Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 9346–9351, September 1996
Colloquium Paper

This paper was presented at a colloquium entitled "Biology of Developmental Transcription Control," organized by Eric H. Davidson, Roy J. Britten, and Gary Felsenfeld, held October 26–28, 1995, at the National Academy of Sciences in Irvine, CA.

Regulation of *Patched* by Sonic hedgehog in the developing neural tube

(central nervous system development/*Drosophila* homolog)

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ABSTRACT **Ventral cell fates in the central nervous system are induced by Sonic hedgehog, a homolog of hedgehog, a secreted *Drosophila* protein. In the central nervous system, Sonic hedgehog has been identified as the signal inducing floor plate, motor neurons, and dopaminergic neurons. Sonic hedgehog is also involved in the induction of ventral cell type in the developing somites. *ptc* is a key gene in the *Drosophila* hedgehog signaling pathway where it is involved in transducing the hedgehog signal and is also a transcriptional target of the signal. *PTC*, a vertebrate homolog of this *Drosophila* gene, is genetically downstream of *Sonic hedgehog* (Shh) in the limb bud. We analyze *PTC* expression during chicken neural and somite development and find it expressed in all regions of these tissues known to be responsive to Sonic hedgehog signal. As in the limb bud, ectopic expression of *Sonic hedgehog* leads to ectopic induction of *PTC* in the neural tube and paraxial mesoderm. This conservation of regulation allows us to use *PTC* as a marker for Sonic hedgehog response. The pattern of *PTC* expression suggests that Sonic hedgehog may play an inductive role in more dorsal regions of the neural tube than have been previously demonstrated. Examination of the pattern of *PTC* expression also suggests that PTC may act in a negative feedback loop to attenuate hedgehog signaling.**

During early vertebrate development, signaling centers from the midline direct the dorsal-ventral fate of cells in neural tube and somites. Recent studies (1) have identified Sonic hedgehog as a signaling molecule that is responsible for establishing aspects of dorsal-ventral polarity in the vertebrate central nervous system and somites.

In the developing neural tube, contact-mediated signaling from the underlying notochord is responsible for inducing the formation of the floor plate along the ventral midline (2, 3). In addition the floor plate and the notochord induce the formation of motor neurons lateral and dorsal to the floor plate in a contact independent manner (2, 4). These two signaling centers, the floor plate and notochord, also produce signals responsible for inducing ventral fates in the somites (5, 6). *Sonic hedgehog* is expressed in both the notochord and the floor plate (7–11). Ectopic expression of *Sonic hedgehog in vivo* as well as application of Sonic hedgehog protein to explants *in vitro* result in induction of both floor plate and motor neuron marker genes (7, 8, 11–14). This raises an apparent paradox: a single signal is capable of generating both contact-dependent and -independent responses. A possible resolution is that different thresholds of the protein can trigger induction of different cell fates. In fact, low concentrations of Sonic hedgehog induce motor neurons but not floor plate markers, whereas high concentrations induce floor plate cells (12, 13). Hence the experimental demonstration of contact dependence likely reflects the requirement for extremely high concentrations of Sonic hedgehog present on the surface of cells producing it rather than a need for direct contact per se. In addition to acting at a distance in motor neuron induction, several lines of evidence have implicated Sonic hedgehog as the long-range factor from the floor plate and the notochord mediating ventral sclerotomal cell fate in the somites (15, 16). Finally, Sonic hedgehog has also been identified as the signal inducing dopaminergic neurons, a ventral cell type in the midbrain (17, 18).

How Sonic hedgehog acts to regulate embryonic patterning remains unknown and needs not be direct. In fact, the assays described above have not definitively established whether these inductive activities require secondary signals. If Sonic hedgehog is directly responsible for induction of motor neurons and sclerotome, it would need to diffuse over many cell diameters *in vivo*. However, no evidence for diffusion of the protein was observed by immunohistochemistry, perhaps due to limited sensitivity of the technique (12, 19).

