Development 122, 2835-2846 (1996)
Printed in Great Britain © The Company of Biologists Limited 1996
DEV1084

Spatial regulation of a zebrafish *patched* homologue reflects the roles of *sonic hedgehog* and protein kinase A in neural tube and somite patterning

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SUMMARY

Signalling by members of the Hedgehog family of secreted proteins plays a central role in the development of vertebrate and invertebrate embryos. In *Drosophila*, transduction of the Hedgehog signal is intimately associated with the activity of protein kinase A and the product of the segment polarity gene *patched*. We have cloned a homologue of *patched* from the zebrafish *Danio rerio* and analysed the spatiotemporal regulation of its transcription during embryonic development in both wild-type and mutant animals. We find a striking correlation between the accumulation of *patched1* transcripts and cells responding to *sonic hedgehog* activity both in the neurectoderm and mesoderm, suggesting that like its *Drosophila* counterpart, *patched1* is regulated by *sonic hedgehog* activity. Consistent with this interpretation, mis-expression of *sonic hedgehog* results in ectopic activation of *patched1* transcription. Using dominant negative and constitutively active forms of the protein kinase A subunits, we also show that expression of *patched1* as well as of other *sonic hedgehog* targets, is regulated by protein kinase A activity. Taken together, our findings suggest that the mechanism of signalling by Hedgehog family proteins has been highly conserved during evolution.

Key words: induction, midline signalling, *patched*, *sonic hedgehog*, protein kinase A, zebrafish

INTRODUCTION

In *Drosophila*, cell patterning is controlled by the segment polarity genes, a molecularly heterogeneous group that includes the components of two signal transduction pathways (Klingensmith and Nusse, 1994; Ingham, 1995). One of these pathways is responsible for transducing the activity of the secreted protein Hedgehog, itself encoded by a member of the segment polarity class (Lee et al., 1992; Mohler and Vani, 1992; Tabata et al., 1992; Tabata and Kornberg, 1994). Several genes closely related to *hedgehog* (*hh*) have now been described in various vertebrate species, the best characterised of these being *sonic hedgehog* (*shh*) (Echelard et al., 1993; Krauss et al., 1993; Riddle et al., 1993; Roelink et al., 1994). The spatiotemporal deployment of *shh* is highly conserved from fish to mouse: expression is initiated during gastrulation in the embryonic shield or node and persists in the midline mesoderm, the notochord precursor, as the main body axis extends. Subsequently, expression of *shh* is activated in the floorplate cells of the ventral neural tube that overlie the notochord, where it continues to be expressed throughout somitogenesis (reviewed by Fietz et al., 1994).

Experimental manipulations have identified both the notochord and floorplate as sources of inducing activities that control the patterning of the neural tube and somites (van Straaten et al., 1989; Placzek et al., 1990, 1991; Yamada et al., 1991; Pourquié et al., 1993; Goulding et al., 1994) and several lines of evidence suggest that *shh* is a major component of such activities. First, in the zebrafish, loss of midline signalling in various mutants is closely correlated with loss of *shh* expression (Krauss et al., 1993; Ekker et al., 1995; Macdonald et al., 1995). Second, ectopic expression of *shh* in zebrafish (Krauss et al., 1993; Barth and Wilson, 1995; Macdonald et al., 1995), as well as in mouse and frog (Echelard et al., 1993; Ruiz i Altaba et al., 1995), leads to the inappropriate expression of floorplate and/or ventral brain markers. Such ectopic expression has also been shown to result in the inappropriate activation of the sclerotomal and myotomal markers *pax1* and *myoD*, respectively, in the developing somites of the chick (Johnson et al., 1994) as well as of *myoD* in paraxial mesoderm of the fish (Weinberg et al., 1996). Finally, and most definitively, recombinant Shh protein is itself capable of inducing floorplate and motor neuron differentiation in neural plate explants (Marti et al., 1995; Roelink et al., 1995) and sclerotomal or myotomal differentiation in explants of presomitic mesoderm (Fan et al., 1995; Munsterberg et al., 1995).

At later stages of development, transcription of *shh* is activated in the posterior mesenchyme of the developing limb buds,

a region corresponding to the signalling centre known as the zone of polarising activity (ZPA). Mis-expression of *shh* in the anterior of the limb bud results in digit duplications similar to those induced by heterotopic grafts of ZPA material (Riddle et al., 1993; Chang et al., 1994), suggesting that *shh* mediates the signalling activity of this region of the vertebrate limb.

Although the importance of *shh* during vertebrate embryogenesis is thus well established, little is known about the way in which the activity of the Shh protein is transduced. In *Drosophila*, by contrast, genetic analysis has identified a number of putative components of the *hh*-signalling pathway, such as the cAMP-dependent protein kinase (PKA) (Jiang and Struhl, 1995; Lepage et al., 1995; Li et al., 1995; Pan and Rubin, 1995; Strutt et al., 1995) as well as the products of several segment polarity genes, including *patched* (*ptc*) (Hidalgo and Ingham, 1990; Ingham et al., 1991; Ingham and Hidalgo, 1993; Capdevila et al., 1994), a novel multipass membrane spanning protein (Hooper and Scott, 1989; Nakano et al., 1989). Inactivation of either Ptc or PKA in the developing limbs of *Drosophila* has remarkably similar consequences; in both cases, various *hh* target genes are inappropriately activated, suggesting that Ptc and PKA normally act to suppress the Hh response pathway. Whilst PKA most likely acts by phosphorylating other components of this pathway, the molecular mechanism of Ptc activity has remained enigmatic. One suggestion, based upon its predicted topology and membrane localisation, is that it functions as a receptor for Hh, binding of the latter antagonising Ptc activity and thus relieving the repression of different target genes (Ingham et al., 1991). Although consistent with the spatial deployment of the two proteins and the interactions between them as deduced by genetic analysis, this model has so far received no direct biochemical support.

Whatever the identity of the Hh receptor, it seems likely to have been highly conserved through evolution since vertebrate *hh* homologues are capable of activating the response pathway when expressed in transgenic *Drosophila* (Krauss et al., 1993; Chang et al., 1994; Ingham and Fietz, 1995). Moreover, the discovery that the response of tissue explants to Shh activity can be attenuated by drugs that activate PKA (Fan et al., 1995; Hynes et al., 1995) suggests that at least some of the intracellular components of the pathway may have also been conserved. In this study, we have explored the extent of this conservation further by cloning a zebrafish homologue of *ptc* and analysing its expression during embryogenesis. In *Drosophila*, *ptc* is itself one of the principal targets of *hh* activity, its transcription being upregulated in *hh*-responding cells. Using midline mutants and overexpression strategies, we have investigated the relationship between *shh* activity and transcription of the zebrafish *ptc1* gene. Our results support a role for *shh* and *ptc1* in the specification of both neural and mesodermal cell fates in the zebrafish; in addition, we show that both aspects of *shh* signalling are mediated by PKA activity.

MATERIALS AND METHODS

In situ hybridisation

In situ hybridisation was performed as described by Oxtoby and Jowett (1993) with the following modifications: hybridisation were performed at 70°C and post-hybridisation washes were as suggested by Henrique et al. (1995). Stained embryos were dehydrated through an ethanol: butanol series, embedded in Fibrowax and sectioned (6-10 μm). Double stainings were performed essentially as described (Jowett and Lettice, 1994).

Probes used for in situ hybridisations were synthesized using the following templates: *shh*, (Krauss et al., 1993); *pax-[b]* (Krauss et al., 1991); *nk2.2*, (Barth and Wilson, 1995); *MyoD* (Weinberg et al., 1996). Unincorporated DIG-UTP was removed by centrifugation through a Nuc50 column (Kodak). Specimens were analysed using a Zeiss Axioplan microscope and photographed with Kodak Ektachrome 64T film. Images were scanned on a Sprintscan 35 slide-scanner and processed using Adobe Photoshop software.

zebrafish stocks

Wild-type *Danio rerio* were bred from a founder population obtained from the Goldfish Bowl, Oxford. The *flh*^{n1} strain was obtained from T. Jowett (Newcastle University, UK). The *cyc* and *ntl* strains were obtained from C. Kimmel (University of Oregon, USA). Fish were maintained in a constant recirculating system at 28°C on a 14 hours light/10 hours dark cycle.

