Conservation of the hedgehog/patched signaling pathway from flies to mice: induction of a mouse patched gene by Hedgehog

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The signaling protein Hedgehog (Hh) controls cell fate and polarizes tissues in both flies and vertebrates. In flies, Hh exerts its effects by opposing the function of a novel transmembrane protein, Patched, while also locally inducing patched (ptc) transcription. We have identified a mouse homolog of ptc which in many tissues is transcribed near cells making either Sonic or Indian hedgehog. In addition, ectopic Sonic hedgehog expression in the mouse central nervous system induces ptc transcription. As in flies, mouse ptc transcription appears to be indicative of Hedgehog signal reception. The results support the existence of a conserved signaling pathway used for pattern formation in insects and mammals.

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Intercellular signaling plays a major role in establishing pattern in developing vertebrate embryos. For example, signals from the notochord, a rod of mesoderm along the center of the embryo, induce the floor plate in the adjacent neural tube [Placzek et al. 1990, 1991]. The floor plate, a group of morphologically distinct cells in the most ventral region of the neural tube, induces motor neurons and orients commissural axon outgrowth [Tessier-Lavigne et al. 1988; Placzek et al. 1991]. A signal from the notochord also appears to determine ventral somite cells to become sclerotome, which will form the axial skeleton [Pourquié et al. 1993]. Another signaling center is the zone of polarizing activity (ZPA) in the posterior of the limb buds. The ZPA is able to induce mirror-image duplications of limbs when placed in the anterior limb bud [Saunders and Gasseling 1968] and is believed to normally organize the anterior–posterior axis of the limb bud by causing nearby cells to form posterior structures.

Although the existence of these inductive tissues has been known for years, the molecular identities of the intercellular signals are only now being elucidated. An important step forward was the discovery that the secreted protein Sonic hedgehog (Shh) is produced in several tissues with organizing properties, including the notochord, floor plate, and ZPA [Echelard et al. 1993; Krauss et al. 1993; Riddle et al. 1993; Chang et al. 1994; Roelink et al. 1994; Bitgood and McMahon 1995]. Misexpression of Shh mimics the inductive effects of an ectopic notochord on the neural tube and somites [Echelard et al. 1993; Fan and Tessier-Lavigne 1994; Johnson et al. 1994; Roelink et al. 1994] and also mimics ZPA function in the limb bud [Riddle et al. 1993; Chang et al. 1994]. These studies suggest that Shh is normally responsible for these inductive events.

The Drosophila homolog of Shh, hedgehog (hh), also organizes pattern during development. In the fly, hh establishes the anterior–posterior axis of the embryonic segments and patterns the larval imaginal discs. Hh genes encode a secreted protein with a signal sequence [Lee et al. 1992; Mohler and Vani 1992; Tabata et al. 1992] and a region homologous to serine proteases [Lee et al. 1994]. A crystal structure of the amino-terminal signaling portion of Shh reveals a zinc hydrolase-like domain as well [Hall et al. 1995].

Hh induces transcription of target genes by opposing the repressive activity of the gene patched (ptc). Ptc is a novel protein with multiple transmembrane domains [Hooper and Scott 1989; Nakano et al. 1989] and is present at the plasma membrane (Capdevila et al. 1994b). Ptc inactivates the transcription of hh target genes such as wingless (wg), a member of the Wnt group [Nussel and Varmus 1992], decapentaplegic (dpp), a transforming growth factor-β (TGF-β) family member [Kingsley 1994],

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and ptc itself. In ptc mutants these genes are ectopically activated [Martinez-Arias et al. 1988; Hidalgo and Ingham 1990; Capdevila et al. 1994a], whereas overexpression of ptc represses their transcription [Ingham et al. 1991; Sampredo and Guerrero 1991; Schuske et al. 1994; Johnson et al. 1995]. In contrast, hh overexpression causes ectopic embryonic wg expression [Ingham 1993] and ectopic dpp and ptc expression in wing discs [Basler and Struhl 1994; Capdevila and Guerrero 1994; Tabata and Kornberg 1994]. Thus, a balance between the opposing activities of these two genes determines the level of target gene expression. Hh may act directly on Ptc as an antagonist [Ingham et al. 1991] or indirectly through a separate signal transduction pathway.