In *Drosophila*, hedgehog (hh), the fly homolog of Sonic hedgehog, has been implicated in short-range (e.g., in the wing imaginal discs and in establishing early segment borders) and long-range inductions (e.g., cellular patterning of the dorsal epidermis) (20–24). Supporting the idea that hh is a diffusible factor, in the fly the protein can be detected a few cell diameters beyond cells transcribing the *hh* gene (24–26). The regulation of gene expression by hh is mediated by the gene *patched* (*ptc*) (27, 28). ptc constitutively represses downstream targets, including its own transcription (29). The hh signal relieves ptc repression, thus inducing transcription of target genes (27, 29). Low levels of *ptc* transcription are therefore indicative of cells capable of responding to hh, since without ptc hh has no effect on transcription. High levels of *ptc* transcription, on the other hand, serve as a marker for cells that are directly responding to hh, since *ptc* is strongly derepressed in all cells receiving the hh signal.

We previously reported the cloning of a chicken homolog (*PTC*) of the *Drosophila patched* gene and demonstrated it to be in the Sonic hedgehog signaling pathway during vertebrate limb development (30). Here we report the expression pattern of *PTC* during chicken neural and somitic development. *PTC* is highly expressed in chicken tissues responsive to Sonic hedgehog signal, including the ventral neural tube and the ventral somites. We also demonstrate that *PTC* is genetically downstream of *Sonic hedgehog* in these tissues being induced in the neural tissue and paraxial mesoderm by ectopic expres-

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sion of *Sonic hedgehog*. Similar results have recently been obtained with Sonic hedgehog misexpression in the mouse dorsal hindbrain and spinal cord (31). Therefore, *PTC* appears to be a common downstream gene in the Sonic hedgehog pathways, patterning different tissues, and gives important insights into the nature of Sonic hedgehog signaling.

MATERIALS AND METHODS

Chicken Embryos. Chicken embryos were obtained by incubation at 37°C of pathogen-free White Leghorn chicken embryos (SPAFAS). Embryos were staged according to Hamburger and Hamilton (32).

***In Situ* Hybridizations.** Whole mount *in situ* hybridizations were carried out as described by Riddle *et al.* (9). The chicken *PTC* digoxigenin-labeled probe, from the 3.8 kb clone 200 (30), was prepared by T3 RNA polymerase transcription following *Sal*I linearization. Following the whole mount procedure, the embryos were dehydrated in 30% sucrose in PBS, embedded into 7.5% gelatin in PBS, and sectioned at the cryostat.

Retroviral Misexpression. A retrovirus expressing the full coding sequence of *Sonic hedgehog* (9) was injected on the right side of the paraxial mesoderm of stage 11–13 chicken embryos. The virus titer was 1–2 × 10^8 colony-forming units per ml. Embryos were harvested 48 hr after injection, washed in PBS, and processed for whole mount *in situ* hybridization (9). Control injections were performed using RCASBP/AP(A) retrovirus expressing Alkaline phosphatase (33).

RNA Analysis. mRNA from stage 32 chicken heart, lung, intestine, and liver and from stage 23 chicken limb bud were isolated with the PolyATtract mRNA isolation system (Promega). mRNA (3 μg) was electrophoretically separated on a 0.8% agarose gel in formaldehyde, transferred to a GeneScreen Plus membrane (DuPont), and UV cross-linked. The filter was hybridized either with a ^{32}P-labeled chicken *PTC* clone-200 probe or with a ^{32}P-labeled chicken GAPDH probe in 50% formamide, 5× SSPE (standard saline phosphate/EDTA; 0.18 M NaCl/10 mM phosphate, pH 7.4/1 mM EDTA), 10× Denhardt's solution (0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin), 2% SDS, and 100 μg/ml salmon sperm DNA at 42°C. The blot was washed at room temperature with 2× SSC and 0.05% SDS, and at 60°C with 0.1% SSC and 0.1% SDS.

