific markers at various stages of development.

Expression of the winged-helix transcription factor, *HNF-3β*, marks the ventral midline of the CNS, from the diencephalon to the spinal cord, and is the earliest marker of ventral CNS development in the mouse (Ruiz i Altaba et al., 1993; Sasaki and Hogan, 1993; Martí et al., 1995a). Both in vitro and in vivo studies indicate that *HNF-3β*, and related genes in other vertebrates, can activate and be maintained by *Shh* (Echelard et al., 1993; Ruiz i Altaba et al., 1995). Whole-mount antibody staining of 10.5 dpc *Wexp3-dnPKA* transgenic embryos showed ectopic HNF-3β broadly distributed in the region of the dorsal midbrain, mid/hindbrain junction and dorsal hindbrain (compare Fig. 2A,B), where the *Wnt-1* enhancer is known to be expressed at its highest levels (Echelard et al., 1994). At the mid/hindbrain junction, where the transgene was expressed in a complete circle, HNF-3β expression was no longer restricted to its normal ventral

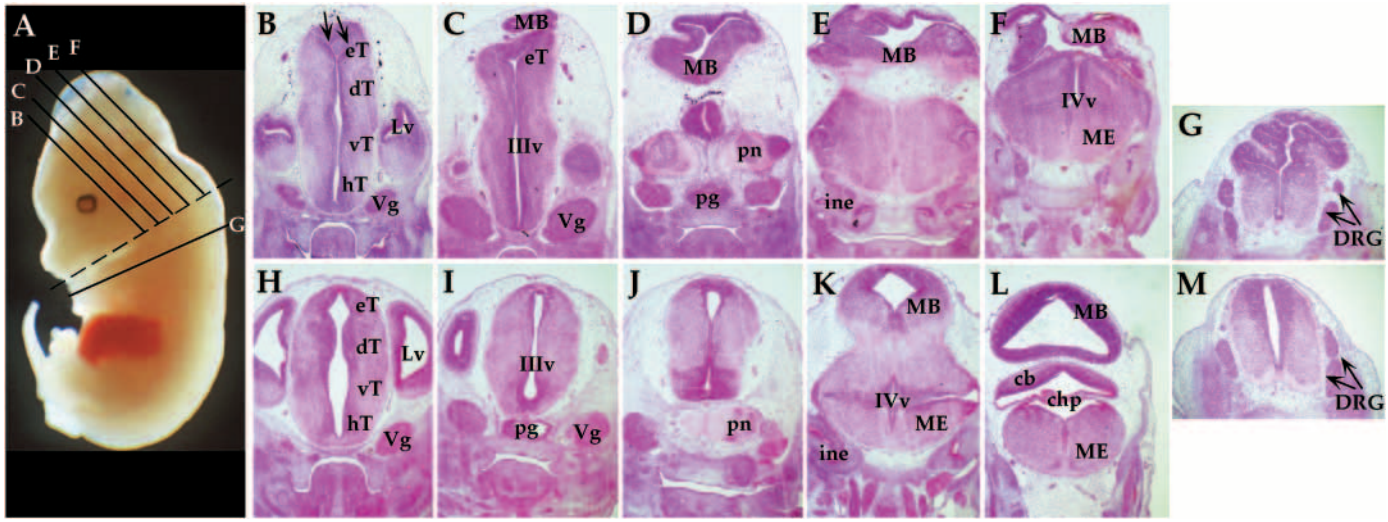

Fig. 3. CNS morphology of a 12.5 dpc *Wexp3-dnPKA* transgenic embryo compared to its wild-type littermate. (A) Transgenic embryo showing planes of section for the accompanying panel. In all panels dorsal is up. (B) Section through the diencephalon showing the splitting of the dorsal part of the third ventricle (arrows) as the most rostral aberration observed in the *dnPKA* transgenic brain. Caudal ends of the telencephalic vesicles are seen at each side of the diencephalon. (C) Section through the caudal diencephalon showing midbrain overgrowth (MB) overlaying the dorsal most diencephalon (epithalamus, eT). (D,E) Sections through the rostral (D) and caudal (E) midbrain showing aberrant morphology of the midbrain, while ventral tegmental areas appear to have normal morphology (pontine nuclei, pn). (F) Section through the midbrain-hindbrain junction showing the highly disrupted morphology of the midbrain (MB) and absence of the cerebellar anlage. (G) Section through the cervical spinal cord showing a prominent overproliferation of the dorsal ventricular zone. (H-M) Corresponding sections through a 12.5 dpc wild-type mouse brain. All sections were stained with haematoxilin and eosin. Abbreviations: cb, cerebellum; chp, choroid plexus; DRG, dorsal root ganglia; dT, dorsal thalamus; eT, epithalamus; hT, hypothalamus; ine, inner ear (semicircular canals); ME, medulla; pg, pituitary gland; pn, pontine nuclei; vT, ventral thalamus; Vg, trigeminal ganglion; Lv, lateral ventricle; IIIv, 3rd ventricle; IVv, 4th ventricle.