Cloning and sequence analysis

Two pairs of primers (rev2, rev4 and genie1, genie2, see below) were used separately to perform PCR starting from random-primed cDNA samples synthesised using either bud stage or somitogenesis stage zebrafish RNA.

Rev2: ggacgaattcTSYTCNRGCCARTGCAT
Rev4: ggacgaattcYTNGANTGYTTYTGGGA
Genie1: ggacgaattccGAYGGNATNATNAAYC
Genie2: ggacgaattcRTAYTGYTCCCARAANA

(large lettering corresponds to sequences conserved between mouse and different insect *ptc* sequences, small lettering corresponds to an *Eco*RI site and arbitrary flanking sequence introduced to facilitate subsequent cloning of PCR products). Amplification was performed using 35 cycles of 94°C 30 seconds; 50°C 30 seconds; 72°C 90 seconds. Fragments of the expected size were subcloned into Bluescript KS and sequenced. One fragment was obtained with the Genie1 and Genie2 primers (called Genie) and two with the Rev2 and Rev4 primers (called Rev1 and Rev3). Two primers identical to Rev1 and Rev3 fragments, respectively, a primer complementary to the Genie fragment and the Genie2 primer were used in different pairwise combinations to perform PCR starting from the same original cDNA samples. Two fragments of 1.8 and 2 kb were amplified using the Rev1- and Genie-derived primers and the Rev3-derived and Genie2 primers respectively, which after sequencing proved to be homologous to both mouse and *Drosophila ptc*.

2×10^6 plaques of a λgt11 library made from 33-hour-old zebrafish embryos (K. Zinn) were screened at low stringency according to standard procedures using the 1.8 and 2 kb fragments as probes. We did not obtain any positive clones with the 2 kb probe using either the 33 hour library or libraries from other stages. 20 positive plaques from the screen using the 1.8 kbp fragment were purified and analysed by PCR using oligonucleotides against the 1.8 kb probe and against the lambda vector. The longest clone, λ105, was sequenced on both strands using either Pharmacia or Applied Biosysytems automated sequencers with primers from the cloning vector and internal primers. Sequence analysis was performed using Geneworks (Intelligenetics) and GCG software packages.

The nucleotide sequence of the zebrafish *ptc1* gene described here is deposited in the EMBL database under the accession number: X98883.

Plasmids

The open reading frames of a dominant negative mutant cDNA of the regulatory subunit of *Drosophila melanogaster* cAMP-dependent protein kinase (Li et al., 1995) and of a constitutively active subunit of the mouse PKA catalytic subunit (Orellana and McKnight, 1992) were subcloned into the CS2 expression plasmids where cDNA transcrip-

tion is driven by the ubiquitous CMV enhancer/promoter region (Turner and Weintraub, 1994) to create pCS2dnPKA and pCS2cPKA, respectively. The open reading frame of *shh* was subcloned into the CDM8 expression vector (Seed, 1987).

Embryo injections

Injections were performed on 2- to 4-cell-stage embryos using backfilled capillaries (Clarks Microelectrical Instruments, Reading) and a pressure-pulsed Narishige microinjector. RNA, synthesized in vitro from linearized plasmids p64T*shh* (Krauss et al., 1993), pCS2dn PKA and pCS2cPKA, respectively, was injected at empirically determined concentrations which were of approximately 100 µg/ml. Plasmid DNA, purified using Qiagen columns, was injected at a concentration of approximately 100 µg/ml.

RESULTS

Isolating sequences homologous to *Drosophila patched* from zebrafish

Vertebrate homologues of *ptc* were first identified in mouse using a polymerase chain reaction (PCR) strategy based upon sequence comparisons of *ptc* homologues from distantly related insect species (Goodrich et al., 1996). Two pairs of degenerate oligonucleotide primers based upon sequences conserved between the mouse and insect genes were used to amplify related sequences from zebrafish cDNA (see Materials and Methods). Two fragments of approximately 320 bp and one fragment of around 330 bp, whose sequences proved to be related to both mouse and insect *ptc* sequences, were amplified. Oligonucleotides based on sequences within these three PCR fragments were then used to amplify

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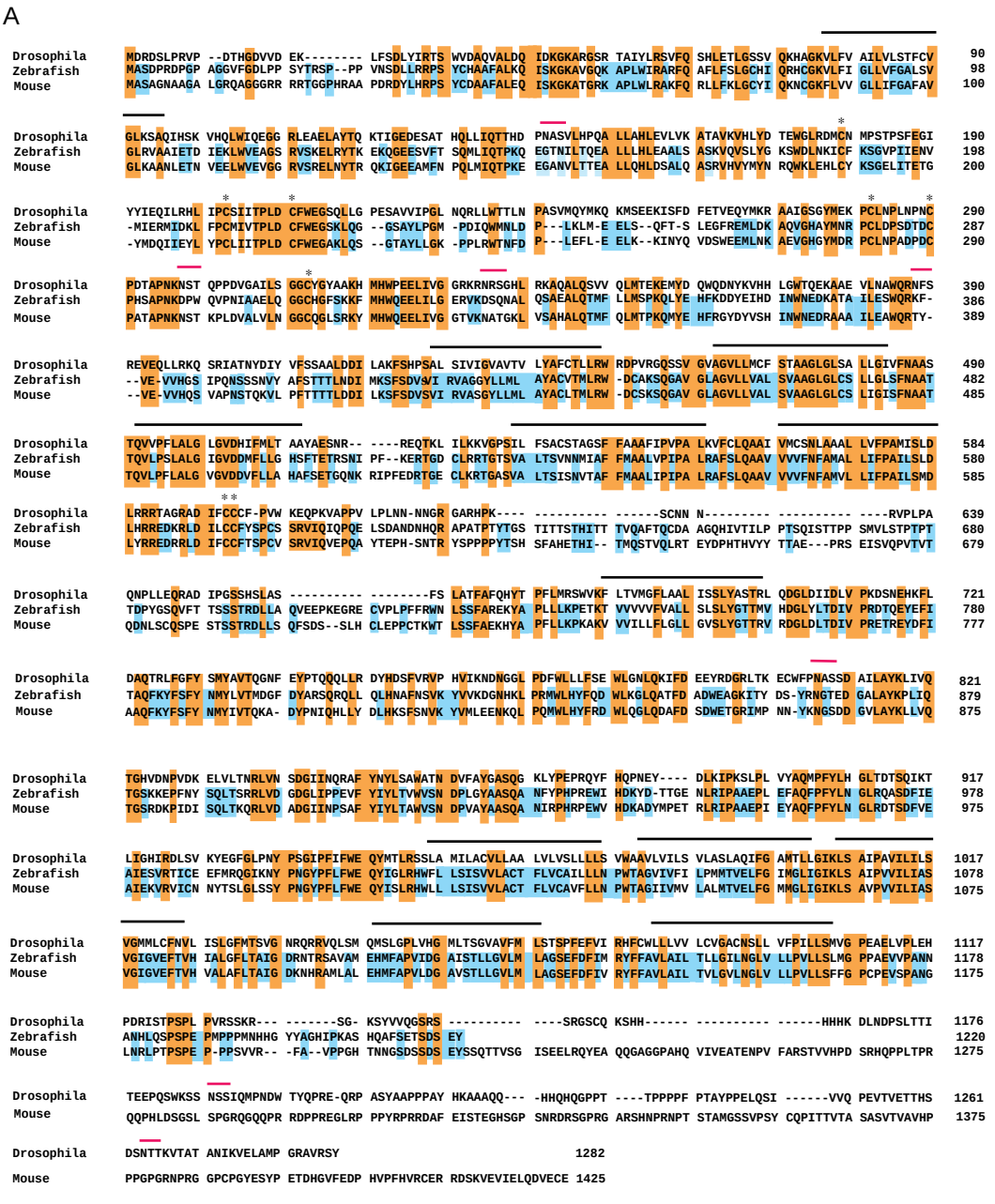
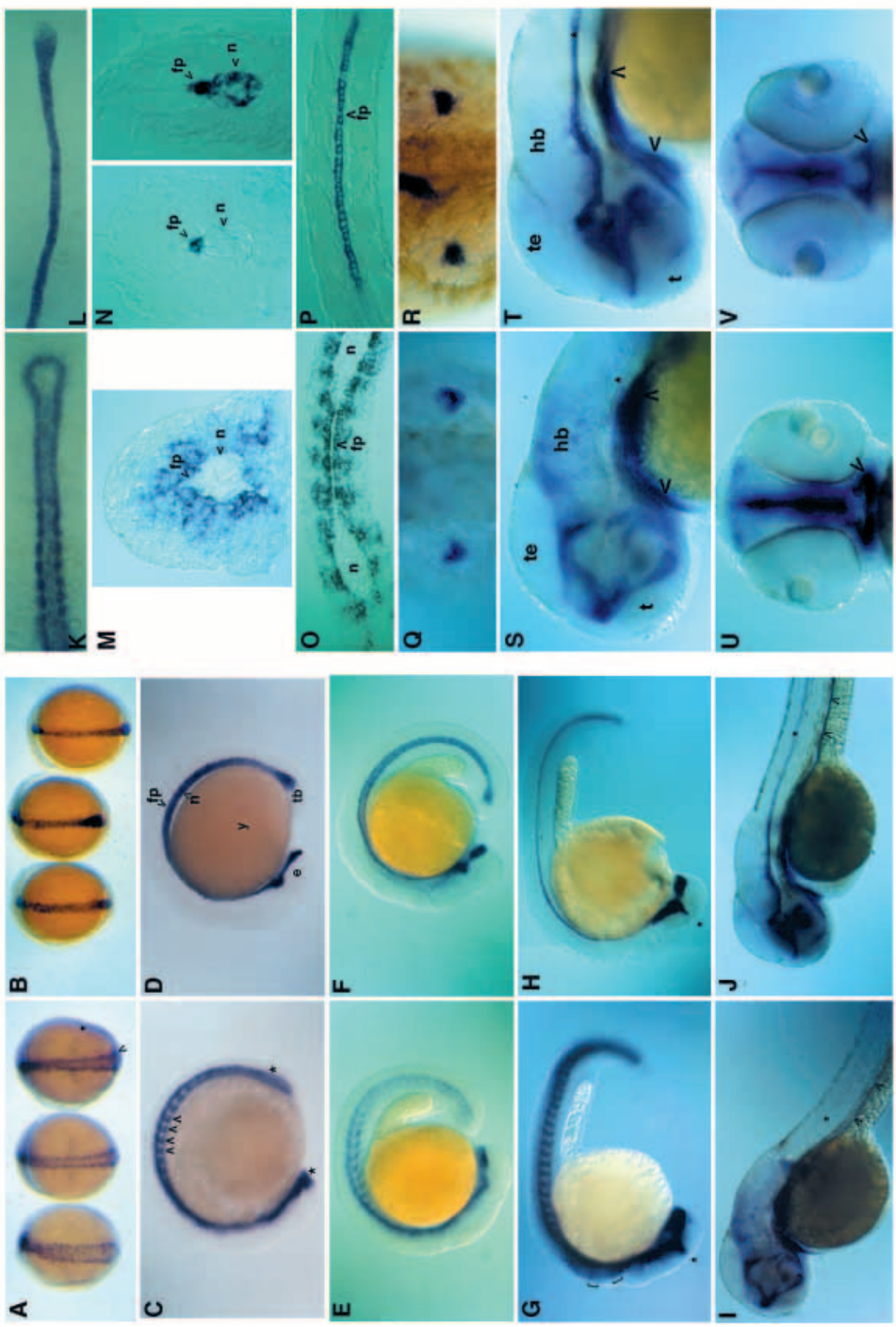