RESULTS

***PTC* mRNA Expression.** *PTC* expression in the chicken limb bud has previously been characterized (30). To analyze the range of tissues expressing *PTC*, *PTC* mRNA expression in chicken embryonic tissues was analyzed by Northern blotting. A single *PTC* transcript, ≈9.5-kb long, is present in mRNA prepared from stage 32 chicken lung and from stage 23 limb bud (Fig. 1). Low levels of *PTC* mRNA can be detected in mRNA purified from stage 32 chicken intestine by longer exposure of the blot (data not shown). No *PTC* transcript is detectable in the developing heart or liver using this technique (Fig. 1).

PTC expression in the neural tube was noted previously (30). To examine this expression more closely, the pattern of *PTC* expression during chicken development was analyzed at various stages by whole mount *in situ* hybridization. *PTC* is first detectable in stage 4 chicken embryos around Hensen's node (data not shown). By stages 10–13, *PTC* transcripts are found in neural tissue, from the caudal end of the neural tube through the diencephalon (Fig. 2*A*). In the developing hindbrain, *PTC* is expressed at stage 15 in the rhombomeres in a gradient that is higher ventrally and lower dorsally (Fig. 2*C*). Cells of the floor plate do not express *PTC* (Fig. 2*C*, arrowhead). *PTC* expression in the neural tube is dynamic. By stage 17, *PTC* mRNA fades in the hindbrain from dorsal to ventral within each rhombomere, but is still strongly expressed at each segment boundary (Fig. 2*D*) and is still excluded from the floor plate (Fig. 3*D*, arrowhead). As development proceeds, expression within the spinal cord retracts caudally (Fig. 2*E*), whereas the expression in the brain is maintained at least through stage 32 (data not shown).

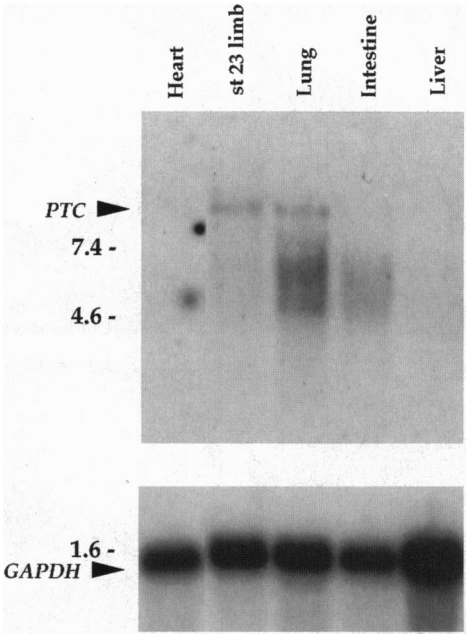

FIG. 1. Characterization of the chicken *PTC* mRNA. Northern blot analysis of chicken embryonic mRNA. Each lane contains ≈3 μg of poly(A)$^+$ RNA extracted from stage 32 chicken embryonic heart, lung, intestine, and liver and from stage 23 chicken limb bud. The filter was hybridized either with chicken *PTC* probe or with chicken GAPDH probe. A single transcript of ≈9.5 kb was detected with the *PTC* probe.

PTC is also expressed in a variety of non-neural tissues. By stages 10–13, *PTC* is expressed in the somites (Fig. 2*A*, arrowhead). At stage 17, *PTC* is also strongly expressed in the posterior mesoderm of the first and second branchial arches (Fig. 2*B*, arrowhead), in the caudal intestinal portal (Fig. 2*B*, arrow), and in the paraxial mesoderm. In a stage 25 chicken embryo, *PTC* transcripts are detected in the posterior mesoderm of the developing limb (Fig. 2*E*). By stage 32, *PTC* mRNA is found in the tongue and buccal region (Fig. 2*F*, arrowhead) and in the feather germs (Fig. 2*F*, arrow) in addition to the brain. Sections through the feather germs revealed that *PTC* expression is restricted to the mesodermal cells (Fig. 2*G*), while *Sonic hedgehog* is expressed in the ectoderm (34). Sections of whole mount *in situ* hybridization preparations reveal a low level of *PTC* expression throughout all mesodermal tissues in addition to the regions of strong expression described above (data not shown).