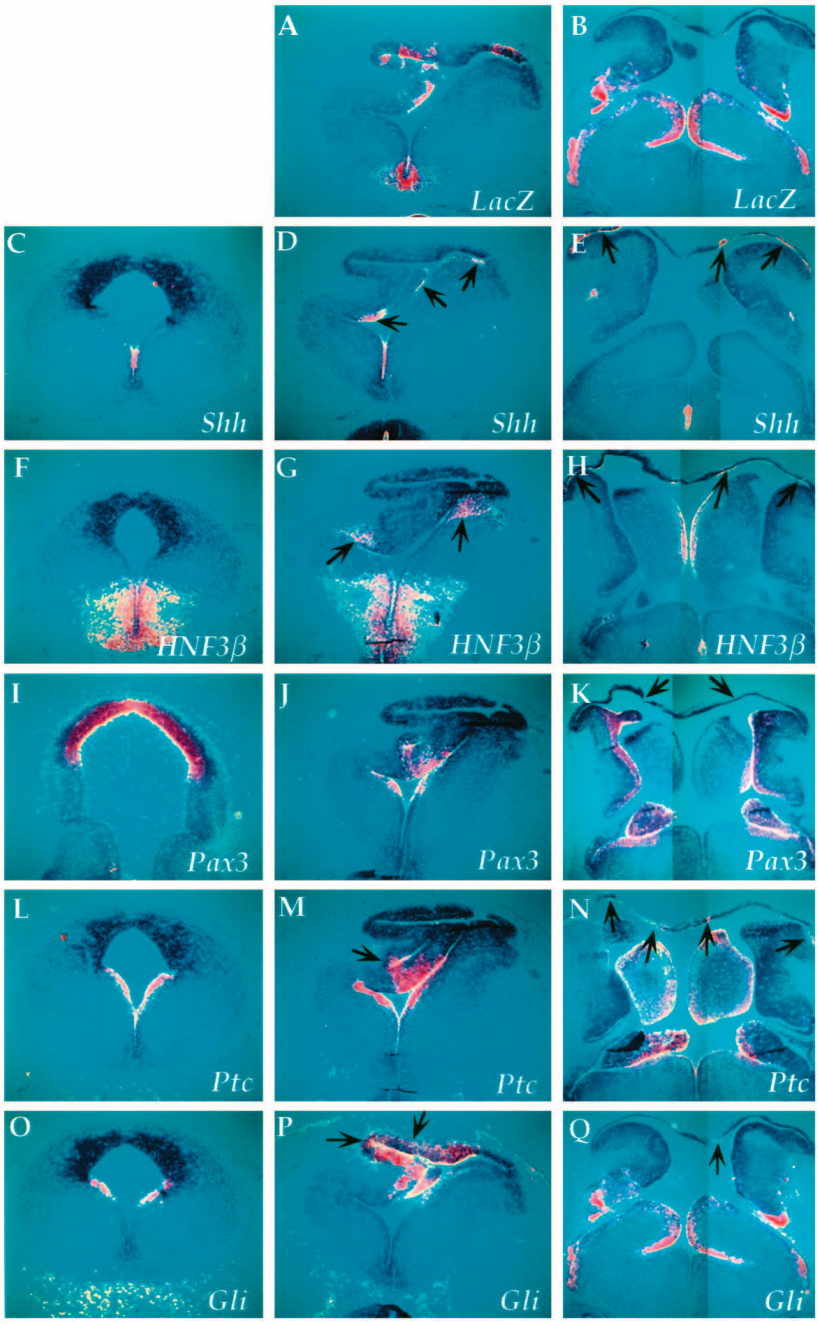

Fig. 4. In situ hybridization of ^{35}S-radiolabelled probes to 12.5 dpc midbrain sections. Sections through the 12.5 dpc midbrain vesicle of a wild-type mouse embryo (C,F,I,L,O), a *Wexp3-dnPKA* transgenic embryo (A,D,G,J,M,P) and through the mid-hindbrain junction of the same transgenic embryo (B,E,H,K,N,Q). In all panels dorsal is up. (A,B) Expression of the transgene as detected by hybridization with the *lacZ* RNA tag. *Wnt-1*, and consequently the transgene, are also expressed in the ventral midbrain at this time. (C) Expression of *Shh* in cells lining the ventral portion of the ventricle. (D,E) Expression of *Shh* in the ventral ventricular zone, as well as areas of ectopic dorsal expression (arrows). (F) Expression of *HNF3β* in the ventral region of a normal midbrain vesicle. (G) Expression of *HNF3β* in the ventral midbrain and areas of ectopic gene expression (arrows). (H) Arrows point to patches of ectopic *HNF3β* activation in the most dorsal part of the mid-hindbrain junction of a transgenic embryo. (I) Expression of *Pax3* in a wild-type midbrain is localized to the dorsal half of the ventricular epithelium. (J) Expression of *Pax3* is repressed from the most dorsal part of the transgenic midbrain. (K) Expression of *Pax3* in the mid-hindbrain junction of the *dnPKA* transgenic embryo is absent in the most dorsal cells (arrows). (L) Expression of *Ptc* in the ventral ventricular border of a wild-type midbrain. (M) Expression of *Ptc* in the ventral ventricular zone and areas of ectopic *Ptc* activation (arrows). (N) Arrows point to patches of ectopic *Ptc* activation in the most dorsal part of the mid-hindbrain junction. (O) Expression of *Gli* in the wild-type midbrain vesicle is restricted to the ventromedial aspect of the ventricular layer. (P) Ectopic expression of *Gli* in the transgenic dorsal midbrain (arrows). (Q) Arrow points to a small patch of ectopic *Gli* activation in the most dorsal part of the mid-hindbrain junction.