B

Mouse	100 (100)				
Chick	89 (94)	100 (100)			
Zebrafish	64 (79)	64 (79)	100 (100)		
Drosophila	39 (59)	40 (60)	39 (62)	100 (100)	
C. Elegans	39 (60)	39 (60)	39 (60)	33 (58)	100 (100)
	Mouse	Chick	Zebrafish	Drosophila	C. Elegans

Fig. 1. Comparison of the amino acid sequences of *ptc* genes from different organisms. (A) Sequence alignment of the *Drosophila* and mouse ptc proteins with zebrafish ptc1. Putative transmembrane domains are indicated by black lines above the sequence. Orange shading indicates identities between all three species. Blue shading indicates identities between the two vertebrate proteins. Putative N-glycosylation sites in *Drosophila* are indicated by pink lines and conserved cysteine residues by asterisks. Zebrafish Ptc1 is significantly shorter at the C terminus than Ptc proteins from other species. We checked the sequence of the cDNA in the region of the stop codon and the position of the stop codon, by amplifying this region from cDNA prepared from zebrafish embryos at somitogenesis stages. (B) Percentage identity and similarity between *patched* genes from different organisms. Comparisons were done using 'GCG Bestfit'. The first number is the percentage identity and the number in brackets is the percentage similarity.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two fragments of approximately 1.8 kb and 2.0 kb and each of these fragments was used as a probe to screen a 33-hour-old embryonic zebrafish cDNA library. One full-length cDNA, designated *ptc1*, was isolated and is described in this paper. Similar clones corresponding to the other *ptc* homologue, *ptc2*, have yet to be isolated and this gene will not be considered further here.

The deduced amino acid sequence of *ptc1* shows 64% identity to both the mouse and chicken *ptc* genes and 39% identity to *Drosophila ptc*. Comparison of the different Ptc sequences reveals that eight cysteine residues are conserved in all four species. The *Drosophila* protein has seven potential N-glycosylation sites of which three are conserved in mouse and chick but only one is present in zebrafish. Hydropathy analysis predicts that each protein contains 12 potential transmembrane domains (see Fig. 1A). There are two regions in the amino acid sequences that are particularly divergent; in the first of these, the vertebrate sequences have insertions relative to the fly sequence, between the sixth and seventh putative transmembrane domains. This region is also relatively divergent between zebrafish and mouse although it is well conserved between chick and mouse (Marigo et al., 1996). The second significant divergence occurs towards the C-terminus, which in zebrafish is considerably shorter than in either *Drosophila* or mouse; the region absent in zebrafish shows little conservation between the latter two species (see Fig. 1A). Database searches have also revealed homology to a *ptc*-related sequence identified by the *C. elegans* genome project (Wilson et al., 1994); the zebrafish *ptc1* gene is as similar to this gene as it is to *Drosophila ptc* (Fig. 1B).

Spatial expression pattern of *ptc1* during zebrafish embryogenesis

The expression pattern of *ptc1* during zebrafish embryogenesis was analysed by in situ hybridisation and compared to that of *shh*. Transcription of *ptc1* is first apparent at around 70% epiboly in the presumptive mesodermal cell layer in two stripes of cells flanking the axial mesoderm, which at this stage already shows robust expression of *shh*. As the convergence extension movements of gastrulation proceed, low-level *ptc1* expression is apparent throughout the presomitic mesoderm while the two stripes of high-level expression, corresponding to the so-called adaxial cells (Thisse et al., 1993), extend along the entire axis; in addition, high levels of *ptc1* transcript are detectable in the neurectoderm overlying the axial mesoderm and in the ventral part of the future brain (Fig. 2A). With the onset of somitogenesis, high-level expression persists in the tissues surrounding the notochord in the trunk as well as in the ventral neuroectoderm of the brain (Fig. 2C). Distinct patches of high-level mesodermal expression become apparent adjacent to the notochord as the somites form (Fig. 2M), a pattern similar to that of α-tropomyosin at the same stage (Thisse et al., 1993). During later stages of somitogenesis when *shh* expression is lost from the notochord, expression of *ptc1* persists throughout the ventral neural tube, except in the floor-plate, which at this stage still expresses *shh* at high levels. Notably, expression in the mesoderm also persists lateral and ventral to the notochord (Fig. 2G,M-P). In the brain, *ptc1* expression extends dorsally in the diencephalon, paralleling the dorsal extension of *shh* expression in the same region and shows a modulation in the hindbrain, which becomes more defined at later stages (Fig. 2G; see also Fig. 5C).

The relationship between the *ptc1* and *shh* expression domains in the developing brain was analysed directly using double-labelling techniques to visualise both transcripts simultaneously. At all stages, high-level expression of *ptc1* occurs in a domain delimiting the expression of *shh*. By 24 hours, expression of *shh* no longer occupies the most ventral part of the rostral forebrain and *ptc1* expression can now be detected ventrally to *shh*-expressing cells (Fig. 3A). High-level expression of *ptc1* is also detected around the lumen of the neural tube.