***PTC* Expression Pattern in the Developing Neural Tube and Notochord.** Our whole mount *in situ* hybridization revealed that *PTC* has a dynamic expression along the anterior-posterior axis, retracting caudally over time. To examine dorsoventral changes in *PTC* expression in the neural tube, *PTC* expression was analyzed at various developmental stages in sections of whole mount *in situ* hybridizations. Because there is an anterior-posterior gradient of developmental timing in the axial structures of the chicken embryo, with rostral regions being developmentally more advanced, it is possible to examine different developmental stages by sectioning a single embryo at different levels. In sections from the caudal region of stage 12 chicken embryos hybridized with the *PTC* probe, expression was detected in the notochord and at very low levels in the ventral neural tube (Fig. 3*A*). At this stage, *Sonic hedgehog* is first detectable just in the notochord (data not

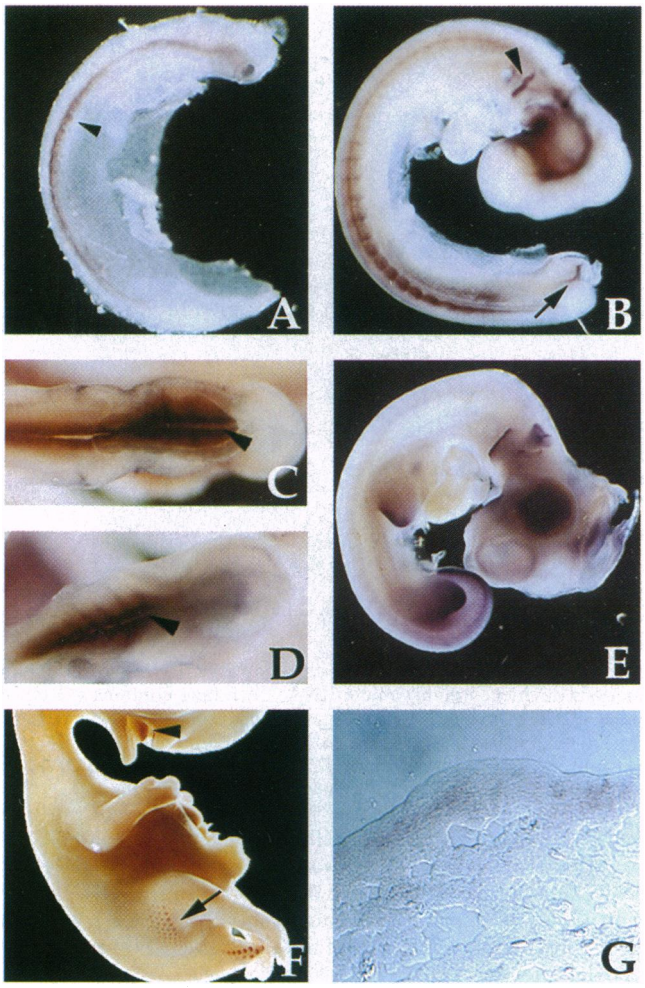

FIG. 2. *PTC* expression during chicken development. Chicken embryos at different embryonic stages were processed by whole mount *in situ* hybridization using a chicken *PTC* probe. (*A*) Stage 13 embryo. *PTC* expression is detected in neural tube and in somites (arrowhead). (*B*) Stage 16 embryo. Staining is observed in central nervous system, somites, stomodeum, posterior branchial arches (arrowhead), and caudal intestinal portal (arrow). *PTC* expression in the hindbrain of a stage 15 chicken embryo (*C*) and in the hindbrain of a stage 17 embryo (*D*). The floor plate (*C* and *D*, arrowhead) does not express *PTC*. (*E*) Stage 25 embryo. *PTC* mRNA is detectable in the posterior mesoderm of the developing limb, posterior mesoderm of the first and second branchial arches, stomodeum, and tail bud. (*F*) Stage 32 embryo shows *PTC* expression in the tongue (arrowhead) and feather germs (arrow). (*G*) Cryosection of a feather germ of the tail. *PTC* expression is restricted to the mesoderm.