domain (Fig. 2C) but was ectopically activated throughout the circumference of the neural tube (Fig. 2D). As we failed to detect significant ectopic expression of Shh at this stage (data not shown), suppression of PKA activity and not direct Shh signaling, was responsible for the induction of dorsal HNF-3β expression. In addition to the phenotypically abnormal embryos, we also detected weak ectopic activation of HNF-3β in one of the transgenic embryos not displaying an obvious phenotype.

At 12.5 dpc, *Shh* was activated in patches of dorsal neuroepithelium in the dorsal midbrain and at the mid/hindbrain junction (Fig. 4D,E). Interestingly, comparison of transgene expression in adjacent sections, indicates that these areas are flanked by, but do not themselves express, the transgene (Fig. 4A,B). The most likely explanation for the apparent non-cell-autonomous activation of Shh is the cell-autonomous extinction of transgene expression after dorsal cells have adopted ventral fates. Dorsal *HNF-3β* expression was also detected at this time. At the level of resolution of our analyis, it would appear that *HNF-3B* expression is both adjacent to and overlapping the domains of ectopic *Shh* expression (Fig. 4G,H). Concomitant with the ventralization of the mid/hindbrain region we observed a restriction of the normally broad domain of dorsal *Pax-3* expression to small patches, which in part overlap regions in which transgene expression is maintained (Fig. 4J,K). Taken together, these findings suggest that dorsal midbrain gene expression in transgenic embryos is compromised, in part, by the activation of the ventral determining

genes *Shh* and *HNF-3β* in response to the reduction of intracellular PKA activity.

