

## Conservation in *hedgehog* signaling: induction of a chicken *patched* homolog by *Sonic hedgehog* in the developing limb

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### SUMMARY

Hedgehog genes have been implicated in inductive signaling during development in a variety of organisms. A key element of the *hedgehog* signaling system is encoded by the gene *patched*. In *Drosophila* hedgehog regulates gene expression by antagonizing the action of *patched*. In addition, *patched* is itself a transcriptional target of hedgehog signaling.

We have isolated a chicken *patched* homolog and find it to be strongly expressed adjacent to all tissues where members of the *hedgehog* family are expressed. As in *Drosophila*, ectopic expression of *Sonic hedgehog* leads to ectopic induction of chicken *Patched*. Based on this regulatory conservation, vertebrate *Patched* is likely to be directly downstream of *Sonic hedgehog* signaling.

An important role of *Sonic hedgehog* is the regulation of anterior/posterior pattern in the developing limb bud. Since *Patched* is directly downstream of the hedgehog signal, the extent of high level *Patched* expression provides a measure of the distance that *Sonic hedgehog* diffuses and directly acts. On this basis, we find that *Sonic hedgehog*

directly acts as a signal over only the posterior third of the limb bud.

During limb patterning, secondary signals are secreted in both the mesoderm (e.g. Bone Morphogenetic Protein-2) and apical ectodermal ridge (e.g. Fibroblast Growth Factor-4) in response to *Sonic hedgehog*. Thus knowing which is the direct target tissue is essential for unraveling the molecular patterning of the limb. The expression of *Patched* provides a strong indication that the mesoderm and not the ectoderm is the direct target of *Sonic hedgehog* signaling in the limb bud.

Finally we demonstrate that induction of *Patched* requires *Sonic hedgehog* but, unlike *Bone Morphogenetic Protein-2* and *Hox* genes, does not require *Fibroblast Growth Factor* as a co-inducer. It is therefore a more direct target of *Sonic hedgehog* than previously reported patterning genes.

Key words: limb development, *Drosophila* homolog, *patched*, hedgehog, *Sonic hedgehog*

### INTRODUCTION

Recent genetic studies have revealed signaling pathways that orchestrate patterning during vertebrate development. *Sonic hedgehog* encodes an important intercellular signal which appears to be responsible for establishing polarized cell fates in the vertebrate central nervous system (Echelard et al., 1993; Krauss et al., 1993; Roelink et al., 1994), somites (Fan and Tessier-Lavigne, 1994; Johnson et al., 1994) and limb (Riddle et al., 1993). In regulating the embryonic patterning of these structures, *Sonic hedgehog* has been implicated in both short- and long-range inductions (Fan and Tessier-Lavigne, 1994; Martí et al., 1995; Roelink et al., 1995).

During limb development, *Sonic hedgehog* is a key signal in patterning the anterior-posterior axis. Grafting experiments demonstrated that the mesoderm at the posterior margin of an early limb bud, called the Zone of Polarizing Activity (ZPA), is responsible for patterning the anterior-posterior axis. When implanted at the anterior margin of a host limb bud, the ZPA

induces mirror-image duplication of the digits (Saunders and Gasseling, 1968). Either misexpression of *Sonic hedgehog* or application of purified *Sonic hedgehog* protein at the anterior margin of a limb bud are sufficient to cause mirror-image duplications identical to those produced by a transplanted ZPA (Riddle et al., 1993; López-Martínez et al., 1995).

There is evidence that secondary signals are produced in response to *Sonic hedgehog* and are likely to be involved in mediating the effects of *Sonic hedgehog* in the limb. For example *Bone Morphogenetic Protein - 2* (*BMP-2*, a member of the TGF- $\beta$  superfamily) is induced in ectodermal and mesodermal cells after ZPA transplantation at the anterior margin of a host limb (Francis et al., 1994) and in response to *Sonic hedgehog* (Laufer et al., 1994).

Integrated signals from the ectoderm and the mesoderm are necessary for *BMP-2* induction. *Sonic hedgehog* is only able to induce *BMP-2* expression in the anterior mesoderm in the presence of members of the *Fibroblast Growth Factor* (FGF) family produced by the Apical Ectodermal Ridge (AER);

Laufer et al., 1994). Several members of the FGF family are produced by the AER and are able to substitute for the ridge in its functions (Niswander et al., 1993; Fallon et al., 1994). One member of this family, *FGF-4*, is asymmetrically expressed in the AER, confined to the posterior half closer to the ZPA (Niswander and Martin, 1992). FGF-4 is itself a secondary signal since misexpression of *Sonic hedgehog* in anterior mesoderm induces *FGF-4* expression in the anterior AER (Laufer et al., 1994; Niswander et al., 1994).

By initiating secondary signals both in the mesoderm (*BMP-2*) and in the ectoderm (*FGF-4*) Sonic hedgehog induces a cascade of gene induction that ultimately results in limb outgrowth and patterning. While little is currently known of the Sonic hedgehog signaling pathway in vertebrates, more is known about the genetic interactions of *hedgehog*, its highly conserved fly homolog (Forbes et al., 1993).

*Hedgehog* (*hh*) is a segment polarity gene that controls body segment pattern and polarity (Nüsslein-Volhard and Wieschaus, 1980; Lee et al., 1992; Mohler and Vani, 1992). *hh* also controls anterior-posterior patterning in imaginal discs, the precursors of fly appendages. *hh* is normally expressed throughout the posterior compartments of discs (Lee et al., 1992). When *hh* is misexpressed in the anterior parts of imaginal discs it induces duplication of the anterior compartment, analogous to the duplications of the vertebrate limb in response to Sonic hedgehog (Basler and Struhl, 1994). In the wing disc this reorganization of the anterior compartment is mediated by the induction of another signaling molecule *decapentaplegic* (*dpp*; Basler and Struhl, 1994; Capdevila and Guerrero, 1994; Tabata and Kornberg, 1994); ectopic expression of *dpp* alone is sufficient to give pattern alterations similar to those caused by ectopic *hh* (Capdevila and Guerrero, 1994; Ingham and Fietz, 1995). Interestingly *dpp* is a homolog of the vertebrate *Sonic hedgehog* target *BMP-2*, underlining a striking parallel between the *hedgehog* signaling pathway in flies and vertebrates.

