Sphedgehog Is Expressed by Pigment Cell Precursors During Early Gastrulation in Strongylocentrotus purpuratus

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We have sequenced the Sphedgehog (Sphh) gene from the sea urchin Strongylocentrotus purpuratus. Sphh transcripts are detected first at the mesenchyme blastula stage, and they accumulate throughout early embryogenesis. The Sphh protein is produced by precursor pigment cells during early and midgastrulation. NiCl2 inhibits pigment cell differentiation in sea urchins. Here, we show that, in S. purpuratus, nickel affects a process(es) between 17 and 24 hr of development, corresponding to the time period when Sphh mRNA is first detected. However, nickel treatment does not alter the early expression of Sphh. Developmental Dynamics 231:370–378, 2004. © 2004 Wiley-Liss, Inc.

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INTRODUCTION

The extreme regulative capacity of sea urchin embryos was first revealed by experiments carried out by Driesch in which four-cell stage embryos were dissociated into individual blastomeres, and each blastomere developed into a small normal larva (reviewed in Horstadius and Wolsky, 1936). More recent experiments have demonstrated that sea urchin embryos maintain their regulative capacity throughout early development. For example, if the skeletogenic mesenchyme cells are removed from the gastrulating embryo, a population of secondary mesenchyme cells are able to switch cell fate and replace the absent skeletogenic mesenchyme, resulting in a larva with a normal skeleton (Ettensohn and McClay, 1988; Ettensohn and Ruffins, 1993). The endoderm also maintains its regulative capacity until after gastrulation. McClay and Logan (1996) carried out a series of experiments in which different amounts of the endoderm were removed. Even in those cases in which the entire archenteron was taken out, the embryos recovered and developed into larvae with all the normal different cell types. These experiments testing the regulative properties of the sea urchin embryo demonstrate that many of the cell fate acquisition events that occur during early sea urchin embryogenesis are driven by cell–cell communication, rather than the inheritance of asymmetrically localized maternal factors, as is the case with mosaic systems such as tunicates (Conklin, 1950). Therefore, a study of the mechanisms and molecules involved in cell–cell communication is central for obtaining a comprehensive understanding of sea urchin development.

The hedgehog family of growth factors has been shown to play a central role in cell specification and patterning in several systems (i.e., reviewed in Hammerschmidt et al., 1997; Ingham and McMahon, 2001). The hedgehog genes code for secreted signaling proteins of ~45 kDa. The hh proteins consist of a signal peptide, a highly conserved N-terminal region, and a more divergent C-terminal region. In addition to signal sequence cleavage, hh proteins undergo an autoproteolytic event that results in the production of a 19-kDa N-terminal peptide and a 26- to 28-kDa C-terminal peptide (Lee et al.,...
1994; Bumcrot et al., 1995; Porter et al., 1995). The autoproteolytic event has a thioester intermediate that is cleaved in a nucleophilic substitution and results in the covalent coupling of cholesterol to the N-terminal peptide (Porter et al., 1996). Autoproteolysis of the hh proteins is driven by sequences in the C-terminal peptide that have similarities to serine proteases (Lee et al., 1994; Porter et al., 1995). The N-terminal peptide is the biologically active form of the hh protein, and it is sufficient for the short- and long-range signaling activities in Drosophila and vertebrates (Marti et al., 1995; Porter et al., 1995; Roelink et al., 1995). In vertebrates, the covalent coupling of cholesterol to the N-terminal peptide seems to be necessary for the long-range signaling function of Shh-N, while the lack of cholesterol coupling to the N-terminal fragment of Shh may account for its accumulation around the cell surface area of the hh-expressing cells (Zeng et al., 2001; Gallet et al., 2003).

In addition to the tethering of cholesterol to the N-terminal of Shh, an additional lipid modification has been identified, the palmitoylation of its N-terminal cysteine. This palmitic modification of Shh may play a role in modulating Shh signaling function (reviewed in Jeong and McMahon, 2002). Also, the crystal structure of the N-terminal peptide suggests that the N-terminal SHH contains a zinc hydrolase domain, which has been proposed to play a role in receptor activation/inactivation or in the cleavage of other N-terminal SHH peptides, thus allowing their diffusion away from the cell surface (Hall et al., 1995).