In contrast to the brain, we failed to observe ectopic activation of either *Shh* or *HNF-3β* in the dorsal spinal cord at 12.5 dpc (Fig. 5F and data not shown). Thus, the dramatic overproliferation, which is restricted to cells expressing the transgene (Fig. 5E,I), occurs in the absence of ectopic floor plate development. However, as in the brain, we observed a marked loss of dorsal *Pax-3* expression in the expanded neuroepithelium (Fig. 5G).

In addition to the induction of floor plate, Shh signaling leads to the induction of motor neuron development in CNS explants. To determine whether the *Wexp3-dnPKA* transgene could induce motor neurons in the absence of ectopic floor plate development, in situ hybridizations were performed with *cRet*, a receptor tyrosine kinase-encoding gene which is a marker of differentiating motor neurons (Pachnis et al., 1993). At 14.5 dpc *cRet* expression in the spinal cord is normally limited to the ventrolaterally located motor neurons (Fig. 6B). In contrast, in *Wexp3-dnPKA* transgenic embryos, *cRet* was also expressed in clusters of dorsal cells (Fig. 6C,D), opposite to sites of transgene expression in the dorsal ventricular region (Fig. 6A). Thus, induction of ectopic motor neurons occurred in the apparent absence of a floor plate-derived signal, presumably as a direct response to the suppression of PKA activity in the ventricular region. Interestingly, we failed to observe ectopic *cRet* expression at 12.5 dpc suggesting that it may be possible to generate additional motor neurons several days after their normal formation.

Aspects of the Shh signaling pathway are evolutionary conserved

Genetic studies in *Drosophila* have demonstrated that *ptc* and ci^D are activated by the hh signaling pathway (Forbes et al., 1993). Activation at the transcriptional level, in the case of ptc, and post-transcriptional level in the case of ci^D, is also seen in clones lacking PKA activity (reviewed in Perrimon, 1995; Kalderon, 1995; Johnson et al., 1995; Slusarski et al., 1995) consistent with the model that PKA normally represses these targets of the hh signaling pathway. In the mouse, *Ptc* and *Gli* (a mammalian homologue of *cubitus interruptus Dominant*) show dynamic patterns of expression during CNS development but, by 12.5 dpc, expression becomes ventrolaterally restricted within the midbrain and spinal cord, close to the normal site of Shh expression in the floor plate (Goodrich et al., 1996; Hui et al., 1994; Figs 4L,O, 5C,D). Ectopic expression of *Shh* in the dorsal CNS of the mouse leads to ectopic activation of *Ptc*, suggesting that *Ptc* is a likely target of the vertebrate Shh signaling pathway (Goodrich et al., 1996). We therefore set out to determine whether *Ptc* and *Gli* could be ectopically activated by the *Wexp3-dnPKA* transgene. Both genes were ectopically expressed in the dorsal mid/hindbrain region of 12.5 dpc *Wexp3-dnPKA* transgenic embryos (Fig. 4M,N,P,Q). Interestingly, ectopic *Ptc* expression appears restricted to cell types that continued to express the dorsal marker *Pax-3* (Fig. 4J,M), whereas *Gli* was also expressed in the most dorsal regions of the cranial hyperplasia (Fig. 4P). In the spinal cord, both genes were strongly induced in the hyperplastic dorsal ventricular region (Fig. 5H,J,K) in a pattern resembling that of transgene expression (Fig. 5E,I). Thus, as in *Drosophila*, *ptc* expression is activated by hh signaling and repressed by PKA. Moreover, it is likely that the regulation of *Gli* expression is also conserved between mouse and flies.