shown and ref. 7). In more rostral sections of the same embryo, where neural tube and somites are developmentally older, *PTC* is strongly expressed in the ventral neural tube with borders of expression well beyond the floor plate (Fig. 3*B* and ref. 30). *PTC* mRNA is also detected in the somites and in the notochord (Fig. 3*B*). At this stage, *Sonic hedgehog* is expressed in both the notochord and the floor plate (7, 30). In sections of a stage 15 embryo, *PTC* expression was detected in the notochord, ventral somites, and in the splanchnic mesoderm (Fig. 3*C*). The strongest expression in the neural tube is in cells adjacent to the floor plate, whereas the levels of *PTC* transcripts are lower in the floor plate itself and in the notochord (Fig. 3*C*). At stage 18, *PTC* mRNA was found in the sclerotome around the notochord, which no longer expresses *PTC*. Similarly, cells of the floor plate do not express *PTC*. However, by this stage *PTC* transcripts are found along the entire dorsoventral axis of the neural tube, most strongly at the luminal surface (Fig. 3*D* and ref. 30).

***PTC* Induction by *Sonic Hedgehog* Misexpression in the Neural Tube and Paraxial Mesoderm.** Sonic hedgehog has been shown to be involved in the induction of motor neurons and other ventral cell fates in the nervous system. We found *PTC* expressed in the floor plate and later in neural cells flanking the floor plate at a time when they are known to be responsive to Sonic hedgehog, consistent with *PTC* being a molecular target of Sonic hedgehog signaling.

To analyze if *PTC* is downstream of *Sonic hedgehog* in the chicken central nervous system, we misexpressed *Sonic hedgehog* at early stages during chicken development using a replication competent retrovirus (9). The retrovirus was injected in the right side of stages 11–13 chicken embryos, infecting the paraxial mesoderm and the adjacent neural tissue. We analyzed *PTC* expression 48 hr after infection by whole mount *in situ* hybridization. Ectopic expression of *Sonic hedgehog* in the paraxial mesoderm and/or neural tube caused induction of *PTC* transcripts in the axial mesoderm (Fig. 4*B*) and in the dorsal neural tube (Fig. 4*B*, arrowhead). Injection of a control retrovirus expressing Alkaline phosphatase did not affect *PTC* expression either in the neural tube or the paraxial mesoderm (data not shown). The induction of *PTC* transcription in neural tissue, paraxial mesoderm, and limb mesenchyme (30) demonstrates that a unique *PTC* gene is genetically downstream of *Sonic hedgehog* in different tissues during chicken development.

DISCUSSION

We conducted a detailed analysis of the expression pattern of *PTC* in the chicken central nervous system at different stages and found it to be very dynamic. At early stages, we detected *PTC* mRNA first in the notochord, and then in the notochord and the ventral neural tube. Interestingly, this expression pattern is strongly reminiscent of the *Sonic hedgehog* expression and, at these stages, the two genes appear to be coexpressed in the same tissues (30). The *in situ* hybridization technique did not allow us to determine whether the two genes are expressed in the same cells of these tissues or in different cell types. An analysis with specific antibodies against the two proteins should distinguish between these two possibilities. We also found that *PTC* and *Sonic hedgehog* are coexpressed in the same tissues only transiently. At later stages, *PTC* expression is maintained and spreads dorsally in the neural tube but decays in the floor plate cells that express *Sonic hedgehog*. Similarly we detect high levels of *PTC* mRNA in the sclerotomal cells around the *Sonic hedgehog*-expressing notochord but *PTC* transcripts become undetectable in the notochord itself.