Although the targets of Hh and Ptc vary between tissues, one target that is always induced by Hh is ptc itself. Low levels of ptc expression in embryos and discs reflect successful Ptc negative autoregulation [Hidalgo and Ingham 1990; Capdevila et al. 1994a]. High levels of ptc transcription occur where Ptc is functionally inactivated by the Hh signal. Thus, the most consistent indicator of Hh signaling in flies is the level of ptc transcription.

We have identified a mouse homolog of ptc and demonstrate that Shh and ptc expression are tightly linked during development. Mouse ptc is expressed near Shh-producing cells during early inductive events and next to Indian hedgehog [hh]-producing cells during later limb growth. Like hh in Drosophila, ectopic Shh in the mouse CNS induces ptc transcription in neighboring cells. These results demonstrate the existence of a conserved Ptc/Hh signaling relationship in fly and mammalian induction events.

Results

Identification and characterization of a mouse homolog of ptc

A mouse homolog of ptc was identified by RT-PCR of embryonic limb bud RNA using primers for regions conserved in ptc homologs from several insect species (see Materials and methods). The full-length coding sequence of mouse ptc was determined by sequencing two overlapping cDNA clones (M2 and M9) isolated from a 8.5 days postcoitus (dpc) embryonic mouse library. Nine other cDNAs were partially sequenced and found to be identical to this ptc homolog. We have not detected additional ptc genes either by low stringency hybridization to genomic DNA, library screening, or degenerate PCR (data not shown).

The M2 and M9 cDNAs [1.9 and 3.0 kb, respectively] comprise 4.6 kb of contiguous sequence that includes the entire open reading frame of mouse ptc (Fig. 1A). Translation is proposed to begin at the first encountered AUG (nucleotide 97 of M2), as this region (GGCCCAUAUGG) fits the translational initiation consensus well (GCCAUCUG; Kozak 1991). In addition, cDNA clones for human ptc, which have high sequence similarity to the mouse, contain a stop codon upstream of this AUG (data not shown), indicating that this is the initiating methionine for both homologs. The mouse ptc cDNA contains an open reading frame of 1434 codons and, when aligned to fly ptc shares 33% identity and 43% similarity. This similarity is evenly dispersed over much of the sequence. However, the carboxyl terminus and an internal region of mouse ptc containing a 54-amino-acid insert are relatively divergent. Two potential N-linked glycosylation sites (Hubbard and Ivatt 1981) and eight cysteines are conserved between the mouse and fly ptc genes.

Hydropathy analyses [Kyte and Doolittle 1982] show that mouse Ptc lacks a signal sequence but has 12 hydrophobic regions each long enough to span a lipid bilayer (Fig. 1B). The hydropathy plots for the mouse and fly proteins are remarkably similar, suggesting that the proteins have a conserved topological structure. Mouse Ptc is composed of a pair of tandem sets of six potential transmembrane (TM) domains each. In each set of six, the first TM domain is separated from the following cluster of five TM domains by a hydrophilic region of ∼300–400 amino acids. The two halves of the protein have similar predicted topology but no obvious sequence similarity.