DISCUSSION

Previous reports have demonstrated that the secreted protein Shh mediates induction of ventral cell types in the vertebrate CNS at distinct concentration thresholds (reviewed in Placzek, 1995). We show here that expression of a dominant negative form of PKA in the dorsal CNS of the mouse leads to a ventralized phenotype which closely resembles that obtained by ectopic expression of Shh under the control of the same enhancer elements (Echelard et al., 1993; B. St-Jacques, D. Rowitch and A. P. M., unpublished data). Thus, Shh appears to act by removing a PKA-mediated inhibition of ventral development in the mammalian CNS; a genetic pathway that has been evolutionary conserved from flies to mice.

In *Drosophila*, hh is responsible for establishing positional identity within the embryonic segment and larval imaginal disks (reviewed in Ingham, 1995). In the embryonic parasegment, hh signaling maintains *wg* expression in adjacent cells. Reciprocal signaling between these rows of cells is necessary for the establishment of the parasegmental border. *wg* and a second signal, *decapentaplegic* (*dpp*), a member of the *TGF-β* superfamily are activated in separate compartments of the leg discs in response to posterior hh signaling. *dpp* is also a target of hh signaling in the eye and wing. Thus, there are distinct targets of the pathway in different tissues.

In contrast, *ptc* appears to be a general target of the *Drosophila* hh pathway. Cells expressing *ptc* are found adjacent to those expressing *hh* in the embryo and imaginal discs. Moreover, *ptc* expression is lost in the absence of hh and ectopically activated on ectopic expression of *hh*. Interestingly, although *ptc* is a transcriptional target of hh signaling, the ptc protein is a negative regulator of the hh signaling pathway. Hence, loss of ptc leads to the derepression of hh targets, including itself, in the absence of a hh signal. Thus, ptc functions to negatively regulate the hh pathway and hh signaling relieves this inhibition (Ingham et al., 1991).

Interestingly, recent studies have demonstrated that PKA also exerts a negative regulation on hh targets, which is also relieved by hh signaling. As with loss of ptc, loss of PKA leads to the hh-independent activation of hh target genes such as *wg*, *dpp* and *ptc*. Currently the details of the intracellular transduction of the hh signal remain sketchy, though at least three segment polarity genes including; *fused*, a serine/threonine kinase, *costal-2* and *cubitus interruptus* (*ci*), a member of the zinc finger-containing family of transcriptional regulators, are likely to play important roles (Forbes et al., 1993). Whether any of these are targets for ptc- and/or PKA-mediated repression remains to be resolved.

Our data in the mouse CNS are consistent with the manner by which PKA functions in the *Drosophila* hh signal transduction pathway. Although the transcriptional targets of the Shh signal may not be the same as *Drosophila* hh, aspects of the pathway that lead to their activation appear to be shared. Previously, we have shown that, by misexpressing chick *Shh* in the dorsal CNS, ectopic floor plate development could be evoked by the activation of *HNF-3β* and mouse *Shh*, two likely targets of the Shh signaling pathway. We demonstrate here that

we can induce an ectopic floor plate, expressing both *HNF-3β* and *Shh*, by antagonizing PKA activity in the dorsal CNS. Similar observations have also recently been reported for zebrafish embryos injected with dominant-negative forms of *PKA* (Hammerschmidt et al., 1996). Ectopic floor plate development only occurs in the dorsal midbrain region close to the midbrain/hindbrain border despite the widespread dorsal expression of the transgene. Most likely this reflects the fact that the transgene is persistently expressed at high levels in this domain from the first somite stage, whereas, in the hindbrain and spinal cord, expression is initiated at later stages. Interestingly, where *Shh* and *HNF-3β* are co-expressed in the dorsal CNS, expression of the transgene and the dorsal marker, *Pax-3*, are lost. Presumably, cells that show *Shh* and *HNF-3β* co-expression, which normally occurs at the ventral midline, denote a full ventralization of the CNS, which is incompatible with dorsal gene expression in the same cells. Not surprisingly ventralization of the dorsal mid/hindbrain region leads to a severe anatomical phenotype, the absence of a recognizable cerebellar anlage. Similarly, expression of *HNF-3β* in the same general region under the control of an *En-2* enhancer leads to a ventralized phenotype and a disruption of dorsal mid/hindbrain structures (Sasaki and Hogan, 1994).