By 36 hours, additional sites of *ptc1* transcription appear in the first branchial arch (not shown) and the posterior mesenchyme of the fin buds (Fig. 2Q), in both cases close to domains of *shh* expression (Fig. 2R; see also Krauss et al., 1993). By 48 hours, both genes exhibit a complex expression pattern in the brain with a persistence of high-level *ptc1* expression adjacent to *shh*-expressing cells. High-level expression of *ptc1*, is, however, also observed some distance away from cells expressing *shh* in a number of places, including most notably a domain of intense expression in the hindbrain (Figs 2S,T, 3B). Expression of both genes is now obvious in the foregut (Fig. 2S-V) as well as in more posterior domains.

Fig. 2. Comparison of the *ptc1* and *shh* expression patterns during wild-type embryogenesis. Transcripts were revealed by in situ hybridization with antisense RNA probes to *ptc1* (A,C,E,G,I,K,M,O,Q,S,U) or *shh* (B,D,F,H,J,L,N,P,R,T,V). (A,B) Dorsal views of embryos at the end of gastrulation. The arrowhead indicates a row of hypoblast cells adjacent to the axial mesoderm which express *ptc1* at high levels. The asterisk indicates the anterior boundary of the *ptc1* low-level expression domain in the presomitic mesoderm. (C,D) Lateral views of 10-somite (C) and 14-somite (D) stage embryos. Arrowheads denote *ptc1* expression in somites. Asterisks delimit the expression of *ptc1* in the neurectoderm all along the anteroposterior axis of the embryo. (E,F) 18-somite stage embryos. (G,H) 26-somite stage. Asterisks indicate the dorsal extension of *ptc1* and *shh* expression domains in the diencephalon. Brackets indicate upregulation of *ptc1* in rhombomeres 2, 4 and 6, a modulation of expression that is unrelated to *shh* expression (see also Fig. 5c). (I,J) Embryos at 48 hours of development. Asterisks indicate the position of the floor plate of the neural tube. Arrowheads indicate expression in the gut. *golden* mutant embryos, which are defective in pigmentation, were used to facilitate visualisation of *ptc1* and *shh* expression. (K,L) 8-somite stage embryos. Dorsal view of flat preparations showing the complementarity between the paraxial expression of *ptc1* and the axial expression of *shh* in the notochord and the tailbud. (M,N) Transverse sections through the trunk of embryos at 24 hours of development. (N) Sections at two different levels along the axis, the section on the right-hand side being more posterior. (O,P) Horizontal sections through the trunk of embryos at 24 hours of development. (Q,R) Dorsal view of the trunk of 36-hour-old embryos showing the expression of *ptc1* and *shh* in the posterior region of the developing fins. (S,T) Detail of embryos shown in I and J, respectively. Asterisks indicate the position of the floor plate of the neural tube. Arrowheads indicate expression in the foregut. Expression in the hindbrain, when observed dorsally, was found to be restricted to periventricular cells in the midline (data not shown). (U,V) Frontal views of embryos shown in I and J, respectively. Arrowheads indicate expression in the foregut surrounding the developing mouth. Abbreviations : e, eye; fp, floor plate; hb, hindbrain; n, notochord; te, tectum; t, telencephalon; tb, tail bud; y, yolk.

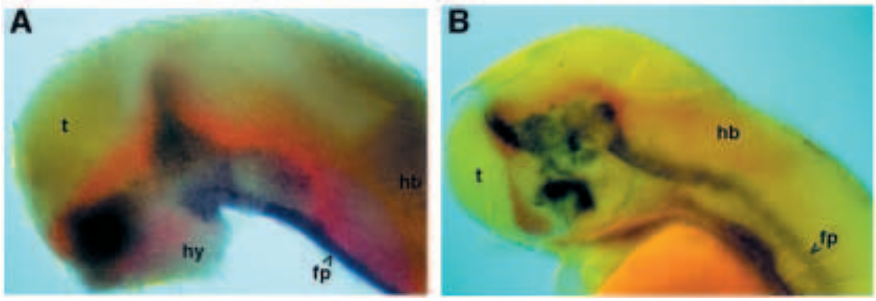

Fig. 3. Simultaneous localisation of *ptc1* and *shh* transcripts in the head. Whole embryos were hybridized simultaneously with riboprobes to *ptc1* and *shh* revealing *ptc1* expression in red staining and that of *shh* in blue. Lateral views are shown with anterior to the left; yolk and eyes were removed to improve brain visualisation. (A) Embryo at 36 hours of development. (B) Embryo at 48 hours of development. Abbreviations: fp, floor plate; hb, hindbrain; hy, hypothalamus; t, telencephalon.

Disruption of midline signalling affects both neural and mesodermal expression of *ptc1*

The relationship between the patterns of expression of *shh* and *ptc1* described above is highly reminiscent of that between their *Drosophila* counterparts (Taylor et al., 1993; Tabata and Kornberg, 1994) suggesting that, as in *Drosophila*, transcription of *ptc1* may be induced in response to *hh* signalling. Since no mutation of *shh* is currently available in the zebrafish, we took advantage of a number of mutants that eliminate *shh* expression at different levels along the anteroposterior axis to investigate this possibility.

Mutation of the *cyclops* (*cyc*) gene disrupts the specification of the prechordal plate mesoderm (Thisse et al., 1994) and concomitantly the induction of the overlying neurectoderm, resulting in defective midline signalling in the developing brain which gives rise to the cyclopic phenotype (Hatta et al., 1994; Ekker et al., 1995; Macdonald et al., 1995). In addition, *cyc* mutants lack a floorplate, apparently due to a defect in the response of cells to the inductive signal from the underlying notochord (Hatta et al., 1991). In line with these phenotypic effects, expression of *shh* is completely absent from both the prechordal plate mesoderm and the overlying neurectoderm that gives rise to the ventral floor of the brain (Krauss et al., 1993) while, posterior to the midbrain, expression is normal in the axial mesoderm but absent from the ventral neural tube.

Expression of *ptc1* in *cyc* embryos mirrors these changes in *shh* expression precisely: thus by the time the body axis is fully extended, no *ptc1* transcripts are detectable anterior to the domain of expression of *pax[b]* (Fig. 4A), which marks the future midbrain (Krauss et al., 1991), and the forebrain and midbrain remain devoid of *ptc1* expression throughout the rest of embryogenesis (Fig. 4B). The only exception is a short dorsoventral stripe in the abnormal neural fold of the diencephalon that appears at around 24 hours of development surrounding a stripe of *shh*-expressing cells that appears at the same stage (Fig. 4B-D). Posterior to the midbrain/hindbrain boundary, *ptc1* is expressed normally in the somites and ventral neural tube (Fig. 4B), except that it persists along the ventral midline of the latter, presumably reflecting the failure of the floorplate to differentiate.

In contrast to *cyc*, mutation of the *floating head* (*flh*) gene has no effect on prechordal plate mesoderm but instead disrupts notochord specification; this leads to a premature loss of *shh* expression from the axial mesoderm posterior to the midbrain-hindbrain boundary and the resultant disruption of floor plate induction is reflected in patchy expression of *shh* along the ventral midline of the neural tube (Talbot et al., 1995; see Fig. 5D). In addition, muscle pioneer cells that derive from the adaxial cells adjacent to the notochord (Felsenfeld et al., 1991; Thisse et al., 1993) fail to differentiate (Halpern et al., 1995; Talbot et al., 1995). A similar effect on muscle pioneer differentiation is caused by mutation of the *notail* (*ntl*) gene, which also lacks a notochord. Unlike *flh*, however, *ntl* mutants do not lack the floorplate (Halpern et al., 1993). These differences in phenotype seem to reflect differences in the effects of each mutation on *shh* expression, which persists longer in the axial midline of *ntl* embryos (Krauss et al., 1993) than in that of *flh* embryos (Talbot et al., 1995).