The comparison of *PTC* and *Sonic hedgehog* expression patterns suggests that the domain of *PTC* expression might be regulated by Sonic hedgehog in the central nervous system at early stages during chicken development. We addressed this hypothesis by misexpressing *Sonic hedgehog* at early stages of chicken development using the avian retroviral system. Indeed we detected up-regulation of *PTC* mRNA in the dorsal neural tube and paraxial mesoderm in response to Sonic hedgehog. This demonstrates that PTC is a member of the Sonic hedgehog signaling pathway in patterning different tissues: limb bud (30), neural tube, and somites.

Sonic hedgehog is known to act both as a short- and a long-range signal. The induction of floor plate markers from the notochord requires cell contact (2, 3), while the differentiation of motor neurons and sclerotome involves a long-range induction (2, 4–6). Sonic hedgehog is responsible for both these types of inductions (7, 8, 11–16). Interestingly, *PTC* is expressed in floor plate, neural cells lateral to the floor plate, and sclerotome. Therefore, a common downstream gene, *PTC*, appears to mediate Sonic hedgehog signaling pathway both in short-range and in long-range inductions. Further-

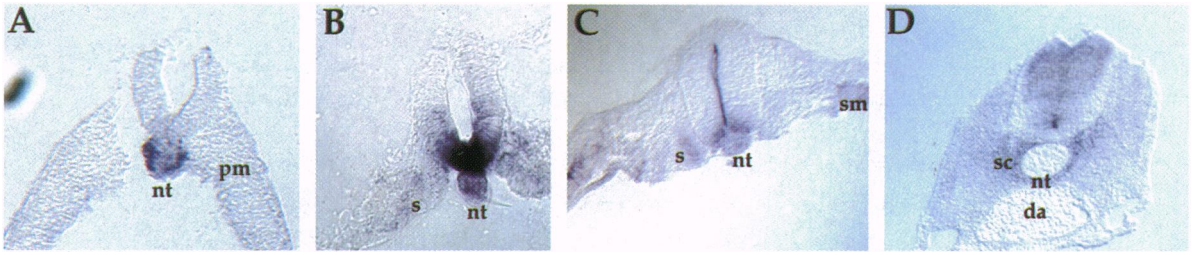

FIG. 3. *PTC* expression in the developing neural tube and notochord. Cryosections of whole mount *in situ* hybridizations hybridized with the *PTC* riboprobe. (*A*) Section at a posterior level of a stage 12 embryo: *PTC* is expressed just in the notochord. (*B*) Section at the trunk level of a stage 12 chicken embryo. *PTC* mRNA is detectable in the notochord, ventral neural tube, and somites. (*C*) Section at the trunk level of a stage 15 embryo. *PTC* is found in the notochord, ventral part of the somites, and splanchnic mesoderm. The neural tube shows lower levels of *PTC* expression in the floor plate cells. The highest *PTC* expression is found in cells adjacent to the floor plate. (*D*) In a section of a stage 18 embryo at the level of the trunk, *PTC* is excluded from the notochord and floor plate. It is expressed in the neural tube, strongly at the luminal side, and in the sclerotome. da, Dorsal aorta; nt, notochord; pm, paraxial mesoderm; s, somite; sc, sclerotome; sm, splanchnic mesoderm.

more, the finding of *PTC* expression in all the cells that are known to respond to the hedgehog signal suggests that PTC is probably part of the transduction machinery, like its homolog has been suggested to be in *Drosophila* (27).

If, as we suggest, *PTC* is a marker for Sonic hedgehog response, then a particularly striking aspect of our expression studies is that *PTC* transcripts are detected throughout the dorsal-ventral extent of the late neural tube and hindbrain. This would appear to represent an extreme example of long-range action of the ventral midline signal. It is formally possible that the more dorsal expression could be activated in response to another unknown member of the hedgehog family or in response to hedgehog-independent signals. However, we favor the hypothesis that this *PTC* expression is a direct response to Sonic hedgehog because the late dorsal expression evolves in a continuous manner from the earlier, ventrally localized expression domain. Moreover, the spread of *PTC* expression is focused around the lumen of the neural tube, which gives a potential conduit for facilitating Sonic hedgehog diffusion. Consistent with this possibility, Sonic hedgehog has been localized to the apical, luminal surface of the floor plate cells producing it (12, 19).