As well as the dorsal activation of *Shh* and *HNF-3β*, we also observed a more widespread ectopic expression of cells only expressing *HNF-3β*, as observed in transgenics ectopically expressing *Shh* (Echelard et al., 1993). Thus, reduction of PKA activity also mimics this aspect of Shh signaling. However, *HNF-3β* is activated in the absence of a Shh signal. In these brain regions, dorsal gene expression is variably repressed, but not completely eliminated, suggesting that the expression of *HNF-3β* is not sufficient to fully ventralize the dorsal CNS. Whether the differences in the degree of ventralization in the dorsal brain reflects the timing and/or levels of transgene expression is unclear. In addition to being expressed in dorsal regions of the CNS, the *Wnt-1* enhancer also directs expression to the ventral midbrain. Interestingly, we have not detected any morphological abnormalities or irregularities in the expression of a variety of ventrally defined genes including *Shh*, *HNF-3β*, *Tyrosine hydroxylase*, *Ptc* and *Gli* (data not shown). This observation suggests that expression of the *dnPKA* in the ventral midbrain, where *Shh* is normally expressed, does not appear to result in the perturbation of ventral patterning.

In contrast to the brain, *HNF-3β* was not ectopically activated in the dorsal spinal cord. However, we observed strong induction of *Ptc* expression in the dorsal spinal cord. Several lines of evidence suggest that *Ptc* regulation by Hh signaling is conserved in mice. Firstly, in the mouse embryo, *Ptc* expression is always observed adjacent to the sites of *Hh* [*Shh, Indian hedgehog* and *Desert hedgehog(Dhh)*] expression, as would be expected for a common target of Hh signaling (Bitgood et al., 1996; Goodrich et al., 1996). Further, in *Dhh* mutants, *Ptc* expression is specifically lost in the gonads where *Dhh* signaling is required for spermatogenesis (Bitgood et al., 1996). Finally, ectopic expression of *Shh* using the *Wnt-1* enhancer leads to the ectopic induction of *Ptc* (Goodrich et al., 1996). Interestingly, *Ptc* expression alone is not sufficient to cause the down regulation of dorsal gene expression in transgenic embryos as it is expressed in overlapping cell populations with that of *Pax-3* and the transgene. Given that during normal floor plate development *Ptc* is only transiently expressed in the ventral midline of the CNS and later is restricted from floor plate cells at a time when *HNF-3β* and *Shh* expression is initiated (Goodrich et al., 1996), its normal function may be in the suppression of floor plate development. Only by antagonizing Ptc through the activation of Shh would floor plate development be able to proceed. An analogous role for Ptc has previously been described in imaginal disk and segmental patterning in Drosophila, where hh is required to antagonize ptc in order to activate the hh signaling pathway (Ingham et al., 1991).