In both fly embryos and larval imaginal discs a key gene in the hh signaling pathway is *patched* (*ptc*; Ingham et al., 1991; Capdevila et al., 1994). *ptc* is a novel transmembrane protein (Hooper and Scott, 1989; Nakano et al., 1989). It is believed to be part of the machinery for the transduction of the hh signal, possibly as a receptor (Ingham et al., 1991) since epistasis analyses place it downstream of *hh* (Hidalgo and Ingham, 1990; Ingham et al., 1991) and upstream of all other genes in the pathway in hh responding cells, including *smoothened* (Hooper, 1994), *fused* (Forbes et al., 1993), and *cubitus interruptus* (Forbes et al., 1993). *ptc* protein has been shown to be required to obtain a change in target gene transcription in response to hh both in the ventral cuticle during embryogenesis and in the larval imaginal discs (Ingham et al., 1991; Capdevila et al., 1994). Thus low levels of *ptc* transcription marks cells capable of responding to hh. In contrast, high levels of *ptc* transcription are indicative of cells actively receiving the hh signal. *ptc* constitutively represses downstream targets and the hh signal relieves *ptc* repression, thereby inducing transcription of the downstream genes (Hidalgo and Ingham, 1990; Ingham et al., 1991; Capdevila et al., 1994). In addition to its role in transduction of the hh signal *ptc* is itself a target gene of hh. While in different tissues hh induces different targets, *ptc* transcription is strongly induced in cells responding to hh signal (Hidalgo and Ingham,

1990; Ingham, 1993; Basler and Struhl, 1994; Tabata and Kornberg, 1994).

Considering the many roles the hedgehog signals play in organizing vertebrate development, it is critical to learn how these signals are received and interpreted. The isolation of a vertebrate homolog of *ptc* allows us to examine the conservation of the hh signal transduction pathway between arthropods and vertebrates and to dissect the hedgehog signaling pathway. Here we report the cloning of a chick *patched*-related gene (*PTC*). The analysis of *PTC* expression and regulation is revealing about the mechanism by which Sonic hedgehog patterns the vertebrate limb.

## MATERIALS AND METHODS

Unless otherwise noted, all standard cloning techniques were performed according to the method of Ausubel et al. (1989). All enzymes and molecular biology reagents were supplied by Boehringer Mannheim Biochemicals except as noted.

### PCR cloning of chicken *Patched*

Based on the comparison of fly and butterfly *ptc*, and mouse *Ptc* amino acid sequences (Goodrich et al., 1996), two pairs of degenerate oligonucleotides were designed with an *EcoRI* site on their 5' ends to facilitate subcloning. The nucleotide sequences of these oligos are:

R4: 5'-GGACGAATTCYTIGAYTGYYTGGGA-3'  
 R2: 5'-GGACGAATTCT(CG)YTCI(TG)GCCARTGCAT-3'  
 G1: 5'-GGACGAATTCGAYGGIAT(TAC)AT(TAC)AAAYC-3'  
 G2: 5'-GGACGAATTCRTAYTGYTCCCARAAIA-3'

I represents inosine, R purine, and Y pyrimidine.

Total RNA isolated from stage 23 chick limb buds was used as a template for reverse transcription with R2 and G2 oligos. 10 µg of RNA were heated at 65°C for 5 minutes, then chilled on ice. 45 picomoles of oligonucleotides were used for the reaction with murine reverse transcriptase (Stratagene) in the buffer supplied by the company. After 1 hour at 37°C the enzyme was inactivated 5 minutes at 95°C and 1/25 of the reaction was amplified by PCR using either the R2 and R4 or G1 and G2 primer pair respectively. The PCR reaction conditions were as follows: 4 minutes at 94°C followed by 30 cycles of 94°C for 15 seconds, 50°C for 30 seconds and 72°C for 90 seconds and last cycle was an extension at 72°C for 10 minutes. The 346 bp and the 353 bp PCR products with R2 and R4 primers and with G1 and G2 primers respectively were cloned into pBlue-script SK(+) (Stratagene) and analyzed by sequencing using Sequenase v2.0 (US Biochemicals).

### Isolation of chicken *patched* cDNA clones

About 10<sup>6</sup> colonies of an amplified pBluescript KS(+) stage 22 chick limb bud cDNA library were transferred to nylon filters (Colony/Plaque screen, NEN) and regrown on ampicillin plates at 37°C for 6 hours. The filters were treated with 0.5 N NaOH and neutralized with 1 M Tris-HCl, pH 7.5, then the DNA was fixed by air drying. The filters were hybridized in 50% formamide, 10% dextran sulfate, 2× SSC and 1% SDS at 42°C with a mixture of the two <sup>32</sup>P-labeled PCR products. Then they were washed twice at room temperature with 2× SSC for 10 minutes, twice at 42°C in 2× SSC, 1% SDS for 30 minutes and twice at 65°C in 0.2× SSC, 1% SDS for 30 minutes. One positive colony, clone 1-3, identified by exposure of the hybridized filters on Kodak XAR-5 film, was purified and verified by sequencing to be a partial cDNA of the chicken homolog of the *patched* gene. This clone and the PCR product amplified with the two primers R2 and R4 were used to screen a λZAPII (Stratagene) stage

32 chick limb cDNA library. Two of the 13 positives, clone 20 and clone 200, were completely sequenced. They contained the start codon and the stop codon, respectively, and they overlapped between nucleotide 949 and 3200 of the open reading frame.

### In situ hybridizations

Whole-mount in situ hybridizations were performed as described by Riddle et al. (1993).

For in situ hybridization to histological slides, 5 µm sections were processed and hybridized with <sup>35</sup>S-labeled riboprobes essentially as described by Tessarollo et al. (1992). Sections were photographed with a combination of bright-field and dark-field optics with a red filter using a Zeiss Axiophot microscope and Kodak 64T film.

The chick *PTC* antisense digoxigenin-labeled and <sup>35</sup>S-labeled riboprobes were generated by *SalI* linearization and T3 RNA polymerase transcription of the 3.8 kb clone 200. Clone 200 was linearized with *XbaI* and transcribed with T7 RNA polymerase for the sense control probe. The *Sonic hedgehog* probe, a 1.7 kb fragment of pHH2 clone, was prepared as described by Riddle et al. (1993). The *BMP-2* probe, a 1.5 kb clone, was prepared as described by Laufer et al. (1994).

### Chick embryos, surgeries and retroviral infections

All experimental manipulations were performed on standard specific pathogen-free white Leghorn chick embryos provided by SPAFAS (Norwich, Connecticut). Eggs were incubated at 37°C and staged according to Hamburger and Hamilton (1951).