Mouse ptc is transcribed in many embryonic and adult tissues

The embryonic and adult expression of mouse ptc was examined on RNA blots. A ptc probe hybridizes to an 8-kb transcript in mRNA from developing and adult tissues (Fig. 2). Because the open reading frame is 4.5 kb long, ptc mRNA contains ∼3.5 kb of untranslated sequence. ptc is expressed at very low levels at 7 dpc, is most strongly transcribed between 11 and 15 dpc, and persists at moderate levels at 17 dpc. In the adult, ptc RNA is abundant in the lung, brain, liver, and kidney. Upon overexposure of the blot, ptc RNA is detected faintly in the heart, skeletal muscle, and testis. ptc transcription is therefore widespread in the embryonic and adult mouse.

ptc is expressed near Shh in axial tissues during development

In flies, ptc is transcribed at high levels in cells that receive Hh signal. In situ hybridization reveals a similar relationship in mice. Antisense probes from three different parts of the ptc cDNA yield identical results, and no specific staining is observed with a control probe from the sense strand (data not shown). Our observations of Shh expression are in accord with earlier reports [Echclard et al. 1993; Chang et al. 1994; Roelink et al. 1994; Birgood and McMahon 1995].

Shh and ptc are expressed in neighboring cells from the earliest stages of neurulation. ptc RNA is first detected faintly along the anterior axis of 7.5-dpc embryos at the early head-fold stage [data not shown]. By 8 dpc, hybridization is intense down the middle of the head folds, with weak staining in the neuroepithelium extending to the node where Shh is expressed (Fig. 3A,B). Sections of
hybridized whole mounts show that ptc is transcribed strongly in the ventral-most cells of the neural tube and weakly in the ventral presomitic mesoderm (Fig. 4A). These tissues abut the Shh-expressing chordamesoderm, which forms the head process and notochord (Figs. 3B and 4B).

Slightly later, at 8–8.5 dpc, ptc transcription is generally limited to the midline of the neural tube (Fig. 3C,E), except for two transverse domains of expression in the developing brain (Fig. 3E). ptc RNA continues to be present at low levels in the ventral presomitic mesoderm and is at high levels in the ventral halves of the somites and in the lateral plate mesoderm (Fig. 3E). Shh expression persists in the axial mesoderm and commences in the ventral midbrain in a region thought to be analogous to the floor plate (Fig. 3D,F).

With the onset of Shh transcription in the floor plate, ptc and Shh expression briefly overlap before resolving into a complementary pattern. By day 9, ptc RNA begins to disappear from the floor plate where Shh is strongly expressed (Fig. 3G,H). Sections through posterior, less developmentally advanced, tissues reveal a gradient of ptc staining in the neural tube. The highest levels are in the floor plate adjacent to the Shh-expressing notochord (Fig. 4C,D). Similarly, in the somite ptc is expressed most strongly in the cells close to the notochord, with lower expression levels in more distant cells of the sclerotome (Fig. 4C,E). In more anterior tissues, ptc expression has disappeared from the Shh-expressing floor plate but persists in regions along both sides of the floor plate (Fig. 4E,F). Although there may be a brief period when both genes are transcribed in the floor plate, the changes in ptc and Shh transcription occur coordinately, so that the two genes are expressed in complementary regions most of the time. An exception is seen in the hindbrain, where briefly between days 9 and 9.5, strong stripes of ptc staining extend dorsolaterally within each rhombomere (Fig. 3G, inset). Shh RNA remains restricted to the ventral midline cells of the rhombomeres (Fig. 3H, data not shown).

Expression of ptc and Shh does not change much in the spinal cord or somites of 10-dpc embryos (Fig. 3J). In the spinal cord, ptc is widely expressed, extending up the sides of the neural tube, whereas Shh expression is lim-
show that ptc RNA is present in both the endoderm and mesoderm while Shh expression is restricted to the ventral endoderm [data not shown]. However, by 13.5 dpc, ptc RNA is limited to the mesoderm, adjacent to Shh RNA in the endoderm (Fig. 5A–D). ptc and Shh are also found in complementary sites in the hair and whisker follicles, where beginning on day 12.5 ptc is expressed in rings surrounding a central domain of Shh (Fig. 5A,B). Other regions of ptc expression include the branchial arches [Fig. 3G,I], palate, tongue, toothbuds, and genital eminence [data not shown].