In the fly, ci^D, is an essential component of the hh signaling pathway (Forbes et al., 1993). Our data suggest that *Gli*, one of its vertebrate counterparts, may be regulated by Shh signaling. *Gli* expression is normally restricted to the ventral CNS, in a similar distribution to *Ptc*. However, suppression of PKA activity leads to the dorsal activation of *Gli* in both the brain and spinal cord. Interestingly, regulation of ci^D by Hh in *Drosophila* is post-transcriptional, whereas *Gli* appears to be a transcriptional target in the CNS of mice. As all the other responses that we observe to expression of the *dnPKA* transgene are mimicked by expression of Shh itself, it is reasonable to suppose that regulation of *Gli* in the CNS is normally mediated by Shh signaling. Interestingly, we observed ectopic motor neuron induction in the dorsal spinal cord where *Gli* is ectopically activated. It is interesting to speculate that transcriptional regulation by Gli may play a role in this aspect of Shh-mediated ventralization. Whatever the mechanism, our results indicate that, as with other aspects of the Shh pathway, inhibition of PKA activity is sufficient to initiate the development of motor neurons in the absence of a Shh signal. It is particularly intriguing that inhibiting PKA activity is sufficient to activate both motor neuron and floor plate fates independently of each other, an observation previously reported with reference to Shh (Tanabe et al., 1995). This finding may further reflect the distinct concentration requirements of Shh in the induction of motor neuron and floor plate cell types (Roelink et al., 1995) whereby high levels of PKA inhibition in the dorsal mid/hindbrain region may result in floor plate development and low levels in the dorsal spinal cord may result in motor neuron development. Presumably, other molecules downstream of PKA inhibition or in independent pathways must also be implicated in distinguishing what should become a motor neuron versus a floor plate cell.

The final aspect of the phenotype that we observe, which is shared by transgenics expressing *Shh* (Echelard et al., 1993), is a pronounced proliferative effect in the dorsal CNS. In the midbrain, this gives rise to an ectopic out-pocketing and, in the spinal cord, to a dramatic expansion of the ventricular epithelium. As the ventral CNS is known to produce mitogenic signals (Placzek et al., 1991), our data suggest that Shh may play a role in regulating mitotic activity in the CNS, independent of its patterning function, and this role is normally antagonized by PKA signaling. There is suggestive evidence that this growth response is cell autonomous. Expression of the *Wnt-1* enhancer is normally restricted to a small population of cells localized to the dorsal midline (Echelard et al., 1994). However, we observe a broad dorsal domain of expression of the *dnPKA* transgene in the spinal cord at 12.5 dpc, which encompasses the expanded ventricular zone. Thus, cells expressing the transgene appear to over proliferate. How this mitogenic response is regulated remains to be determined but