Concentrated retrovirus expressing either *Sonic hedgehog* (RCAS-A2; Riddle et al., 1993) or an *Alkaline phosphatase* control (RCASBP/AP(A); Fekete and Cepko, 1993) were injected at the anterior margin of stage 20-22 right wing buds beneath the AER. Embryos were harvested 16 hours after infection, washed in PBS, fixed in 4% paraformaldehyde in PBS and processed for whole-mount in situ hybridization.

For extirpation, the AER was visualized in stage 20-22 right wings by staining with Nile blue sulfate (0.01 mg/ml in Ringer's solution) and then removed with electrolytically sharpened tungsten wire needles. The exposed mesoderm was subsequently infected with either the *Sonic hedgehog* RCAS-A2 retrovirus or the control RCASBP/AP(A) retrovirus.

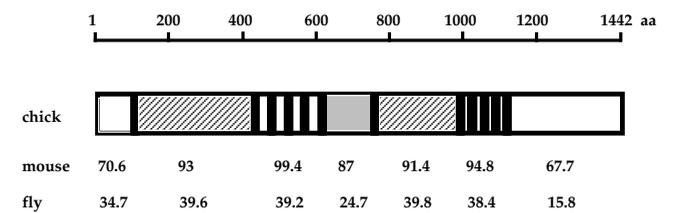
## RESULTS

### Isolation of a *patched* homolog from chick

In order to examine the *hedgehog* signaling pathway in the chick embryo, we isolated a homolog of the *Drosophila* gene *ptc* (sequence GenBank accession number: U40074). The predicted open reading frame is 1442 amino acids long and is 86.2% identical to the mouse homolog and 33.4% identical to the fly homolog. The identity between the two vertebrate proteins is lower at the amino terminus (70.6%) and at the carboxy terminus (67.7%) and higher than 90% in between, including two very hydrophobic regions that may span the plasma membrane (Fig. 1).

### Comparison of *PTC* and *Sonic hedgehog* expression patterns

In *Drosophila*, *ptc* is highly expressed in cells directly responding to the hh signal (Ingham, 1993; Basler and Struhl, 1994; Tabata and Kornberg, 1994). If its function were conserved in vertebrates we would expect the chick homolog to be expressed in all *Sonic hedgehog* target tissues such as the ventral neural tube (Echelard et al., 1993; Krauss et al., 1993; Roelink et al., 1994), the sclerotome (Fan and Tessier-Lavigne,



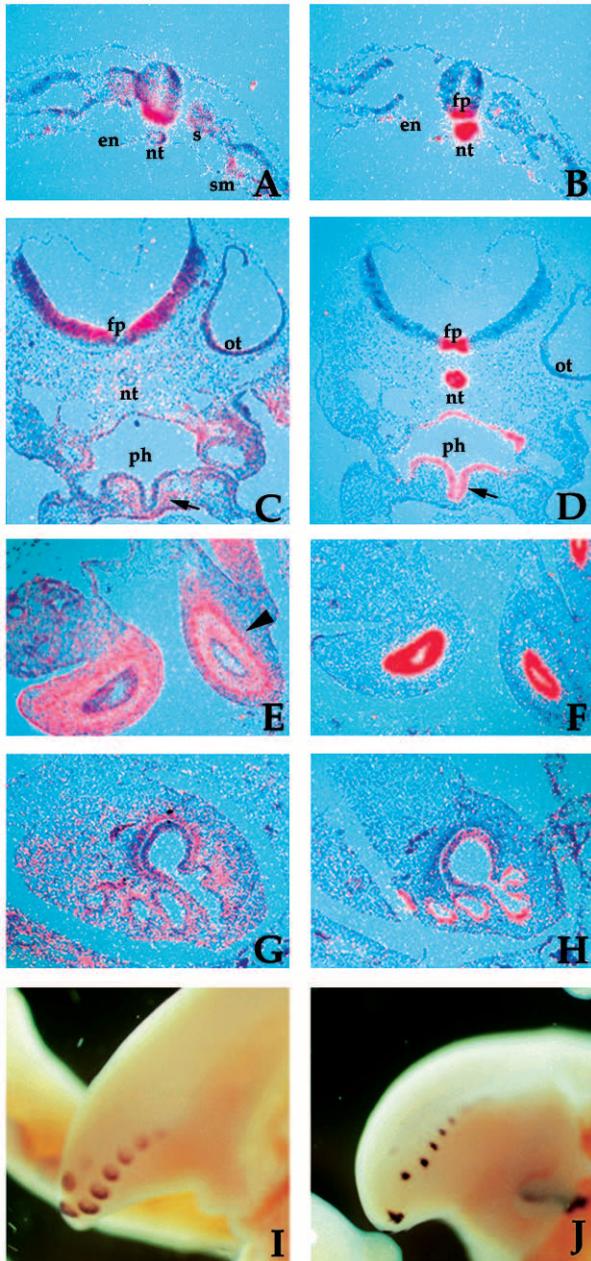
**Fig. 1.** Scaled schematic representation of the predicted PTC protein structure. Amino acid analysis of the predicted PTC protein sequence suggests that PTC is a multiple transmembrane protein. Analysis of the mouse and *Drosophila* sequences leads to the prediction of 12 transmembrane domains (Goodrich et al., 1996) as modeled here. Hatched boxes represent extracellular loops, black stripes transmembrane domains, and the gray box an intracellular loop. Amino acids are numbered above. Percentage identity between different domains of the chick and mouse proteins and of the chick and fly proteins are shown.

1994; Johnson et al., 1994), the visceral mesoderm (Roberts et al., 1995), and the limb bud (Riddle et al., 1993).

*PTC* and *Sonic hedgehog* expression patterns during chick development were compared by in situ hybridization. We hybridized adjacent sections with specific probes for *Sonic hedgehog* and *PTC*. In sections through the trunk of a stage 10 embryo, *PTC* transcripts are found in the ventral part of the neural tube with a domain broader than just the floor plate (compare Fig. 2A and 2B) and at lower levels in the notochord, epithelial somites, endoderm and splanchnic mesoderm (Fig. 2A). *Sonic hedgehog* at this stage is expressed specifically in the notochord, floor plate and endoderm (Fig. 2B).