To date, invertebrates appear to have only one member of the hedgehog gene family, whereas vertebrates have several hh gene family members (Chang et al., 1994; reviewed in Hammerschmidt et al., 1997). For example, mice have three members of the hh gene family: Sonic hh (Shh), Indian hh (Ihh), and Desert hh (Dhh) (Echelard et al., 1993). We report here the identification of the S. purpuratus hh gene, which is the only member of the hh gene family present in the S. purpuratus genome. During early gastrulation, Sphh is expressed by the precursor pigment cells, and its expression is not repressed by nickel, a known inhibitor of pigment cell development.

**RESULTS**

**Cloning of Sphedgehog**

A previous polymerase chain reaction (PCR) screen of the S. purpuratus genome using hedgehog de-generate primers isolated a partial fragment of a Sphedgehog (Sphh) gene (Chang et al., 1994). Based on the sequence of the Sphh fragment Sphh primers were generated and used in combination with bacteriophage vector primers in a semi-nested PCR library screen. The PCR library screen of a 44 hr S. purpuratus cDNA library resulted in the isolation of two fragments: one containing the 5’ Sphh sequence and the other containing the 3’ Sphh sequence from the previously isolated Sphh fragment (Fig. 1A). Taken together, the sequences of these two fragments and the sequence obtained from the genomic screen (Chang et al., 1994) code for the entire Sphh protein (Fig. 1A). Extensive PCR screening of the 44 hr library failed to amplify any other fragments that corresponded to additional members of the hh gene family.

Protein sequence comparison between SPHH and mouse SHH (sonic hedgehog protein) showed that these two proteins are 64% similar and 49% identical. When the N-terminal peptides, corresponding to the biologically active portion of the proteins, are compared, the percent similarity increases to 74%. Very similar results were obtained when Sphh was compared with IHH (Indian hedgehog protein) and DHH (desert hedgehog protein). The hh homolog in Lytechinus variegatus, another sea urchin species, has been cloned and sequenced (Lvhhedgehog; Hertzler and McClay, unpublished results—sequence available in GenBank). Sequence comparison between Sphh and LVVHH show that HH is very well conserved within echinoderms (Fig. 1B). The two proteins are 91% identical.

**Sphh Is a Single Copy Gene**

Protostome invertebrates are known to contain only a single member of the hh gene family, while vertebrates have several members of the hh gene family (Chang et al., 1994; Hammerschmidt et al., 1997). Although invertebrates, echinoderms are deuterostomes. Thus, it was of interest to investigate the number of hh gene family members that are present in echinoderm genomes. To test whether S. purpuratus has one or more members of the hh family in its genome, Southern blot analysis was carried out with S. purpuratus genomic DNA cut with several different restriction enzymes and hybridized with a probe encoding the N-terminus of the Sphh protein. The hybridization of the probe to the digested genomic DNA samples was performed under high and low stringency. At both hybridization temperatures, the probe reacted with a single band in the three genomic DNA samples cut with three different restriction enzymes (Fig. 2A). These results indicate that Sphh is a single copy gene.

**Sphh Is Expressed During Gastrulation and the Prism and Pluteus Stages**

To establish the temporal expression pattern of Sphh during early S. purpuratus embryonic development, RNase protection analyses were carried out on RNA samples obtained from embryos at different developmental time points (Fig. 2B). The results from these experiments show that Sphh is not a maternal message. The accumulation of the Sphh transcripts is first evident at the mesenchyme blastula stage and transcript levels increase and are present throughout the rest of early embryogenesis.

To characterize the spatial expression pattern of Sphh expression, RNA in situ hybridizations using a digoxigenin-labeled Sphh probe were carried out. The results from these experiments were inconclusive.
**SPHH Is Expressed in Precursor Pigment Cells**