**ptc is transcribed adjacent to two hh family members during limb development**

ptc transcription in the limb bud corresponds to the expression patterns of two hh homologs, Shh and Ihh. ptc RNA is first detected at the posterior edge of the forelimb bud on day 9.5. This expression becomes stronger and broader by 10.5 dpc (Figs. 3I and 6A). Low levels of ptc RNA are present in a similar position in the less advanced hindlimb [data not shown]. By 10.5 dpc, strong Shh transcription appears in the posterior mesenchyme of the forelimb bud (Fig. 6B). This site of expression presumably corresponds to the murine ZPA, since in chicken embryos Shh products are located in the ZPA (Riddle et al. 1993). Widespread ptc staining persists in the posterior half of 11-dpc limb buds, partially overlapping with Shh RNA in the most posterior mesenchyme (Fig. 6C,D). At 11.5 dpc, ptc expression fades in a distal domain near the edge of the limb bud (Fig. 6E). Low levels of Shh are still present in a narrow band of cells in the posterior (Fig. 6F). At no time is ptc RNA found in the apical ectodermal ridge (AER), which is a critical signaling source for maintenance of limb outgrowth (Summerbell 1974). In 12.5- and 13.5-dpc embryos ptc is transcribed in cells adjacent to a source of a second hh family member, Ihh. At this stage, ptc RNA is detected in the periphery of the digital condensations and later in the perichondrium surrounding the hypertrophic digit cartilage (Fig. 6G). Ihh is transcribed in digital condensations neighboring the ptc-expressing cells (Fig. 6H), but Shh is absent after 12.5 dpc (Fig. 5B). Whereas ptc and Shh are transcribed together early in limb bud development, the expression of ptc correlates with Ihh transcription during cartilage differentiation.

**ptc is expressed ectopically in Strong's Luxoid mice**

Mice homozygous for the semidominant mutation Strong’s Luxoid (Ist\(^{5}\)) develop extra digits on their fore- and hindlimbs. Although the product of the Ist\(^{5}\) locus is unknown, the limb phenotype appears to be caused by an ectopic Shh-expressing ZPA in the anterior of the limb bud (Chan et al. 1995). This ectopic expression of Shh is stronger in the hindlimb than the forelimb. If ptc is downstream of Shh signaling in the limb bud, then ptc should also be expressed ectopically in Ist\(^{5}\) mice.

Homozygous Ist\(^{5}\) embryos (11.5–12 dpc) were identi-
fied by the presence of ectopic outgrowths on all four of their limbs and were examined by in situ hybridization with the ptc probe. In each mutant limb, ptc RNA is found in the proximal region of the anterior outgrowths, as well as in the normal posterior domain [Fig. 6,i]. Like Shh, ectopic ptc is transcribed most strongly in the hindlimbs, at levels comparable to normal ptc expression whereas ectopic ptc signal is weak in the forelimbs. Thus, ectopic expression of ptc in lsr2 embryos correlates with ectopic expression of Shh.

Ectopic Shh induces ptc in the CNS

The tight spatial and temporal correlation between the expression of ptc and hh homologs in wild-type and mutant mice suggests that, as in flies, Hh induces transcription of the ptc gene. Ectopic expression of chick Shh under the control of a Wnt-1 enhancer was found previously to cause the activation of ventral gene expression in the dorsal CNS [Echelard et al. 1993]. We now show that ptc also is expressed ectopically in Wnt-1–Shh transgenic embryos.