Later in development, at stage 18, *PTC* is broadly expressed in the neural tube but is excluded from the cells of the floor plate (Fig. 2C). We also noticed that in the central nervous system *PTC* is more strongly expressed near the ventricular surface, including the ventricular zone of neural proliferation. *Sonic hedgehog* expression at the midline remains restricted to the notochord and floor plate (Fig. 2D). *PTC* is also expressed in the sclerotomal cells around the notochord, while at this stage it is excluded from the notochord itself (Fig. 2C). In the pharynx, *PTC* is expressed in the mesenchymal cells (Fig. 2C, arrow), while *Sonic hedgehog* is expressed in the overlying epithelial tissue (Fig. 2D, arrow). Thus at stage 18 *PTC* and *Sonic hedgehog* are expressed near each other in a variety of tissues, and in the neural tube and in the pharynx the two genes show a complementary pattern.

The complementary relationship between the expression patterns of the two genes is even more evident at later stages. At stage 32 in the gastrointestinal tract, mesodermal cells express *PTC* (Fig. 2E) while *Sonic hedgehog* is detected in the endodermal cells (Fig. 2F). *PTC* mRNA is transcribed in the submucosa directly adjacent to the endoderm and accumulates to high levels in the developing muscular mucosa (Fig. 2E, arrowhead) which may reflect the relative density of cells in this tissue. At this same stage *PTC* is also transcribed in the mesenchymal cells of the developing lung, most strongly in the cells adjacent to the epithelium, while *Sonic hedgehog* mRNA is in the epithelium (Fig. 2G, H). The two genes are also expressed in complementary patterns in the feather germs of stage 32 embryos, where *PTC* is expressed in the mesoderm



**Fig. 2.** Relationship between *PTC* and *Sonic hedgehog* expression. Adjacent sections at the trunk level, of a stage 10 embryo hybridized either with the *PTC* probe (A) or with the *Sonic hedgehog* probe (B) revealed overlapping expression in the notochord and floor plate of the neural tube as well as expression of *PTC* in the ventral somites and splanchnic mesoderm. Sections through the hindbrain of a stage 18 embryo hybridized with the *PTC* (C) or *Sonic hedgehog* (D) probes shows that at later stages the expression of the two genes becomes complementary such that tissues expressing *Sonic hedgehog* no longer express *PTC*. *PTC* expression in the pharyngeal mesenchyme (C) is denoted with an arrow and *Sonic hedgehog* expression in the pharyngeal epithelium (D) with an arrow. The complementary relationships are maintained later in development such that at stage 32 in sections of the intestine, *PTC* can be detected in the mesoderm (E) and *Sonic hedgehog* in the endoderm (F). Arrowhead in E denotes the muscular mucosa. Similarly, in sections of stage 32 lung, *PTC* is expressed in the mesenchyme (G) subjacent the epithelium expressing *Sonic hedgehog* (H). Complementary expression of *PTC* (I) and *Sonic hedgehog* (J) can be seen in whole-mount hybridizations to stage 32 feather germs of the tail. en, endoderm; fp, floor plate; nt, notochord; ot, otocyst; ph, pharynx; s, somite; sm, splanchnic mesoderm.

specifically expressed in a more restricted domain at the posterior margin of a stage 20 limb bud, in the ZPA tissue (Fig. 3B). By stage 24 the strong *PTC* expression divides into two domains: a more distal domain which spreads anteriorly and a more proximal, posteriorly restricted domain (Fig. 3D). In principle the two domains of *PTC* expression could be induced by separate sources of hedgehog signal(s) or a single *Sonic hedgehog* source spatially restricted in its ability to activate the *PTC* target. Hybridizing the contralateral limb of the same embryo with a *Sonic hedgehog* probe revealed that both *PTC* domains overlap with the *Sonic hedgehog* expression domain at stage 24 (Fig. 3E). At stage 29, when *Sonic hedgehog* expression fades (Riddle et al., 1993), *PTC* expression also decreases (data not shown).

The onset of a second pattern of *PTC* expression associated with bone development is visible in a stage 29 hindlimb, where *PTC* mRNA is found in a domain around the developing skeletal elements including the perichondrium (Fig. 3F). By stage 32 the cells around the cartilage of the phalanges express *PTC* (Fig. 3G). At these later stages *Sonic hedgehog* is no longer expressed in the developing limb but another member of the *hedgehog* family, *Indian hedgehog*, is expressed specifically in the pre-hypertrophic regions of the cartilage (A. Vortkamp and C. Tabin, unpublished data). Thus the expression pattern of *PTC* is closely related to two different members of the vertebrate *hedgehog* family during limb development.

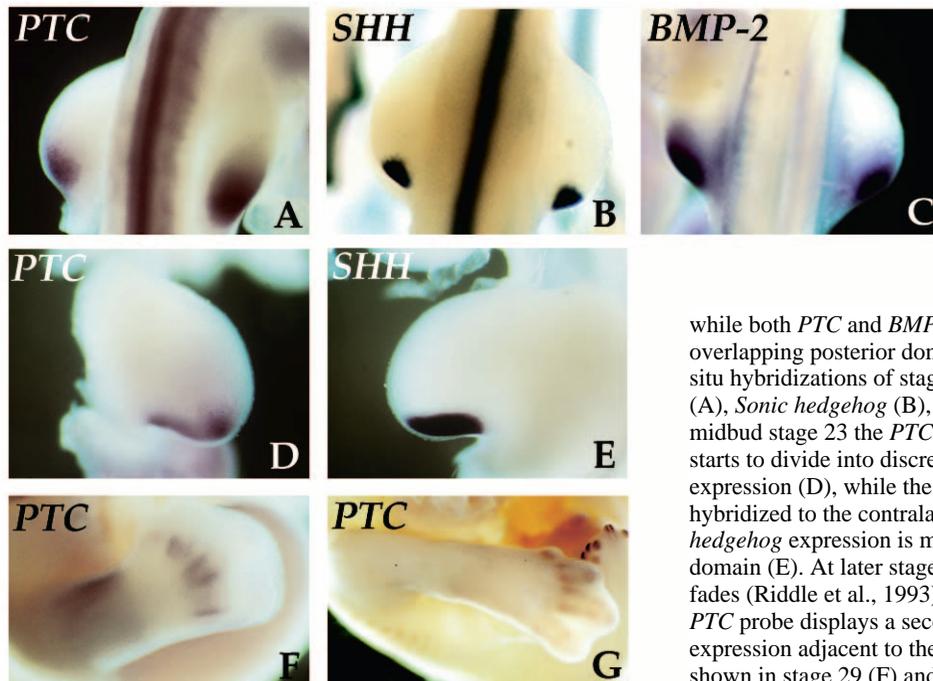
#### ***PTC* as a marker for *Sonic hedgehog* target cells in the limb**

The complementary expression pattern of *Sonic hedgehog* and *PTC* in many developing tissues strongly suggests that the *hedgehog* signaling pathway is conserved from insects to vertebrates. Therefore the chick homolog can be used as a marker to address several important questions concerning *Sonic hedgehog* signaling during limb development. One open question concerns the range of direct action of *Sonic hedgehog* in limb patterning. Immunohistochemistry has been used to detect *Sonic hedgehog* protein in the cells synthesizing it

around ectodermal foci of *Sonic hedgehog* RNA producing cells (Fig. 2I, J).