A commercially available goat polyclonal antibody raised against a peptide sequence from mouse Indian hedgehog (IHH) N-terminal section was used to analyze the spatiotemporal expression of SPHH protein. The Ihh antibody was raised to a sequence that differs in only two amino acids from the equivalent sequence in SPHH. The Ihh antibody reacted against protein extracts obtained from embryos at different time points gave inconclusive results on Western blot analyses. When the Ihh antibody was used in immunocytochemistry experiments with 42 hr *S. purpuratus* embryos, the results demonstrated that SPHH is expressed by a group of cells located on the ectoderm of the embryo (Fig. 3A,C). Treatment of the same stage embryos with a sample of the Ihh antibody preabsorbed with the IHH peptide used to raise the antibody resulted in no ectodermal cell specific signal (Fig. 3B). The same immunocytochemistry analysis on 42 hr embryos was carried out by using a monoclonal antibody raised against the entire mouse Sonic hh (Shh) protein. The mouse Shh monoclonal antibody also recognizes a group of cells located on the ectoderm of the embryos (data not shown; Fig. 4B).
double-label immunocytochemistry was carried out using the Shh and Ihh antibodies, it was observed that both antibodies recognized the same cells, indicating that both antibodies recognized SPHH in the same group of cells located on the ectoderm of the gastrulating embryo (Fig. 4A, B). Double-label control embryos that were treated with only goat polyclonal Ihh antibody, but with both anti-goat rhodamine-labeled antibody and antimouse fluorescein isothiocyanate (FITC)-labeled antibody showed ectoderm cells labeled only in red. Conversely, only green-labeled ectodermal cells were present in embryos treated with the monoclonal Shh antibody and both the secondary antibodies (data not shown).

The morphology of the cells expressing SPHH and their location at 42 hr postfertilization suggested that they are precursor pigment cells. To test this hypothesis, immunocytochemistry was carried out on 42 hr S. purpuratus embryos using the Ihh antibody and the monoclonal antibody SP-1/20.3.1. SP-1/20.3.1 antibody specifically recognizes an epitope expressed by pigment cells and their precursors (Gibson and Burke, 1985).

The results of the double-label experiment presented here clearly demonstrate that the SP-1/20.3.1 antibody reacts with the same group of cells as those stained by the Ihh antibody (compare Fig. 4C with D). These data indicate that, at 42 hr postfertilization, precursor pigment cells express SPHH protein in S. purpuratus embryos.

**Treatment of S. purpuratus Embryos With Nickel Results in the Inhibition of Archenteron Elongation and Pigment Cell and Skeletogenic Mesenchyme Differentiation**

The treatment of sea urchin embryos with compounds (i.e., lithium) that disrupt embryogenesis has proven to be a very effective way to unveil the molecular pathways that drive early sea urchin development. Other investigators found that treatment of L. variegatus embryos with NiCl2 causes a radialized phenotype that is characterized by the presence of primary mesenchyme cell clusters all around the perimeter of the embryo instead of the two ventral clusters found in normal embryos (Hardin et al., 1992). The aberrant location of the primary mesenchyme clusters has been the most extensively studied aspect of the NiCl2 phenotype. However, NiCl2-treated embryos also fail to differentiate pigment cells (Hardin et al., 1992). The effect of NiCl2 treatment on the differentiation of pigment cells has not been analyzed in S. purpuratus embryos. We were particularly interested in determining the window of time during which NiCl2 affects embryogenesis and any possible relationship between nickel’s effect on pigment cell differentiation and SPHH expression.

To establish the developmental time window during which nickel affects pigment cell differentiation, embryos were treated with 1 mM NiCl2 beginning at different times postfertilization. Previous studies done on the effect of NiCl2 on spicule formation in L. variegatus indicated that nickel affected some process(es) that occurred during the hatching blastula to early gastrula stages (Hardin et al., 1992). Embryos were treated with NiCl2 at 5 hr, 17 hr, and 24 hr postfertilization. Greater than 90% of the embryos treated with NiCl2 at 5 hr and 17 hr did not develop pigment cells, whereas embryos treated at 24 hr did (Fig. 5A). It should be pointed out that, in all embryo cultures treated with NiCl2 at 5 hr and 17 hr postfertilization,

**Fig. 1. A:** DNA sequence and predicted amino acid sequence of Sphedgehog gene and protein. A seminested polymerase chain reaction approach was used to screen a 44 hr S. purpuratus library with SPHH-specific primers obtained from a previously reported partial SPHH genomic sequence. Two fragments were amplified during the library screen. The first fragment (underlined in purple) encodes the complete predicted N-terminal open reading frame sequence upstream of the published sequence (underlined in red). The second fragment (underlined in green) encodes the complete predicted C-terminal open reading frame sequence downstream of the published sequence. The C-terminal fragment contains the putative processing site (underlined in blue) where the cholesterol unit would get tethered to the cleaved N-terminal SPHH peptide. **B:** The predicted SPHH protein from the DNA sequences obtained from our cDNA screen (protein sequence predicted by 5—underlined in purple—and 3—underlined in green—DNA sequences) and the genomic screen (in red) were compared to the predicted Lvhh protein sequence. Both proteins are 92% similar and 91% identical. Both the putative N-terminal and C-terminal peptides are very similar in both proteins. GenBank accession no. AV02192.