Ventralization of the dorsal CNS is strongest in the midbrain and rostral hindbrain [myelencephalon], presumably reflecting the early activation of Wnt-1 in these regions of the brain (McMahon et al. 1992). Some embryos appear to develop a morphologically normal ectopic dorsal floor plate [asterisk in Fig. 7A]. Whereas most transgenic embryos ectopically expressing the transgene have a similar phenotype, an open midbrain

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**Figure 3.** In situ hybridization of ptc and Shh probes to mouse embryos. Mouse embryos were hybridized with probes to ptc (A,C,E,G,I) and Shh (B,D,F,H,J). All views are lateral except A, which is ventrolateral, and E and F, which are ventral. (A–F) Anterior is to the left. The developmental stages are 8 dpc, presomitic (A,B); 8.5 dpc, 8–10 somites (lateral C,D, ventral E,F); 9 dpc, 15–20 somites (G,H); 9.5 dpc (inset, G) and 10.5 dpc, 30–40 somites (I,J). The red arrows in E point to transverse expression of ptc in the developing brain. The inset in G is a dorsal view of the hindbrain, with rhombomere expression indicated by the red arrow. In I, the red arrows demarcate the ventrolateral expression of ptc in the midbrain. [d] Diencephalon; [fp] floor plate; [h] heart; [hd] head fold; [ig] foregut; [hg] hindgut; [hp] head process; [im] lateral mesoderm, [m] mandibular arch; [mb] midbrain; [nc] notochord; [nd] node; [nt] neural tube; [pm] presomitic mesoderm; [s] somite.
levels of Shh may be required for induction of HNF-3β compared to ptc, consistent with the fact that different cell fates are determined by different concentrations of Shh protein (Roelink et al., 1995).

Discussion

The pattern of mouse ptc transcription supports the existence of a conserved signaling pathway with at least two hh genes as inducing signals and a single ptc gene as a target. ptc is expressed most highly in cells close to Shh-expressing cells. The dynamic pattern of ptc transcription follows closely changes in Shh expression during development so that transcripts of the two genes are found mostly in adjacent, nonoverlapping tissues. Furthermore, Shh is able to activate ptc transcription in the mouse CNS, implying that high levels of ptc transcription in other developing tissues are also induced by cells expressing one of the hh family members. Because the relationships between these mouse genes are much like hh modulation of ptc expression during Drosophila development, similar signaling mechanisms may be employed in mammals and insects.

ptc expression reflects regulation by Shh

In flies, Hh increases ptc transcription levels by preventing negative autoregulation of ptc itself, thereby causing ptc to be transcribed strongly in cells nearest the Hh signal. The intense expression of ptc in Shh- and hh-responsive tissues is consistent with both genes regulating ptc expression. For example, in 9- to 9.5-dpc embryos, ptc is expressed throughout the ventral two-thirds of the neural tube but at particularly high levels in the floor plate that contacts the Shh-expressing notochord. Similarly, ptc staining is strongest near the ZPA in the limb and near the notochord in the somites. In addition to these sites of high transcription, ptc appears to be expressed at low levels throughout most tissues, as the background staining observed in embryos hybridized with the antisense probe is not observed with the sense or other control probes (data not shown). We propose that as in flies, the elevation of high ptc levels in mouse embryos may be attributable to Shh inactivation of Ptc activity, leading to ptc derepression.

Two experiments suggest that Shh induces these high levels of ptc RNA in adjacent cells. First, the lstD mutation, which causes a second ZPA and high Shh levels to develop in the anterior of the limb buds (Chan et al., 1995), leads to ectopic ptc transcription. Second, ectopic expression of Shh in the dorsal neural tube activates ptc transcription. Although it is unclear whether Shh induces ptc transcription directly or through secondary signals, it is intriguing that this relationship exists in mice, as a balance in the expression levels of these two genes is crucial for correct gene expression in flies (Johnson et al., 1995). Additional experiments are under way to determine whether mouse Ptc opposes Shh induction of target genes.