In *flh* embryos at 24 hours, expression of *ptc1*, like that of

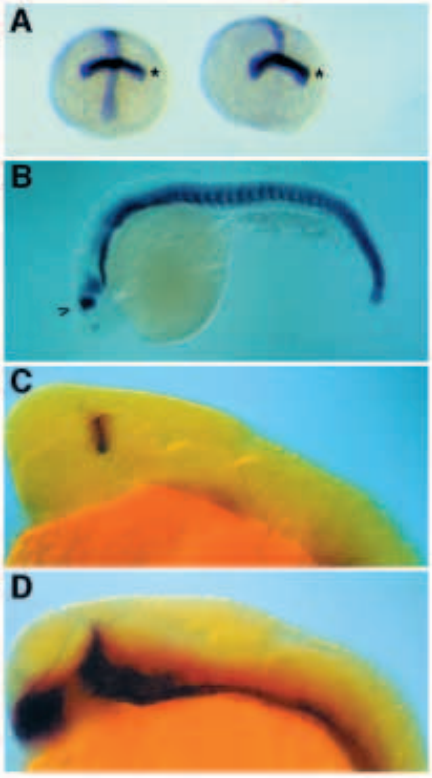

Fig. 4. Expression of *ptc1* and *shh* in *cyclops* mutants. *ptc1* expression is detected in blue (A,B) or in red (C,D) and *shh* in blue (C,D). (A) Frontal view of embryos at the 1- to 2-somite stages. Expression of *ptc1* is shown in a wild-type sib on the left and in a *cyclops* mutant on the right. To provide a landmark along the anteroposterior axis, embryos were simultaneously hybridized with a probe for *pax-2* highlighting the position of the future posterior midbrain (asterisk). (B) *ptc1* expression in a 24-hour-old *cyclops* mutant. The arrowhead indicates re-expression of *ptc1* at high levels in a dorsoventral stripe in the diencephalon. (C) 24-hour-old *cyclops* embryos. *ptc1* expression is revealed in red, *shh* in dark blue. (D) 24-hour-old wild-type embryo.

shh, is completely normal in the brain and anterior neural tube (Fig. 5C,D). More caudally, however, expression is restricted to small clusters of cells distributed sporadically along the ventral neural tube in a manner reflecting the intermittent expression of *shh* (Fig. 5; compare C to D and E to F). In addition to the effects on neural expression, the broad stripes of *ptc1* expression typical of wild-type somites are absent from *flh* homozygotes, with only scattered mesodermal cells expressing the gene in close association with expressing cells in the neural tube (Fig. 5E). Thus expression of *ptc1* in the mesoderm, as in the neural tube, seems to depend upon proximity to a source of *shh* activity. To investigate this relationship further, we analysed the expression of *ptc1* at earlier stages of development in *flh* embryos. At the bud stage, *shh* is still expressed along the axial midline in *flh* embryos, in cells of uncertain origin (Fig. 5B); at the same stage, *ptc1* is expressed in a single stripe of cells along the midline instead of in the two stripes typical of wild type (Fig. 5A). A similar change in the early pattern of *ptc1* expression is seen in *ntl* homozygotes (data not shown). However, at 24 hours, in *ntl* mutants high-level expression persists in distinct stripes in each somite adjacent to the floor-plate that expresses *shh* (Fig. 5G,H).

Induction of *ptc1* transcription by ectopic *shh* activity mirrors its effects on neural and adaxial-specific genes

The analysis of *ptc1* expression in midline mutations suggests that expression of *shh* is necessary for the induction of high-level *ptc1* transcription. To test whether *shh* is sufficient to induce such transcription, we next examined the effects of ectopic *shh* activity on *ptc1* expression. Previous studies have shown that injection of synthetic *shh* mRNA into the 2- to 4-cell-stage embryo results in the ectopic activation of genes such as *axial* and *nk2.2* whose expression domains are normally restricted to the ventral region of the neural tube and brain (Krauss et al., 1993; Barth and Wilson, 1995). In the brain, the distribution of *ptc1* transcripts around the *shh* expression domain closely mirrors the expression domain of *nk2.2* (Fig. 6A,B). To determine whether *ptc1* expression can similarly be ectopically induced by *shh* in the nervous system, embryos from the same cohort were injected with *shh* mRNA, fixed after 27 hours of development and hybridised with probes for *ptc1* and *nk2.2* As expected, *nk2.2* is expressed ectopically in dorsal and lateral regions of the brain as well as in the eye rudiment of such embryos (Fig. 6D). Similarly, high-level expression of *ptc1* is also detected ectopically in the diencephalon and midbrain of *shh*-injected embryos as well as in the eye rudiments (Fig. 6C). Notably, ectopic expression of *ptc1* was never detected in the telencephalon. Similar effects on *ptc1* expression were observed in small groups of cells following injection of a *shh* expression plasmid (data not shown) supporting the interpretation that the induction of transcription is a direct consequence of *shh* activity.

To examine the effects of ectopic *shh* activity in the mesoderm, embryos from the same injection cohorts were fixed at the onset of somitogenesis and hybridised with probes for *myoD* and *ptc1*. Like *ptc1*, expression of *myoD* is restricted to the adaxial cells in the presomitic mesoderm (Fig. 6F) and recent studies have suggested that its expression is regulated by *shh* (Weinberg et al., 1996). In a significant proportion of injected embryos, we found that the expression domains of both *ptc1* (64%; *n*=112) and *myoD* (71%; *n*=90), are expanded so that they occupy most of the lateral mesoderm on one or both sides of the midline (Fig. 6G,H). In addition, *ptc1* is ectopically expressed throughout much of the neurectoderm at this stage following *shh* injection (Fig. 6G).

Transcription of *ptc1* is regulated by protein kinase A

In *Drosophila*, removal of PKA activity from cells mimics their response to *hh* signalling, activating the transcription of *ptc* and other effectors of *hh* activity, suggesting that PKA normally acts to repress expression of *hh* target genes (Jiang and Struhl, 1995; Lepage et al., 1995; Li et al., 1995; Pan and Rubin, 1995; Strutt et al., 1995). Since the relationship between

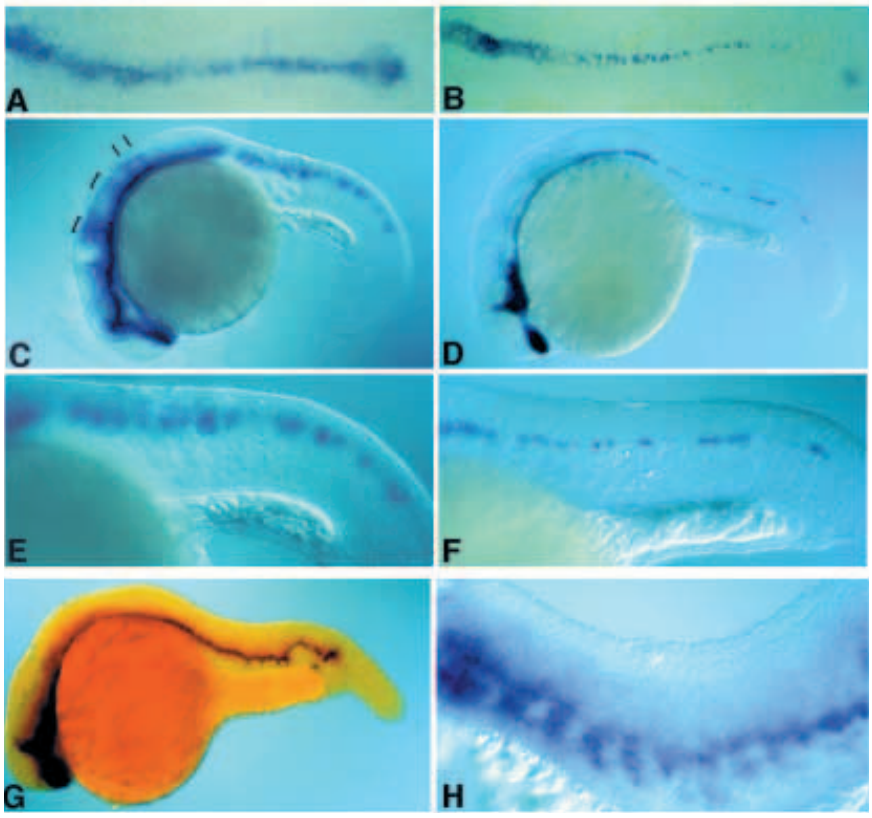

Fig. 5. Comparison of *ptc1* and *shh* expression in *floating head* and *no tail* mutant embryos. Expression of *ptc1* (A,C,E) and *shh* (B,D,F) in *flh* mutant embryos. (A,B) Embryos at the 5-somite stage. (C,D) Embryos at 24 hours of development. Brackets and bars indicate modulation of *ptc1* in different rhombomeres which is unrelated to *shh* expression. (E,F) Lateral view of the trunk of embryos shown in C,D. Expression of *ptc1* and *shh* in *ntl* mutant embryos. (G) *ntl* mutant embryos at 24 hours of development. *ptc1* expression is revealed in red, *shh* in blue. (H) Lateral view of the trunk of a 24-hour-old *ntl* embryo in which *ptc1* expression is revealed in blue.