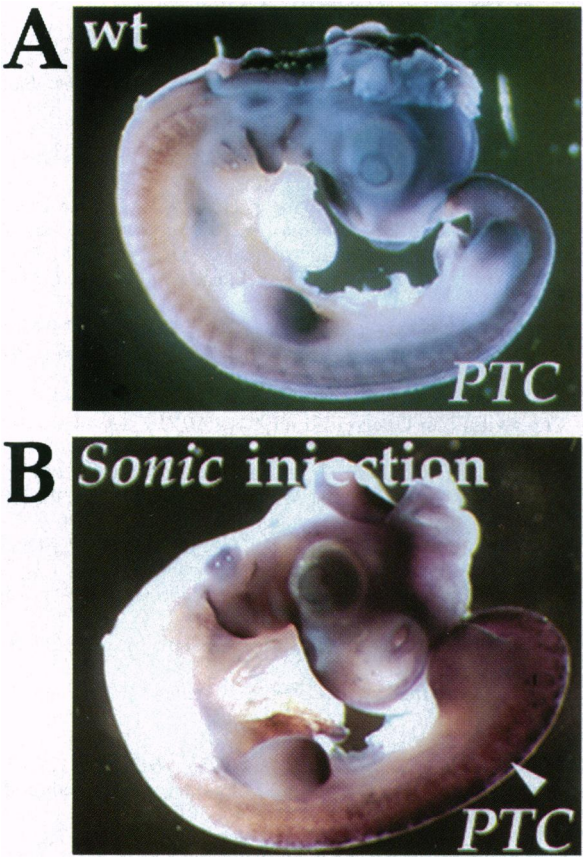

FIG. 4. Induction of *PTC* by *Sonic hedgehog*. (*A*) Stage 23 chicken embryo processed by whole mount *in situ* hybridization with a *PTC* probe. (*B*) Stage 23 chicken embryo injected at stage 10 with a *Sonic hedgehog* expressing retrovirus in the right side. *PTC* expression is compared with the uninfected animal (*A*). *PTC* induction is detected in the dorsal neural tube (arrowhead) and the axial mesoderm.

The implication of this data is that cells of the dorsal neural tube are actively responding to Sonic hedgehog late in neural tube development. Ventral midline signals are only thought to direct patterning of the ventral half of the neural tube, the dorsal half being patterned by an independent mechanism (35). However, this specification of the neural dorsoventral polarity takes place relatively early (11), when *PTC* expression is localized to the ventral neural tube. By the time *PTC* is expressed in the dorsal neural tube, this region is committed to a dorsal fate and ventral signals can no longer transform those cells to a floor plate or motor neuron fate. Thus, rather than dorsoventral specification, the late *PTC* expression in the dorsal neural tube may reflect a second role not previously appreciated of Sonic hedgehog in the induction or elaboration of late dorsal neural phenotypes.

One of the most interesting aspects of the regulation of the vertebrate *PTC* gene is that *PTC* and *Sonic hedgehog* are only transiently coexpressed in the notochord, floor plate, endoderm, and posterior limb bud (30). Several mechanisms could account for the repression of *PTC* in cells where it is initially expressed and its subsequent restriction to cells complementary to those expressing *Sonic hedgehog*.

In *Drosophila*, three mechanisms restrict *ptc* expression. First, there are tissues where it is apparently not expressed simply by virtue of a lack of positively acting transcription factors (36, 37). This could be the basis for the lack of *PTC* expression in the limb bud apical ectodermal ridge (AER) (30). Second, there are *Drosophila* tissues, such as the anterior compartment of the imaginal discs, where *ptc* transcription is kept at a very low level due to a negative feedback loop mediated by *ptc* activity (28). This is likely to explain the low level of *PTC* expression throughout the vertebrate mesoderm. Finally, transcription of *Drosophila ptc* is completely repressed in tissues expressing *hedgehog* by another segment polarity gene, *engrailed* (29, 38). This repression is independent of *ptc* activity. There could similarly be a transcription factor coexpressed with *Sonic hedgehog* in the notochord, floor plate, endoderm, and posterior limb bud that represses *PTC* activity. Because of transient coexpression of *Sonic hedgehog* and *PTC*, such a negatively acting transcription factor would have to be activated in those tissues with kinetics that lag behind the induction of *Sonic hedgehog*.