As in flies, ptc seems to be a unique target of Shh.
signaling. Several other genes are activated by Shh, but ptc is the only known target gene expressed near all Hh sources. Furthermore, in the CNS, ptc induction is more sensitive than induction of another Shh target gene, HNF-3β. ptc transcription thus appears to be a general indicator of Shh signaling. If this is true, then the gradient of ptc transcription normally observed along the dorsoventral axis of the neural tube may reflect the control of cell differentiation by graded levels of Hh [Rechlin et al. 1995]. ptc and Hh homologs are coexpressed in many tissues patterned by inductive interactions, so this conserved Hh–Ptc pathway may be a general mechanism for cell–cell communication during development. The basis for tissue-specific responses to Hh and Ptc signaling remains to be explored.

ptc may respond to multiple hh genes

ptc is expressed near three different hh homologs during development, suggesting that ptc is a common target of multiple Hh signaling pathways. For example, the expression of Ihh and ptc in adjacent tissues during cartilage differentiation suggests that ptc responds to Ihh as well as to Shh. ptc is also expressed at the same time as Desert hedgehog (Dhh) in the germ line [Bitgood and McMahon 1995]. The one site of ptc expression that does not correlate with transcription of known hh genes is in the rhombomeres of 9- to 9.5-dpc embryos (Fig. 3C; Bitgood and McMahon 1995). Because ptc expression is tightly linked to the expression of hh homologs else-

where, the presence of ptc RNA in the rhombomeres may indicate an unidentified hh family member that is transcribed away from the midline. Alternatively, ptc may have some Hh-independent functions. Although there may be multiple ptc genes, only single genes have been found in mouse, chick, and human despite hybridization searches at low stringency (this work; Marigo et al. 1996, R.L. Johnson, L.V. Goodrich, and M.P. Scott, unpublished).

Additional components of the Hh signaling pathway may be conserved between flies and vertebrates

In flies, cubitus interruptus (ci), which encodes a zinc finger-containing protein [Orenic et al. 1990], is an activator of ptc transcription and is required for Hh signaling [Forbes et al. 1993]. Hh signaling elevates Ci protein levels post-transcriptionally [Motzny and Holmgren 1995; Slusarski et al. 1995], whereas ptc has the opposite effect [Johnson et al. 1995]. As in the fly, where ptc and ci are expressed in many of the same cells, mouse Gli, a ci-related gene, and to a lesser extent, Gli2 and Gli3, are transcribed in ptc-expressing tissues [Hui et al. 1994]; ci and its homologs all contain five zinc finger domains [Kinzler et al. 1988; Orenic et al. 1990; Hui et al. 1994] and may act as transcription factors, as human Gli binds specific DNA sequences [Kinzler and Vogelstein 1990]. Gli could activate ptc transcription in response to a Shh signal

Another conserved component of the Hh–Ptc pathway
is the cAMP-activated protein kinase A (PKA). Like ptc, PKA negatively regulates dpp, wg, and ptc transcription and ci protein levels, and is genetically downstream of hh [Jiang and Struhl 1995; Johnson et al. 1995; Lepage et al. 1995; Li et al. 1995; Pan and Rubin 1995; Strutt et al. 1995]. Similarly, PKA antagonizes Shh signaling during development of the somites and neural tube [Fan et al. 1995; Hynes et al. 1995]. Because the relationship between ptc and PKA is not well understood in flies, it is uncertain how these genes interact in vertebrates.

Possible Ptc protein functions

The sequence analysis and hydropathy plot leads to a possible model of mouse Ptc (Fig. 8). The two conserved glycosylation sites and the six conserved cysteines suggest that the two large hydrophilic loops are extracellular, whereas the amino and carboxyl termini and the central hydrophilic loop are cytoplasmic. Neither the amino acid length nor the composition of the proposed intracellular domains is well conserved except near the membrane. In contrast, the 12 putative transmembrane domains and the extracellular loops are more highly conserved, suggesting that important functional domains of the protein reside within or outside the plasma membrane.