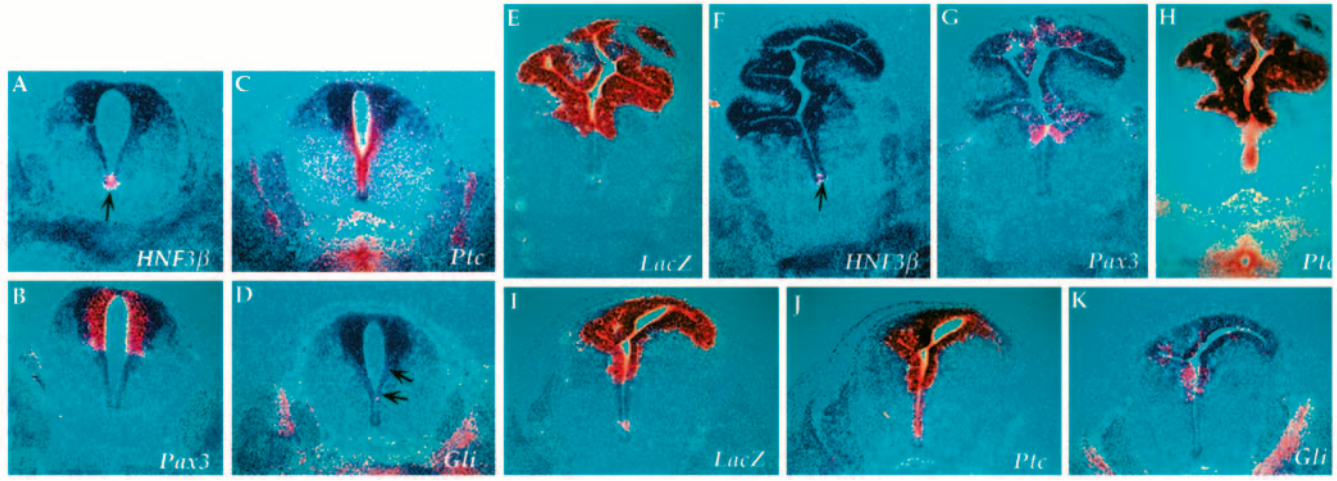

Fig. 5. *In situ* hybridization of ^{35}S-radiolabelled probes to 12.5 dpc spinal cord sections. Transverse sections through wild-type (A-D) and *Wexp3-dnPKA* transgenic (E-H) spinal cords at the cervical level. (I-K) Transverse sections through a *Wexp3-dnPKA* transgenic spinal cord at the thoracic level. Dorsal is up in all panels. (A,F) Expression of *HNF3β* in wild-type and transgenic spinal cords restricted to the floor plate (arrows). (B) Strong expression of *Pax3* in the dorsal half of the ventricular layer. (G) Weak discontinuous expression of *Pax3* in the dorsal half of the transgenic spinal cord. (C) Expression of *Ptc* in the ventral ventricular border. (H,J) Endogenous *Ptc* expression in the ventral ventricular zone in addition to the strong dorsal activation of *Ptc* expression in overlapping cells with that of the transgene (shown in E and I as *lacZ* expression). (D) Expression of *Gli* in the medial ventricular zone (area between arrows). (K) Ectopic expression of *Gli* in patches of cells overlapping transgene expression (shown in I as *lacZ* expression).

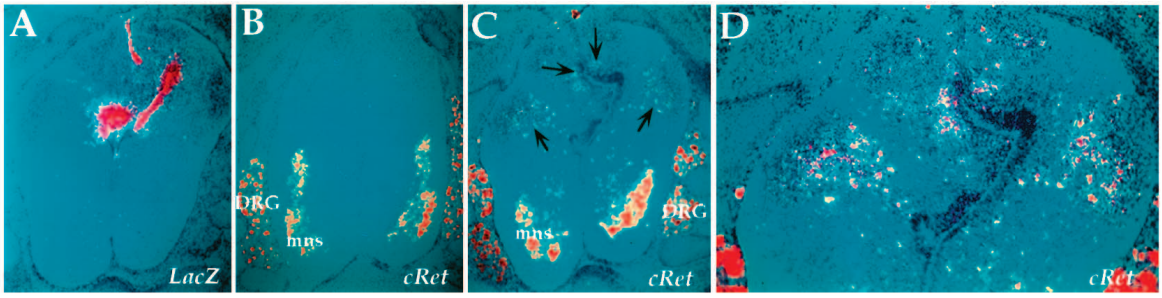

Fig. 6. In situ hybridization of ^{35}S-radiolabelled probes to 14.5 dpc spinal cord sections. (A) Transverse sections at the cervical level of a 14.5 dpc *Wexp3-dnPKA* transgenic spinal cord showing *lacZ* expression. (B) *cRet* expression in a wild-type spinal cord which is restricted to the motor neuron populations (mns) in the ventral horns of the spinal cord as well as the dorsal root ganglia (DRG). (C) Adjacent section to A, showing expression of *cRet* in the ventral motor neurons, DRGs, and ectopically in the dorsal spinal cord (arrows). (D) Higher magnification of C, showing *cRet* expression in the dorsal spinal cord, in cell populations close to the dorsal ventricular zone where the transgene is expressed.

it is interesting to note that Gli possesses oncogenic properties (Ruppert et al., 1991) and is amplified in several human tumors (Roberts et al., 1989).

In conclusion, our data together with published reports of hh signaling in *Drosophila* development (reviewed in Perrimon, 1995; Kalderon, 1995; Ingham, 1995), in zebrafish embryos (Hammerschmidt et al., 1996) and in mesodermal and CNS explants from chick and mice (reviewed in Placzek, 1995) support the general model that PKA plays an important role in repressing targets of hh signaling pathways. At least two of these, *Gli* and *Ptc*, are common targets of both fly and vertebrate hh signaling. Determining where PKA-mediated regulation fits into the pathway will require a more thorough characterization of the transduction of hh signals and the elucidation of key components, in particular the receptor.

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