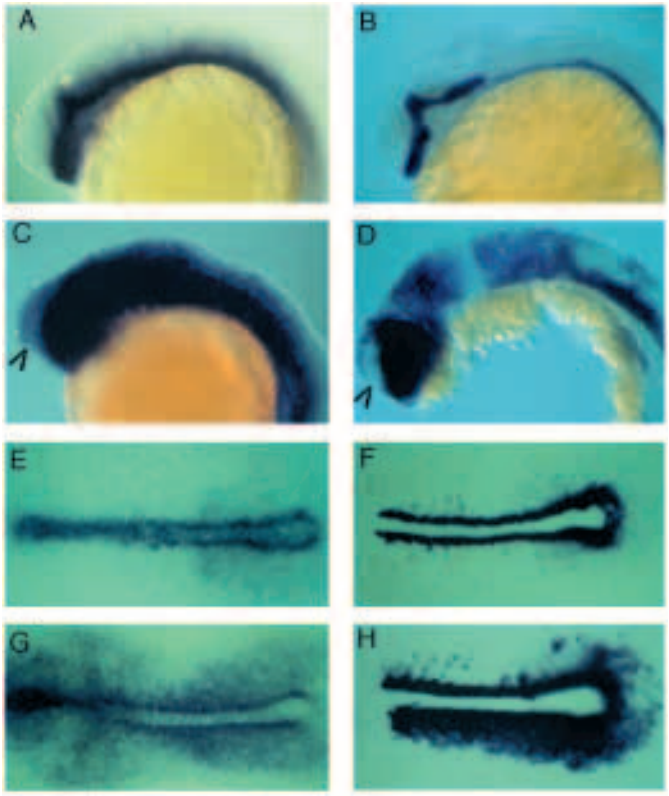

Fig. 6. Induction of *ptc1*, *myoD* and*nk2.2* expression by *shh* in the brain and early somitogenesis stage embryos. (A,C) Lateral views of *ptc1* expression in the brain of 27-hour-old embryos. (A) Uninjected control embryo. (C) *shh*-injected embryo. (B,D) Lateral views of *nk2.2* expression in the brain of 27-hour-old embryos. (B) Uninjected control embryo. (D) *shh*-injected embryo. Arrowheads in C, D show the telencephalon which is devoid of ectopic *ptc1* and *nk2.2* expression. (E,G) Dorsal views of *ptc1* expression in flat preparations of embryos at early somitogenesis. (E) Uninjected embryo. (G) *shh*-injected embryos. No ectopic *ptc1* expression was detected in the ectoderm. (F,H) Dorsal views of *myoD* expression in flat preparations of embryos at early somitogenesis. (F) Uninjected embryo. (H) *shh*-injected embryo.

hh signalling and *ptc* expression appears to be conserved from flies to fish, we wondered whether regulation of *ptc1* and other *shh* targets might also depend upon PKA activity. PKA exists as an inactive heterodimer comprising a catalytic and a cAMP-binding regulatory subunit; binding of cAMP to the latter dissociates the heterodimer allowing the catalytic subunit to phosphorylate its target substrates. To modulate PKA activity, we made use of two previously described dominant mutations of PKA subunits. In the first of these, the cAMP-binding sites in the regulatory subunit are mutated such that it cannot be dissociated from the catalytic subunit (Li et al., 1995). Expression of this mutated subunit, which we refer to as dnPKA, thus results in the inactivation of the catalytic subunit and hence in the reduction or loss of PKA activity. In the second case, the catalytic subunit is mutated such that it cannot bind the regulatory subunit, thus rendering it constitutively active (Orellana and McKnight, 1992); we refer to this mutant form as cPKA.

Amongst embryos injected at the 2- to 4-cell stage with synthetic mRNA encoding the dnPKA mutant and fixed at early somitogenesis stages, more than half (55% n=120) exhibit high-level expression of *ptc1* transcription throughout the lateral mesoderm on one or both sides of the midline (Fig. 7C). In addition, a similar proportion of embryos from the same injection cohort exhibit ectopic expression of *myoD* either unilaterally or bilaterally (Fig. 7D). At later stages (27 hours), injected embryos express both *ptc1* (Fig. 7G) and *nk2.2* (Fig. 7H) at ectopic locations in the diencephalon and mesencephalon. All of these effects are similar to, though somewhat less robust than, those induced by ectopic *shh* expression (compare Figs 6 and 7). Similarly, embryos injected with dnPKA mRNA also consistently show ectopic activation of *pax[b]* in the eye, again similar to, though less extensive than, that induced by ectopic *shh* expression (Fig. 8A-C).

By contrast, injection of embryos with mRNA encoding the cPKA mutant has the opposite effect on the expression of *hh* target genes. At 27 hours, expression of *ptc1* and *nk2.2* is almost totally eliminated from the brain and ventral neural tube of injected embryos, with only a stripe of expression of both genes persisting in the diencephalon (Fig. 9D,E). This stripe corresponds to the dorsal extension of the normal diencephalic expression domain, which also persists in *cyc* mutant embryos (compare with Fig. 4). Like the latter, most cPKA-injected embryos (60%, n=90) also exhibit varying degrees of cyclopia, involving fusion of the retina alone or both the retina and lens (Fig. 9F), a further indication that midline signalling is attenuated or eliminated by the unregulated activity of PKA.

To explore the relationship between *shh* signalling and PKA

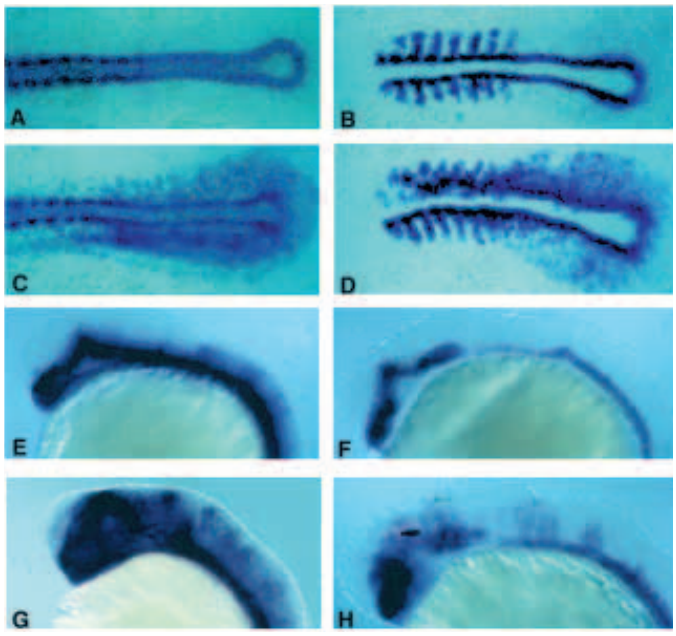

Fig. 7. Induction of *ptc1*, *nk2.2* and *myoD* expression in the brain, and in early somitogenesis stage embryos, by inhibition of PKA activity. (A,C) Dorsal views of *ptc1* expression in flat preparations of embryos at early somitogenesis. (A) Uninjected embryo. (C) *dominant negative PKA*-injected embryos. (B,D) Dorsal views of *myoD* expression in flat preparations of embryos at early somitogenesis. (B) Uninjected embryo. (D) *dominant negative PKA*-injected embryos. (E,G) Lateral views of *ptc1* expression in the brain of 27-hour-old embryos. (E) Uninjected control embryo. (G) *dnPKA*-injected embryo. (F,H) Lateral views of *nk2.2* expression in the brain of 27-hour-old embryos. (F) Uninjected control embryo. (H) *dnPKA*-injected embryo.