Ptc has been proposed as a receptor for Hh protein [Ingham et al. 1991], but the structure suggested by the sequence conservation does not resemble known receptors. Instead, the topology suggests that Ptc is related to transporters that contain 12 transmembrane domains grouped into 6+6 arrays. These transporters facilitate the movement of small molecules such as sugars, ions, or small peptides across the membrane. The Ptc proteins lack the ATP binding cassette (ABC) that is characteristic of one 6+6 family, the ABC-type transporters, which include the multidrug resistance transporter, and the cystic fibrosis gene CFTR (Doige and Ames 1993; Saier 1994). If Ptc is a novel transporter or channel, the regulatory relationships imply that movement of small molecules through Ptc causes repression of target genes.

Why is ptc induced by Hh?

In Drosophila, a peculiar aspect of the Ptc–Hh relationship is the boosting of Ptc protein levels in cells where Ptc function seems to be eliminated by the Hh signal. This mechanism is probably conserved in vertebrates, as Shh also induces high levels of ptc expression in adjacent cells. Why should Hh signaling stimulate production of large amounts of inactive protein? In Drosophila, Hh distribution is broader in ptc mutant embryos [Taylor et al. 1993], suggesting that Ptc restricts the movement of the Hh protein. A high level of Ptc could sharpen and delimit the region where target genes are induced by Hh by blocking or lowering further Hh movement.

Alternatively, Ptc protein may be active in the cells where high levels are induced, but its activity masked by a competing, antagonistic pathway under the control of Hh. In this model Ptc buffers Hh signaling by maintaining a basal level of negative transcriptional regulation that can be overcome only by large amounts of Hh protein interacting with a separate receptor. Induction of high Ptc levels by Hh may raise the threshold for tran-
Figure 7. Ectopic expression of Shh in the dorsal CNS induces the expression of ptc in adjacent cells. Sections through the hindbrain (A–F) and rostral spinal cord (G–I) of 10.5-dpc transgenic mouse embryos expressing chick Shh under the regulation of a Wnt-1 enhancer. Adjacent sections were hybridized with probes for mouse Shh, HNF-3β and ptc as indicated. Normal expression of HNF-3β, Shh, and ptc in the ventral CNS is indicated by large arrows. Ectopic activation of these genes in the dorsal CNS is indicated by small arrows. In addition to their CNS expression, HNF-3β and Shh are also normally expressed in the epithelium of the foregut (g) and ptc in the adjacent mesenchyme.

scriptional activation, thereby ensuring that Hh has its effects in limited regions. Misexpressed Hh causes severe pattern deformations in flies and mice (Echelard et al. 1993, Basler and Struhl 1994; Ingham and Fietz 1995), so it is clearly important to limit the action of the potent Hh protein.

The identification of this mouse ptc homolog helps unravel a key signaling pathway underlying induction and morphogenesis of many tissues and organs. The pathway appears to have several conserved components: the signal Hh, a target ptc, PKA, the zinc finger protein Ci/Gli, and TGF-β targets such as dpp in flies and Bone Morphogenetic Protein (BMP) genes in vertebrates. Although it is not yet known whether vertebrate Hh signals exert their effects by opposing Ptc function, the conservation of so many other features of the pathway makes this likely. In this case, we predict that lost ptc function in vertebrates will lead to ectopic expression of many Hh targets. Further experiments are under way to examine ptc function in mice by removing and misexpressing the mouse ptc gene.

Materials and methods

Isolation of a ptc gene from the mouse

Two degenerate PCR primers [P4REV, GGACGAAATTCYTNGAANTGTTYTGGGA; P22, CATACAGGCAAGTTGTTTCCGCCARTC] [underline indicates cloning sequences] were designed based on a comparison of Ptc amino acid sequences from Drosophila melanogaster, mosquito Aedes gambiae, butterfly Precis coenia, and beetle Tribolium castaneum] (L. Goodrich, L. Nagy, R. Johnson, S. Carroll, and M. Scott, in prep.). P22 was used to reverse transcribe RNA from 12.5-dpc mouse limb bud (gift from Dr. D. Kingsley, Stanford University, CA) for 90 min. at 37°C. The cDNA was amplified with the P4REV and P22 primers with 35 cycles of 94°C for 15 sec, 50°C for 30 sec, and 72°C for 90 sec. PCR products of the expected size were subcloned into the TA vector [Invitrogen].