**Fig. 2. A:** Sphedgehog is a single copy gene. S. purpuratus genomic DNA was digested with MspI, EcoRI, and HindIII restriction enzymes. The digested DNA was analyzed for the presence of members of the hh gene family by Southern blotting at 47°C and 65°C using a probe encoding the N-terminal peptide sequence. At both temperatures, the probe reacted with only one band in each digested genomic DNA sample (marked by arrows). **B:** Sphedgehog transcripts begin accumulating at mesenchyme blastula stage and followed by an increased expression that is maintained throughout early development. RNA samples isolated at different developmental time points (marked as hours postfertilization at the top of the figure) were analyzed for the presence of SPHH transcripts by RNase protection using a probe encoding the N-terminal peptide sequence of Sphedgehog. **C:** The lack of SPHH transcripts in mature eggs (0 hr postfertilization) indicates that SPHH is not a maternal transcript. SPHH RNA is first detected at mesenchyme blastula stage (28 hr postfertilization). SPHH expression is maintained throughout gastrulation (31–48 hr postfertilization) and the early prism stage (48–60 hr postfertilization).

**Fig. 3.** SPHH is expressed by cells that are scattered throughout the ectoderm of midgastrula stage embryos. **A:** Low magnification imagine of 42 hr embryos reacted with a Ihh polyclonal antibody (red), showing that cells scattered throughout the ectoderm are expressing SPHH. **B:** A 42 hr embryo reacted with a sample of Ihh antibody preabsorbed with the IHh peptide used to raise the antibody (red) and showing no ectodermal cell-specific signal. This finding indicates that the staining pattern seen in A is antigen-specific. **C:** A high-magnification imagine of a 42 hr embryo reacted with the Ihh antibody (red), showing that ectodermal cells are expressing SPHH. Original magnifications: ×125 in A, ×250 in B, ×500 in C.
Fig. 4. SPHH is expressed by precursor pigment cells. A,B: Double-label experiment using the Ihh and Shh antibodies. A: A midgastrula stage embryo reacted with the Ihh antibody (red), showing that cells located in the blastocoel and ectoderm are expressing SPHH. B: Same embryo as in A reacted with the Shh antibody (green), showing that the same cells as in A express SPHH. C,D: Double-label experiment using the Ihh and SP-1/20.3.1 antibodies. C: A midgastrula stage embryo reacted with the Ihh antibody (red), showing that cells scattered throughout the ectoderm are expressing SPHH. D: Same embryo as in C reacted with the SP-1/20.3.1 antibody, showing that the cells expressing SPHH are precursor pigment cells. Original magnification, ×500.

Fig. 5. A: Embryos treated with NiCl₂ at 5 hr and 17 hr postfertilization do not develop pigment cells, whereas embryos that are treated with NiCl₂ at 24 hr postfertilization do. a–d: Nomarski images of 48 hr embryos. e–h: Nomarski images of 68 hr embryos. a: A control embryo containing pigment cells (one marked by yellow arrow), an elongated archenteron (marked by green arrow), and a ring of primary mesenchyme cells at the base of the archenteron (marked by blue arrow). b,c: Embryos treated with 1 mM NiCl₂ at 5 hr and 17 hr postfertilization, respectively, lack pigment cells and archenteron. Mesenchymal cells are present within the blastocoel (marked by orange arrows). d: Embryo treated with 1 mM NiCl₂ at 24 hr postfertilization contains pigment cells (marked by a yellow arrow) and an elongated archenteron (marked by a green arrow). B: Sphh is expressed in embryos treated with NiCl₂. RNA was isolated from embryos treated with NiCl₂ at 5 hr (1), 17 hr (2), and 24 hr (3) postfertilization. The RNA was analyzed for the presence of Sphh by reverse transcriptase-polymerase chain reaction. The bands shown correspond to the amplified Sphh. In all RNA samples isolated from embryos of the three Ni treatments, Sphh transcripts were present. Original magnification: ×100 in a,e; ×200 in b–d,f–h.
~5% of the embryos developed some pigment cells. The percentage of embryos refractory to NiCl₂ in these two treatments did not diminish when the embryos were treated with 2 mM NiCl₂. The results from the nickel treatment indicate that NiCl₂ affects a process(es) that takes place between 17 and 24 hr of development and is necessary for proper pigment cell differentiation in S. purpuratus.