#### ***PTC* and *Sonic hedgehog* expression patterns in the developing limb**

*Sonic hedgehog* has an instrumental signaling role during limb development (Riddle et al., 1993). The relationship between *Sonic hedgehog* and *PTC* expression was analyzed in chick limbs at different stages by whole mount in situ hybridization. *PTC* expression in the developing limb bud is dynamic. It is first detected at stage 17 in mesodermal cells at the posterior margin of the limb bud (data not shown) which is the same stage and region that *Sonic hedgehog* starts to be expressed (Riddle et al., 1993). In a stage 20 chick limb bud, *PTC* mRNA is most abundant in the mesodermal cells at the posterior region of the bud (Fig. 3A). *Sonic hedgehog* transcripts are



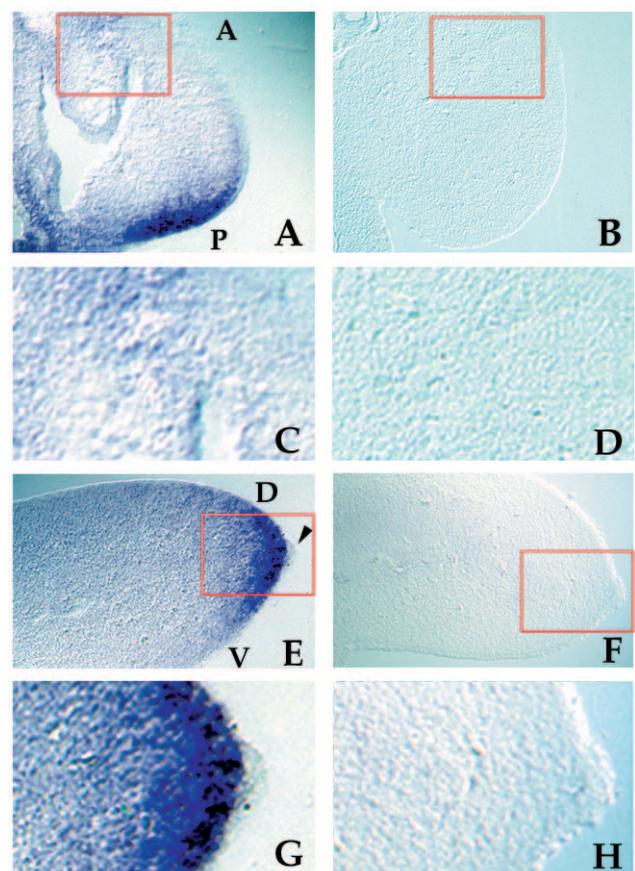
**Fig. 3.** Comparison of *PTC* and *Sonic hedgehog* in limb development. During early stages of limb development *Sonic hedgehog* is expressed in the posterior limb bud

while both *PTC* and *BMP-2* are expressed in larger, overlapping posterior domains. Shown are whole-mount in situ hybridizations of stage 20 limb buds probed for *PTC* (A), *Sonic hedgehog* (B), and *BMP-2* (C). However, by midbud stage 23 the *PTC* probe reveals a domain which starts to divide into discrete posterior and distal regions of expression (D), while the *Sonic hedgehog* probe hybridized to the contralateral limb shows that the *Sonic hedgehog* expression is maintained in a single continuous domain (E). At later stages *Sonic hedgehog* expression fades (Riddle et al., 1993), however hybridization with the *PTC* probe displays a second, independent domain of expression adjacent to the developing skeletal elements, shown in stage 29 (F) and stage 32 (G) hindlimbs.

(Martí et al., 1995). However, as these authors point out, the available antibodies must not be sensitive enough to detect the protein as it diffuses since it is known that *Sonic hedgehog* does act over multiple cell diameters, for example in the induction of sclerotome (Fan and Tessier-Lavigne, 1994). Hence a different measure of the range of *Sonic hedgehog* is required. High levels of *ptc* expression mark cells actively responding to hh in *Drosophila* (Hidalgo and Ingham, 1990; Ingham, 1993; Basler and Struhl, 1994; Tabata and Kornberg, 1994). This suggests that induction of high levels of *PTC* can provide another way of determining the range of *Sonic hedgehog* action in vertebrates. On this basis the direct influence of *Sonic hedgehog* appears to extend beyond the ZPA of a stage 22 limb bud but is limited to a posterior domain (Fig. 3A). This domain of high level *PTC* expression is approxi-

mately the same size as that expressing *BMP-2* in response to *Sonic hedgehog* (Fig. 3C) consistent with *BMP-2* also being a direct target of *Sonic hedgehog* in the limb. Based on *PTC* expression, *Sonic hedgehog* also appears to act directly over a considerable range in the neural tube and sclerotome (Fig. 2C).

**Fig. 4.** Analysis of *PTC* expression in stage 20 limb bud sections (A,B) Cryosections along the anterior-posterior axis of a stage 20 chick limb bud processed by whole-mount in situ hybridization with *PTC* antisense riboprobe (A) and *PTC* sense control riboprobe (B). *PTC* expression is stronger in the mesodermal tissue at the posterior margin of the developing limb and a low level of expression can be detected along the entire anterior-posterior axis when compared with the control. A, anterior; P, posterior. That the expression in the anterior mesoderm is above background can be verified by examination at higher magnification shown in C and D. Regions enlarged in C and D are marked with red boxes in A and B respectively. (E,F) Cryosections along the dorsal-ventral axis of a stage 23 chick limb bud processed by whole-mount in situ hybridization with *PTC* antisense riboprobe (E) and *PTC* sense control riboprobe (F). *PTC* is specifically expressed in the mesodermal tissue and excluded from the AER. Arrowhead indicates the AER. D, dorsal; V, ventral. That the hybridization signal in the AER is at a background level can be verified at higher magnification shown in G and H. The signal in the AER hybridized with the *PTC* probe is below the lower level expression seen in the mesoderm of the same section (G), and is equivalent to the signal seen in both the AER and mesoderm hybridized with the control probe (H).