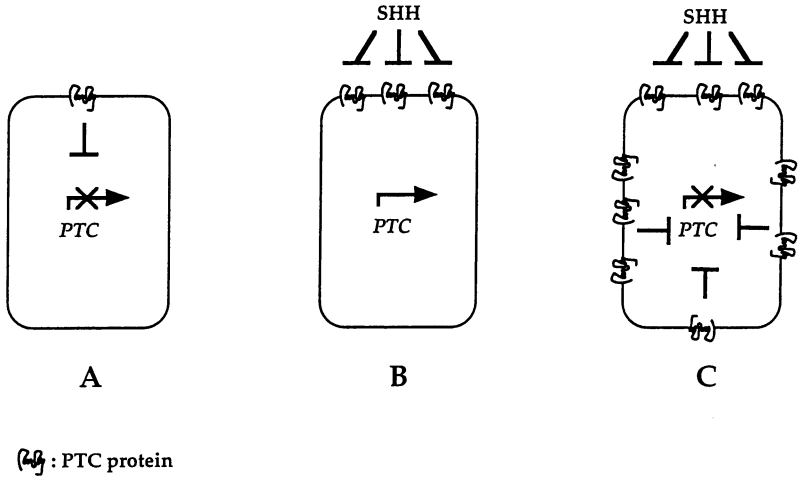

FIG. 5. Role of *PTC* in development. Model for the regulation of *PTC* expression during embryogenesis. (*A*) PTC function negatively affects *PTC* expression. (*B*) In cells receiving Sonic hedgehog signal (SHH), PTC activity is blocked inducing *PTC* transcription. (*C*) In tissues coexpressing *PTC* and *Sonic hedgehog*, PTC expression at the cell surface reaches such high levels that the Sonic hedgehog signal is no longer sufficient to completely antagonize PTC function, resulting in inhibition of *PTC* expression.

Alternatively, the late repression of *PTC* in *Sonic hedgehog*-expressing cells could be a consequence of the negative effects of PTC itself. In this model, *PTC* transcription is repressed by the action of PTC protein, as is known to be the case in *Drosophila* (Fig. 5*A*). Sonic hedgehog protein initially opposes PTC function, relieving the transcription block and inducing *PTC* expression (Fig. 5*B*). However, in tissues expressing *Sonic hedgehog*, most of the Sonic hedgehog protein is known to remain associated with the cells producing it (12, 13, 39). The resultant constitutive expression of *PTC* in those cells might lead to an accumulation of such a high level of PTC protein that the amount of Sonic hedgehog produced is no longer sufficient to block all of the activated PTC and an autoinhibition process takes place (Fig. 5*C*). Thus, we propose that the purpose of high level transcriptional induction of *PTC* in cells where PTC protein activity is repressed by Sonic hedgehog is to ultimately attenuate Sonic hedgehog signaling. The plausibility of this is supported by data in *Drosophila* showing that ectopic induction of high levels of *patched* expression can indeed block response to hedgehog signaling (40). An alternative explanation could be that the up-regulation of *ptc* might act to increase binding to hedgehog and hence limit its diffusion. By this model, *PTC* induction would result in spatial attenuation of hedgehog signaling. However, we favor the model resulting in temporal attenuation which is more consistent with the dynamic expression of *PTC* in the vertebrate central nervous system.

We are grateful to Matthew Scott, Jeff Golden, and Ed Laufer for helpful comments concerning data. This work was supported by grants from the Human Frontier Science Program and the National Institutes of Health.

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