Using the cloned mouse PCR fragment as a probe, 300,000 plaques of a mouse 8.5-dpc kgt10 cDNA library (a gift from Dr. B. Hogan, Vanderbilt University, Nashville, TN) were screened at 65°C [5x SSC, 10% dextran sulfate, 5x Denhardt's solution, 200 ng/ml of sonicated salmon sperm DNA, 0.5% SDS] and washed in 2x SSC, 0.1% SDS, at room temperature. Seven
cDNAs (M1–M6, M8) were isolated. Then 200,000 plaques were rescreened using either a 1.1-kb EcoRI fragment from M2 or a mixed probe (XhoI fragment of M2 and the BamHI–BglII fragment of M9) to isolate additional clones (M9, M10, M12–M15, M17–M21). Of the 18 isolated clones, 11 were subcloned into pBluescript II (Strategene). To determine the coding region of ptc, two clones, M2 and M9, were sequenced on both strands using dideoxy chain termination methods (Sanger et al. 1977). The sequence was analyzed using the MacVector and GCC programs.

RNA blot hybridization

A mouse embryonic RNA blot and an adult multiple tissue RNA blot (Clontech) were probed with a 900-bp EcoRI fragment from an amino-terminal coding region of ptc. Hybridization was performed at 65°C in 5× SSPE, 10× Denhardt’s solution, 100 mg/ml of salmon sperm DNA, and 2% SDS. After several short washes at room temperature in 2× SSC, 0.05% SDS, the blots were washed at high stringency in 0.1× SSC, 0.1% SDS, at 50°C.

Whole-mount in situ hybridization and sectioning

Embryos (7.5 to 15.5) were obtained from superovulated FVB females mated to FVB males and staged as noon of the day of the plug as 0.5 dpf. IstP mutant mice were provided by Dr. P. Leder (Harvard Medical School, Boston, MA).

To detect ptc transcripts, the plasmid M2-3 (which contains the first 841 bp of the cDNA clone M2) was linearized and transcribed with either T3 or T7 RNA polymerase to generate antisense or sense probes, using nonradioactive digoxigenin nucleotides (Boehringer Mannheim). To detect Shh and Ihh transcripts, antisense riboprobes were generated using Hb-14.1 and Hb-16.1 plasmids as templates (Echelard et al. 1993).

Whole-mount in situ hybridization was performed as described [Wilkinson 1992] but with the following modifications. Embryos were proteinase K digested for 6–20 min and pre-blocked for 2.5–3 hr. The posthybridization RNase digestion was omitted.

Whole-mount embryos were equilibrated in 50% sucrose in PBS at 4°C overnight and then dissected in sucrose and oriented in melted gelatin (7.5% gelatin, 15% sucrose, 0.05% sodium azide). After solidification, cubes containing the embryos were cut out, frozen on dry ice until opaque, and stored at −80°C. Sections (20–25 mm) were cut on a Hackett cryostat (−20°C) and collected on slides coated with VectaBond (Vector Laboratories). The sections were dried on slides overnight and then soaked in PBS for at least 15 min before mounting in Aquamount (Lerner Laboratories). Sections were photographed on a Zeiss Axiophot microscope using Nomarski optics. Sections of Wnt1–Shh mice were prepared as described [Echelard et al. 1993]. Slides of whole-mount in situ and sections were scanned and assembled using Adobe Photoshop with modification only of overall brightness and contrast.

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Note added in proof

The sequence data described in this paper have been submitted to the EMBL/GenBank data libraries under accession no. BankIt30589-V46155.

References