A second open question concerns which tissue(s) are the direct targets of Sonic hedgehog signaling. Sonic hedgehog has been shown to induce expression of secreted molecules including *BMP-2* (Laufer et al., 1994) in the mesoderm and *FGF-4* in the overlying AER (Laufer et al., 1994; Niswander et al., 1994). However, whether the ectoderm, the mesoderm, or both tissues are directly influenced by Sonic hedgehog is unknown. In *Drosophila* *ptc* is required at low levels to restrict the expression of hh target genes that are up-regulated in response to hh (Capdevila et al., 1994). The extraordinary conservation between the vertebrate and fly signaling systems suggests that this will be the case in the chick as well. In addition to the high levels of *PTC* in the posterior, we detect a low level of *PTC* throughout the mesoderm of the limb bud (Fig. 4A-D). This is consistent with the limb mesoderm having the signal transduction machinery to be able to directly respond to Sonic hedgehog. However analysis of *PTC* expression also revealed that *PTC* is excluded from the AER (Fig. 4E - H), suggesting that this tissue may not be capable of directly responding to Sonic hedgehog.

### Induction of *PTC* expression by infection with a *Sonic hedgehog*-expressing virus

Interpretations of *PTC* expression based on the *Drosophila* model are only valid if *PTC* is regulated in an analogous way in vertebrates. To directly test whether the relationship between hedgehog signaling and *patched* expression is conserved through evolution we misexpressed *Sonic hedgehog* in the developing limb by infection with a replication-competent retrovirus expressing chick *Sonic hedgehog* (Riddle et al., 1993). The retrovirus was injected into stage 20 chick limb buds in the anterior mesodermal tissue just underneath the AER. 16 hours after infection embryos were harvested and analyzed by whole-mount in situ hybridization. We found that *PTC* transcription is strongly induced in the mesodermal tissue at the anterior margin of the limb where *Sonic hedgehog* is misexpressed (Fig. 5A, arrow). However, we have never observed induction of *PTC* in the overlying AER, again consistent with that tissue not being a direct target of Sonic hedgehog (Fig. 5A and data not shown).

Another aspect of hedgehog signaling which is conserved between *Drosophila* and vertebrates is the induction of the homologs *dpp* and *BMP-2*. In vertebrates, *BMP-2* is expressed in the posterior limb bud mesoderm and in the AER (Fig. 5B and Lyons et al., 1990). As previously reported (Laufer et al., 1994), following ectopic expression of *Sonic hedgehog* in the anterior limb, *BMP-2* expression is ectopically detected in the mesoderm (Fig. 5B, arrow), as well as in the overlying elongated AER (Fig. 5B). In embryos injected with a control retrovirus neither *PTC* nor *BMP-2* is induced at the anterior margin of the limb bud (data not shown).

### The AER is not essential for induction of *PTC* by *Sonic hedgehog*

*Sonic hedgehog* misexpression is sufficient to induce *PTC* expression in anterior limb mesenchyme. We wanted to additionally determine whether Sonic hedgehog is necessary for the normal induction of *PTC* in the posterior mesenchyme. One way of approaching this question is to remove the AER. FGF-4 protein produced by the AER is essential for the maintenance of *Sonic hedgehog* expression in the posterior margin of the limb (Laufer et al., 1994; Niswander et al., 1994). Thus

removal of the AER abolishes the source of both FGF-4 and Sonic hedgehog within the limb bud. To examine the consequence for *PTC* expression, we removed the posterior half of the AER in a stage 20 chick limb. The absence of the AER results in the loss of *Sonic hedgehog* expression in the mesoderm, and in inhibition of limb outgrowth. 24 hours after surgery the *PTC* mRNA level decreased in the posterior mesoderm of the experimental limb (Fig. 6A, right and arrow) while *PTC* was expressed normally in the unoperated contralateral limb (Fig. 6A, left). Expression of *BMP-2*, another gene downstream of *Sonic hedgehog*, similarly decays after AER removal (Laufer et al., 1994).

The decay of *PTC* expression after extirpation of the AER could either reflect the AER being directly required for *PTC* maintenance or be an indirect consequence of the dependence of *Sonic hedgehog* expression on signals from the AER (Laufer et al., 1994; Niswander et al., 1994). To distinguish between these possibilities we maintained *Sonic hedgehog* expression in the posterior mesoderm after posterior AER removal, by infecting the tissue with the *Sonic hedgehog*-expressing retrovirus immediately following the surgery. 48 hours later *PTC* mRNA is still present at high levels in the posterior mesoderm (Fig. 6B, arrowhead) while *PTC* transcripts decay in an embryo injected with a control retrovirus (Fig. 6B). In contrast, under the same conditions *BMP-2* requires signals from the AER to be maintained in the posterior mesoderm (Fig. 6B). This experiment demonstrates that *Sonic hedgehog* expression but not the AER is required for maintenance of *PTC* expression.

While it is not needed for maintenance of *PTC*, the AER could play a role in the initiation of *PTC* expression. To test whether the AER is required for *PTC* induction, the anterior half of the AER from a stage 22 chick limb was removed and then *Sonic hedgehog* was misexpressed in the anterior mesoderm. The removal of the anterior half of the AER results in the limb bud acquiring a flattened shape but does not affect the ability of the retrovirus to infect the mesoderm. 48 hours after the surgery and subsequent infection, the viral message is detectable in the proximal anterior mesoderm (Fig. 6C, arrow). At the same time point *PTC* expression is induced in the anterior mesoderm in the same area as the ectopic *Sonic hedgehog* (Fig. 6C, arrowhead). In contrast, the AER is required for the induction of other downstream genes such as *BMP-2* (Fig. 6C and Laufer et al., 1994). These experiments demonstrate that *Sonic hedgehog* does not require any signal from the AER to induce and maintain mesodermal *PTC* expression.

## DISCUSSION

### Conservation of *patched* sequence and structure from invertebrates to vertebrates

We cloned a chick *patched*-related gene which encodes a protein highly homologous to mouse *Ptc*. The highest similarity between the vertebrate genes is in two highly hydrophobic regions which are predicted to span the plasma membrane multiple times (Goodrich et al., 1996). The suggested structure contains 12 transmembrane domains in a pattern reminiscent of the six plus six transmembrane domains of a family of transporter proteins (Nikaido and Saier, 1992).