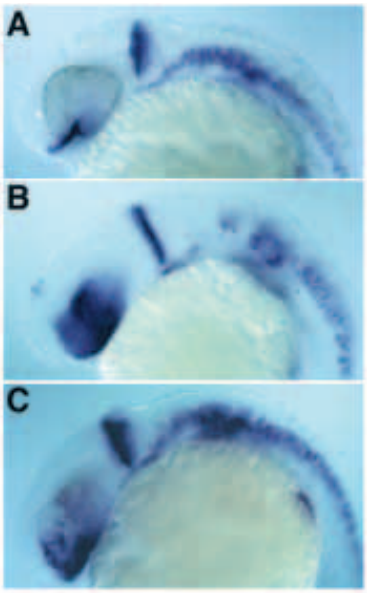

Fig. 8. Expression of *pax[b]* in wild-type, *shh*-injected and dnPKA-injected embryos. Expression of *pax[b]* in uninjected (A), *shh*-injected (B) and dnPKA-injected (C) 27-hour-old embryos. Note expansion of expression into the eyes in the injected embryos in comparison to the restricted expression in the optic stalk (arrowhead) of the normal embryo.

further, we co-injected mRNAs encoding *shh* and cPKA and analysed the eyes of resultant embryos at 27 hours. Injection of *shh* RNA alone leads to a high proportion of embryos with rudimentary eyes (77% *n*=183) as previously described (Krauss et al., 1993; Ekker et al., 1995; MacDonald et al., 1995). Amongst embryos co-injected with both *shh* and cPKA RNAs, only 22% (*n*=70) exhibited such reduction of the eyes. Moreover, the frequency of cyclopia was much reduced (11%) relative to that induced by injecting cPKA alone (60%), suggesting that the activities of *shh* and PKA are mutually antagonistic.

DISCUSSION

ptc1 is a target of *shh* signalling

Signalling by Hh family proteins is used repeatedly and in various contexts during animal development. In *Drosophila*, signalling by *hh* in the embryonic segments as well as in the imaginal discs, retina and ovary is intimately associated with the activity of the segment polarity gene *ptc* (Ingham and Hidalgo, 1993; Ma et al., 1993; Capdevila et al., 1994; Heberlein et al., 1995; Tabata et al., 1995; Forbes et al., 1996a,b). In each instance, the two genes are expressed in complementary domains (Taylor et al., 1993; Tabata and Kornberg, 1994; Capdevila et al., 1994; Forbes et al., 1996b), the activity of *ptc* suppressing the transcription of *hh* target genes wherever it is expressed. Thus when *ptc* activity is eliminated, cells behave as though they have received the *hh* signal (Ingham and Hidalgo, 1993; Capdevila et al., 1994; Lepage et al., 1995; Tabata et al., 1995), even in the total absence of *hh* function (Ingham and Hidalgo, 1993). These properties have led to the notion that *hh* acts by antagonising the activity of *ptc* (Ingham et al., 1991; Ingham and Hidalgo, 1993; Basler and Struhl, 1994; Tabata and Kornberg, 1994) and, because *ptc* encodes a transmembrane protein, it has been suggested that it may act as a receptor for the secreted Hh protein (Ingham et al., 1991).

Since the signalling activity of Hh family proteins has been highly conserved during evolution (Krauss et al., 1993; Chang et al., 1994; Ingham and Fietz, 1995), we anticipated that proteins involved in their reception at the cell surface would similarly be well conserved. The isolation of *ptc* homologues from zebrafish reported here, as well as from mouse (Goodrich et al., 1996) and chicken (Marigo et al., 1996), is in line with this expectation. Whether or not there is a direct interaction between these proteins and the respective Hh family proteins, however, remains a moot point. Although the membrane association of *Drosophila* Ptc (Taylor et al., 1993) is consistent with its hypothesised role as a receptor, the predicted topology of both the invertebrate and vertebrate Ptc proteins is more typical of that of ion channels or transporter proteins (Hooper and Scott, 1989; Nakano et al., 1989; Goodrich et al., 1996).

A defining feature of *Drosophila ptc* is its transcriptional up regulation in response to *hh* signalling. The highest levels of *ptc* expression are typically found in cells immediately adjacent to those expressing *hh* (Hooper and Scott, 1989; Nakano et al., 1989; Taylor et al., 1993; Tabata and Kornberg, 1994; Capdevila et al., 1994; Forbes et al., 1996b) and this pattern of expression depends upon *hh* activity (Hidalgo and Ingham, 1990; Capdevila et al., 1994) whilst ectopic expression of *hh* induces inappropriate levels of *ptc* transcrip-

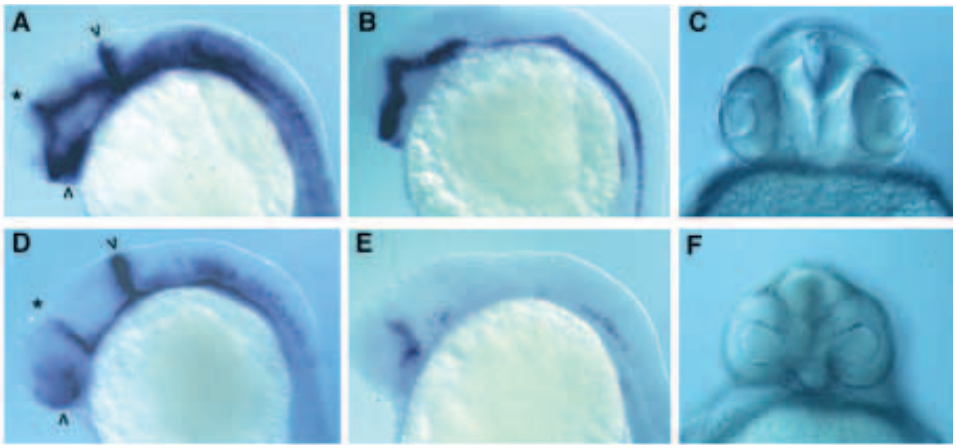

Fig. 9. Suppression of midline signalling by cPKA activity. (A-C) normal (uninjected embryos) (D-F) embryos injected with cPKA RNA at the 2- to 4-cell stage. (A,D) were hybridised with probes for both *pax[b]* and *ptc1*. In D, most of the signal derives from the *pax[b]* probe including the stripe at the midbrain/hindbrain boundary (arrowhead) and the reduced staining in the optic stalks (arrowhead). The asterisk indicates the stripe of *ptc1* expression in the diencephalon - note that in the injected embryo this extends further ventrally compared to the wild-type embryo, indicating a dorsalisation of the brain. (B,E) Hybridised with a probe for *nk2.2*. Note the marked loss of expression following injection of the cPKA mRNA. (C,F) Frontal views of the heads of 27-hour-old embryos, showing the partial fusion of the eyes in the cPKA-injected animal.

tion (Ingham, 1993; Basler and Struhl, 1994; Capdevila and Guerrero, 1994; Diaz-Benjumea and Cohen, 1994; Tabata and Kornberg, 1994; Ingham and Fietz, 1995; Forbes et al., 1996b). High-level expression of *ptc* is thus an indication of a response to *hh* signalling that is independent of cell or tissue type. Although the functional significance of this regulatory relationship is currently unclear, one possibility is that Ptc activity acts as a sink for the Hh protein; in this case, its upregulation in response to Hh would effectively limit the range of the Hh signal. Alternatively, the increase in *ptc* expression could act as a feedback mechanism that attenuates the response of a cell to the *hh* signal.