The comparison of the hydrophobicity plots between the fly and the vertebrate proteins (Goodrich et al., 1996) suggests that

the protein structures are very similar and also their function might be conserved in evolution. The strongest evidence that *PTC* is a vertebrate homolog of *ptc* comes from the demonstration that aspects of its relationship to the vertebrate *hedgehog* genes parallel the regulatory pathways found in flies.

### Expression patterns suggest a close relationship to multiple *hedgehog* family members

We analyzed the expression of *PTC* during chick development. We found an intriguing expression pattern that parallels the timing of onset and embryonic domains of expression of multiple members of the vertebrate *hedgehog* family. In many cases the expression domains of *PTC* and *hedgehog* genes are observed in adjacent embryonic tissues while in other cases they are transiently co-expressed in the same regions. These regions of *PTC* expression include all target tissues where the Sonic hedgehog signal is known to have an important inductive role, such as the neural tube (Echelard et al., 1993), the sclerotome (Fan and Tessier-Lavigne, 1994; Johnson et al., 1994), the visceral mesoderm (Roberts et al., 1995), and the limb bud (Riddle et al., 1993). Moreover the timing of *PTC* induction in these tissues matches when Sonic hedgehog signaling is known to take place. For example, *Sonic hedgehog* is expressed in the notochord synchronously with the presence of floor plate-inducing activity in the notochord (Echelard et al., 1993; Roelink et al., 1994) and concomitant with activation of high expression of *PTC* in the ventral neural tube. A more detailed description of *PTC* expression at various stages of neural development is reported elsewhere (Marigo and Tabin, 1996).

In addition to the endogenous expression of *PTC* being consistent with response to Sonic hedgehog, *PTC* is ectopically induced in response to *Sonic hedgehog* misexpression.

In the chick, a single *patched*-related gene is expressed in a variety of tissue types adjacent to *Sonic hedgehog*-expressing cells. This suggests that the Sonic hedgehog signaling pathway in different organs is mediated, at least partially, by common downstream genes. Moreover, our data suggest that *PTC* might be a common downstream gene of different *hedgehog* family members. This raises the possibility that there is only a single *ptc* homolog transducing hedgehog signaling in higher vertebrates. Consistent with this hypothesis we have not detected other *PTC*-related chick genes by low stringency hybridization, PCR, or DNA analysis (data not shown). We take note of the fact that there appear to be two distinct *ptc*-related genes in zebrafish, however this may be specific to the teleost lineage (Concordet, J. P., Lewis, K., Moore, J., Goodrich, L. V., Johnson, R. L., Scott, M. P. and Ingham, P. W., personal communication).

One of the most interesting aspects of the regulation of the vertebrate *PTC* gene is that *PTC* and *Sonic hedgehog* are only transiently co-expressed in the notochord, floor plate and endoderm. Similarly, *PTC* expression overlaps the entire ZPA region at early stages of limb development, but its expression is subsequently lost in the middle of this region, where *Sonic hedgehog* expression is strongest. These observations can be explained by a feedback mechanism in which the biochemical activity of *PTC*, when *PTC* protein accumulates to a sufficient high level, represses its own transcription in spite of Sonic hedgehog signaling (Marigo and Tabin, 1996). This is consistent with the recent finding that in *Drosophila* forced high levels of *patched* expression are able to block response to hh

signaling (Johnson et al., 1995). This auto-inhibition model would provide a rationale for the otherwise perplexing observation that *PTC* is upregulated specifically in cells where its activity is repressed.

### Possible roles of *PTC* in limb development

The signaling role of Sonic hedgehog during limb development has been particularly well studied (Riddle et al., 1993), but key questions remain. For example, which cells receive Sonic hedgehog signal? Are the effects of Sonic hedgehog on the mesoderm and the AER direct or mediated by other signals? How far does the Sonic hedgehog signal travel? The *PTC* gene sheds light on each of these issues.

We found that *PTC* is expressed at a low basal level throughout the mesoderm, but it is not expressed in the AER. If *PTC* is expressed in cells responding to the *Sonic hedgehog* signal, as *ptc* is in flies, this would imply that the ectoderm is incapable of directly responding to Sonic hedgehog signals. If true, then *Sonic hedgehog* induction of genes like *FGF-4* and *BMP-2* in the AER (Laufer et al., 1994; Niswander et al., 1994) is an indirect effect mediated by mesodermal factors, and the target tissue of the Sonic hedgehog signal in patterning the limb is the mesoderm and not the AER. While we favor this interpretation, it remains possible that Sonic hedgehog affects gene expression in the ectoderm by a *PTC*-independent pathway.

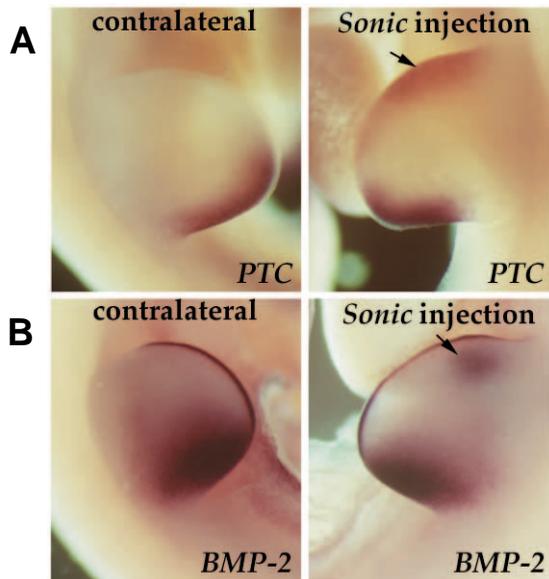
In addition to its low basal level of expression throughout the mesoderm, we observe a high level of *PTC* transcription in the mesenchyme as soon as *Sonic hedgehog* message is detectable in cells of the posterior limb mesenchyme. By analogy to the *Drosophila hh* pathway, it is likely that high levels of *PTC* expression mark cells that are actively responding to *hedgehog* signaling. Thus, Sonic hedgehog appears to act across multiple cell diameters, but its direct influence is limited to perhaps one quarter of the width of the stage 22 limb bud. This domain of high level *PTC* expression is approximately the same size as that expressing *BMP-2* in response to Sonic hedgehog, consistent with *BMP-2* also being a direct target of *Sonic hedgehog*.

The posterior limb domain of high *PTC* expression subsequently divides into separate posterior and distal regions. Interestingly, the expression of the *HOX* genes, an important class of downstream targets of Sonic hedgehog, also evolves into distinct posterior and distal domains which appear to be important in patterning distinct limb elements (Nelson et al., 1996). The repression of *PTC*, and hence of *PTC*-mediated signaling, in the middle of the domain of *Sonic hedgehog* expression may be important in organizing limb pattern.

The expression pattern of *PTC* at later stages of limb development strongly suggests that *PTC* is also in the signaling pathway of *Indian hedgehog*. We detected *PTC* in the tissue surrounding cartilage cells expressing *Indian hedgehog*. Members of the *BMP* family are expressed in this same areas (Lyons et al., 1990; Francis et al., 1994) suggesting that these genes might be in the *Indian hedgehog* signaling pathway as their relative *BMP-2* is in *Sonic hedgehog* pathway in early limb development.

### *Sonic hedgehog* regulates *PTC* expression without requiring an AER signal

Recent studies indicate that a signal from the AER, *FGF-4*, is



**Fig. 5.** Induction of *PTC* and *BMP-2* by *Sonic hedgehog*. Stage 20 chick limb buds were injected with *Sonic hedgehog*-expressing retrovirus at the anterior margin underneath the AER and harvested 16 hours later. *PTC* and *BMP-2* expressions were analyzed by whole-mount in situ hybridization and compared with the contralateral control limb. In (A) *PTC* expression is induced in the anterior mesoderm (arrow) of the injected limb. (B) Induction of *BMP-2* in the mesoderm (arrow) and overlying elongated AER.

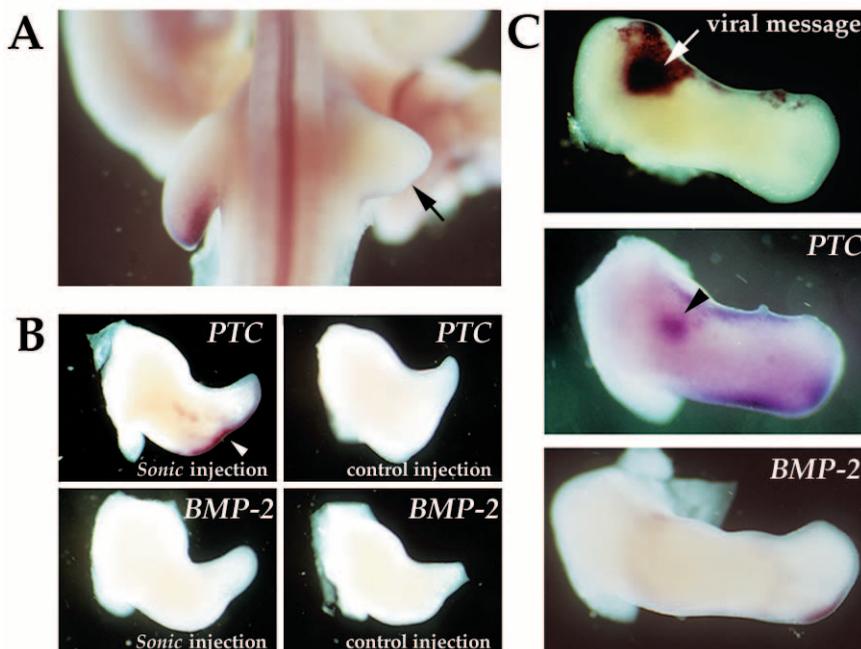
required to give competence to the mesodermal cells to respond to *Sonic hedgehog* signal (Laufer et al., 1994). We find that while FGF-4 is required to activate some downstream genes like *BMP-2*, this AER signal is not necessary to induce or maintain

*PTC* transcription. Thus *Sonic hedgehog* induction of *PTC* seems likely to be more direct than *BMP-2* induction because it does not require signals from the AER. We cannot exclude the possibility of other factors from the mesenchyme cooperating with *Sonic hedgehog* in establishing the *PTC* expression pattern.

Our data provide insight into how different factors are integrated in patterning the developing limb. *Sonic hedgehog* is the signal from the ZPA which is able to induce *FGF-4* expression in the AER (Laufer et al., 1994; Niswander et al., 1994). This induction is likely to be indirect because *PTC* is never detectable in the AER. FGF-4 itself is necessary to maintain *Sonic hedgehog* expression in the posterior margin of the developing limb and FGF-4 is also required for limb proliferation (Laufer et al., 1994; Niswander et al., 1994). The two signals are highly integrated because *Sonic hedgehog* is unable to pattern in the absence of the proliferative signal and, conversely FGF-4, even if it induces proliferation, cannot organize a limb structure in the absence of *Sonic hedgehog*. In *Drosophila* hh acts to inhibit *ptc* protein function, releasing the repression of target genes. One consequence of this is derepression of *ptc* itself. Since *Sonic hedgehog* induces *PTC* in the chick limb bud it is likely that this relationship is conserved. Hence *Sonic hedgehog* may inhibit *PTC* protein activity in mesenchymal cells, ultimately resulting in induction of *PTC* and other downstream genes.

The expression pattern of *PTC*, related to the expression of different members of the *hedgehog* family in many different tissues and the induction of *PTC* by *Sonic hedgehog*, leads us to suggest that the vertebrate *hedgehog* signals are in general mediated by *PTC* in patterning the early vertebrate embryo. The identification of the chicken *PTC* will therefore allow further dissection of the mechanisms by which these important signaling molecules act.

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**Fig. 6.** Effects of the AER on *PTC* expression. (A) The AER was removed from the right limb bud of a stage 20 chick embryo. The embryo was harvested 24 hours after surgery and processed for whole-mount in situ hybridization with the *PTC* probe. *PTC* mRNA is undetectable in the right truncated limb (arrow). (B) The posterior half of the AER of stage 22 chick right limb buds was removed and the exposed mesoderm was infected either with *Sonic hedgehog*-expressing retrovirus or with a control retrovirus. Embryos were harvested 48 hours later and hybridized either with the *PTC* probe or the *BMP-2* probe. Arrowhead points to the *PTC* expression maintained in the limb injected with *Sonic hedgehog*-expressing retrovirus. *BMP-2* expression requires the presence of the AER to be maintained in the mesoderm. (C) The anterior half of the AER of stage 22 chick right limb buds was removed and *Sonic hedgehog*-expressing retrovirus was injected into the exposed anterior mesoderm. 48 hours after surgery and infection, embryos from the same experiment were hybridized with probes either for the viral message or for *PTC* or for *BMP-2*.

Note the induction of *PTC* in the same mesodermal area (arrowhead) where *Sonic hedgehog* was misexpressed (arrow). *BMP-2* is not induced by *Sonic hedgehog* in the absence of the AER.

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