Our analysis of *ptc1* expression in the zebrafish embryo does not allow us to distinguish between these or other possibilities; however, it does provide compelling evidence that this regulatory interaction has itself been conserved during evolution and is therefore likely to be fundamental to the mechanism of *hh* signalling. Thus as in flies, the highest levels of *ptc1* transcription are invariably associated with *shh*-expressing cells; for instance, in the pectoral fin buds and the foregut high-level *ptc1* expression is found immediately adjacent to cells expressing *shh*, while in the CNS, the levels of *ptc1* transcript are at their highest in the ventral region of the neural tube adjacent to the *shh*-expressing cells of the axial mesoderm and floorplate. A similar relationship exists between the expression domains of *ptc* homologues and *shh* both in the chick (Marigo et al., 1996) and mouse (Goodrich et al., 1996). However, in each of these organisms, additional sites of elevated *ptc* expression are observed in tissues associated with the expression of two other *hh* family members, *Indian (Ihh*) and *Desert* (*Dhh*) *hedgehog* (Goodrich et al., 1996; Marigo et al., 1996; Bitgood et al., 1996). In zebrafish, the expression of *tiggy-winkle hedgehog* (*twhh*), the only other *hh* family gene characterised to date in this organism, although initiated slightly earlier, is entirely included within the *shh* expression domain (Ekker et al., 1995). Thus, in contrast to amniotes, there is no expression of *ptc1* that can be specifically associated with the activity of a *hh* family gene other than *shh.* On the contrary, except in the fin buds, where *twhh* is not expressed, it is possible that at least some aspects of the transcriptional regulation of *ptc1* may in part be mediated by *twhh.*

In midline mutants that lack *shh* expression at specific positions along the body axis, we observe changes in the pattern of *ptc1* transcription consistent with a regulatory interaction between the two genes. In *cyc* mutants, high-level *ptc1* expression is completely absent from the brain, consistent with the lack of *shh* expression in the ventral floor of the brain typical of this mutant (Krauss et al., 1993; Ekker et al., 1995; MacDonald et al., 1995). Since *cyc* embryos also lack *twhh* expression along the entire midline (Ekker et al., 1995), we cannot rule out the possibility that this gene may also be necessary (and indeed sufficient) for *ptc1* transcription in the brain. However, the essentially normal expression of *ptc1* along the ventral neural tube of *cyc* mutants, suggests that in this part of the body at least, *shh* activity is sufficient for the regulation of *ptc1* transcription. In *flh* mutants by contrast, *shh* expression disappears from the axial mesoderm at a relatively early stage, many of the cells along the ventral neural tube failing to differentiate into floorplate presumably as a consequence (Talbot et al., 1995). In line with this widespread loss of *shh* expression, transcription of *ptc1* is also severely reduced, being maintained only around the few scattered islands of cells that still express *shh*. Taken with our finding that transcription of *ptc1* can be induced ectopically in the neural tube by mis-expression of *shh*, these data strongly suggest that *shh* is both sufficient and necessary for transcriptional activation of *ptc1* transcription in the neural tube.

ptc1 regulation reflects a role of *shh* in somite patterning in the zebrafish

Whereas low level transcription of *ptc1* is detectable throughout the presomitic mesoderm, its expression is significantly elevated in the adaxial cells that flank the midline mesoderm. Several lines of evidence suggest that this mesodermal expression of *ptc1* depends upon *shh* rather than *twhh* activity. First, at this stage, expression of *twhh* is restricted to the neuroectoderm (Ekker et al., 1995) while *shh* is expressed at high levels throughout the axial mesoderm (Krauss et al., 1993). Second, in *cyc* embryos, which lack *twhh* expression along the entire midline (Ekker et al., 1995) but have normal levels of *shh* expression in the axial mesoderm, expression of *ptc1* in the somitic mesoderm is normal. Conversely, the absence of *shh* expression along the axial midline caused by the *flh* mutation, is accompanied by a significant loss of *ptc1* expression in the mesoderm. When considered with our finding that mis-expression of *shh* induces high-level transcription of *ptc1* throughout the paraxial mesoderm, these data strongly suggest that the mesodermal expression of *ptc1* is regulated by *shh*.

Interactions between the notochord and paraxial mesoderm have previously been well documented in higher vertebrates (Dietrich et al., 1993; Koseki et al., 1993; Pourquie et al., 1993; Goulding et al., 1994) and indeed the activity of Shh has been directly implicated in these interactions (Fan and Tessier-Lavigne, 1994; Johnson et al., 1994; Fan et al., 1995). However, whereas in zebrafish, the induction of *ptc1* expression in the adaxial cells appears to involve a very short-range effect of *shh*, in the chick, Shh protein acts over relatively long distances (Fan and Tessier-Lavigne, 1994; Fan et al., 1995). Moreover, adaxial cells give rise to a myotomal lineage (Felsenfeld et al., 1991; Thisse et al., 1993) and indeed express the muscle-specific transcription factor *myoD*, which can itself be induced by *shh* activity (this report; Weinberg et al., 1996). By contrast, in the mouse and chick, Shh induces expression of the sclerotomal marker *Pax1* (Fan and Tessier-Lavigne, 1994; Johnson et al., 1994; Fan et al., 1995); in line with this, notochord grafts have been shown to induce differentiation of sclerotome at the expense of myotome, actually repressing *MyoD* expression (Goulding et al., 1994). Thus, it appears that the outcome of *shh* signalling on somite patterning differs between lower and higher vertebrates. The different organisation of the somites in amniotes and teleosts may thus be accounted for, at least in part, by a modification of the inductive signals that control their specification. Interestingly recent studies have revealed that, in chick, *MyoD* expression can be induced by Shh in presomitic mesoderm when presented in combination with signals from the dorsal neural tube (Munsterberg et al., 1995), an activity that may reflect its original role in the evolution of the vertebrate body plan.

The role of PKA in *shh* signalling

Intriguingly, as in *Drosophila*, we have found that the effects

of ectopic *shh* activity on *ptc1* expression can be mimicked by reducing or eliminating the activity of PKA. Although PKA has previously been implicated in signalling by Shh protein in the brain and somites of rats and chicks, respectively (Fan et al., 1995; Hynes et al., 1995), our finding that transcription of a *ptc* homologue in both the neuroectoderm and paraxial mesoderm is repressed by PKA provides the most direct indication to date of the extent to which the elements of the *hh* signalling pathway have been conserved. Similar effects are seen on the transcription of other targets of *shh* activity, both in the mesoderm, where *myoD* transcription is induced, and in the neural tube, where *nk2.2* is inappropriately expressed. In addition, we consistently observe the ectopic activation of *pax[b]* in the developing eye, an effect that presages eye abnormalities similar to, though less extreme than, those induced by ectopic *shh* expression (unpublished observations). Analogous effects of a dnPKA on zebrafish eye development have also recently been described by Hammerschmidt et al. (1996).

The implication of PKA in the regulation of *ptc1* is further supported by our finding that constitutive activity of PKA suppresses *ptc1* transcription throughout the brain and neural tube. This effect is accompanied by a similar suppression of *nk2.2* expression and by cyclopia, a condition clearly associated with a loss of midline signalling (Krauss et al., 1993; Hatta et al., 1994; Ekker et al., 1995; MacDonald et al., 1995). Together, all of these effects strongly support the notion that the *shh* signal acts by antagonising the repressive activity of PKA on various target genes, just as in *Drosophila*, *hh* counteracts the repressive effect of PKA on *wg*, *dpp* and *ptc* transcription. In *Drosophila*, it has been argued that PKA acts in parallel to the *hh* signalling cascade (Jiang and Struhl, 1995; Kalderon, 1995). In this regard, it is interesting that co-expression of *shh* and the cPKA mutant results in a mutual suppression of the two contrasting eye phenotypes induced by either activity alone. This result implies a balance between two opposing signals rather than a strictly linear relationship between the two, supporting the conclusions from the *Drosophila* studies. Further analyses will be required to elucidate the precise relationship between these activities.

We are especially grateful to T. Simmonds for running the fish facility, G. Clark and A. Davies for help with the automated sequencing, I. Goldsmith for oligonucleotide synthesis and our colleagues on the 4th floor at the ICRF for interest and advice. We thank T. Jowett and C. Kimmel for providing mutant fish strains, A. Barth, D. Henrique, T. Jowett, D. Kalderon, S. McKnight, D. Simmons and E. Weinberg for various clones and K. Zinn for providing the cDNA library. L. V. G. is an HHMI predoctoral fellow, R. L. J. is a fellow of the Damon Runyon-Walter Winchell Foundation Cancer Research Fund (DRG 1218) and M. P. S. is supported by the HHMI. The experimental part of this study was funded by the Imperial Cancer Research Fund and by grants from the Human Frontiers Science Programme and the European Community Human Capital and Mobility Programme to P. W. I.

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(Accepted 13 June 1996)