Nickel Chloride Treatment Does Not Affect Sphh Expression

The results of our NiCl₂ treatment experiments show that the pigmentless phenotype caused by nickel is mediated by nickel’s effect on developmental processes that occur between 17 and 24 hr of development. It is between 17 and 24 hr of development that the patterning of the secondary mesenchyme begins, and the pigment cell precursors are specified. Because Sphh RNA begins to accumulate between 18 and 24 hr of development and pigment cell precursors express Sphh during early to midgastrulation, we asked the question of whether NiCl₂ has an effect on the expression of Sphh. To answer this, RNA was harvested from embryos that have been treated with NiCl₂ at 5 hr, 17 hr, or 24 hr postfertilization. The presence of Sphh mRNA was analyzed by reverse transcriptase-PCR (RT-PCR). RNA samples obtained from embryos of the three treatment groups contained Sphh mRNA (Fig. 5B). As mentioned above, although most of the embryos that were treated with NiCl₂ at 5 hr and 17 hr postfertilization did not contain differentiated pigment cells, a small percentage of embryos from those treatments developed pigment cells. To establish whether the presence of Sphh in all RNA samples tested was due to the fact that Ni treatment has no effect on Sphh expression or whether the Sphh transcripts detected came from the embryos that were refractory to Ni treatment, semiquantitative PCR was carried out on the same samples. The results of these studies were somewhat variable; however, levels of Sphh transcripts were comparable between control and treated embryos, indicating that Ni does not have an effect in the initial levels of Sphh RNA present in embryos exhibiting the pigmentless phenotype (data not shown).

DISCUSSION

Sea Urchins Have Only One Member of the hh Gene Family

The results from our PCR library screens and the one carried out in the Beachy lab (Chang et al., 1994) using Sphh specific primers suggested that S. purpuratus contains only one member of the hedgehog gene family. This hypothesis was supported by the Southern blot analysis of genomic DNA, which shows that Sphh is a single copy gene. Protostomes have a single member of the hh gene family (Chang et al., 1994), while vertebrates have several members of the hh gene family (Hammerschmidt et al., 1997). Molecular phylogenetic analysis of the hh gene family has shown that the hh gene has undergone two gene duplications. The first one gave rise to Desert hh, whereas the second one resulted in the production of Indian and Sonic hh (Kumar et al., 1996; Zardoya et al., 1996). It has been proposed that these two major duplications occurred before the emergence of vertebrates and probably before the evolution of chordates (Kumar et al., 1996). The data presented here extend our understanding of the evolution of this gene family and argue that the duplications of the hh gene family occurred after the split of echinoderms from the main deuterostome branch.

Sphh Is Expressed by the Precursor Pigment Cells

Analysis of the temporal expression pattern of Sphh demonstrates that Sphh transcripts begin accumulating during the mid/late blastula stage. This mid/late blastula stages correspond to the developmental period when the first patterning events are thought to occur in the vegetal plate (Sherwood and McClay, 1997). Patterning of the germ layers continues throughout gastrulation and early prism stages, and Sphh is expressed throughout this time. Immunocytochemical analyses using two different HH antibodies demonstrated that pigment cell precursors at the midgastrula stage express SPHH. Sphh is the first growth factor shown to be expressed by the pigment cell precursors.

The results presented here beg the question of which other cell population the pigment cells are communicating with through the activity of SPHH. Experiments in which the primary mesenchyme cells (PMCs) are removed from embryos during gastrulation demonstrated that pigment cell precursors communicate with PMCs and are able to substitute for them when PMCs are absent (Ettenson and McClay, 1988; Ettenson and Ruffins, 1993). These results suggest that the PMCs send an inhibitory signal to the precursor pigment cells that prevents them from becoming PMCs (Ettenson and McClay, 1988; Ettenson and Ruffins, 1993). Likewise, there could be some cross-talk between precursor pigment cells and PMCs. Preliminary studies in which gastrulating sea urchins are treated with the hh signaling inhibitor cyclopamine point to this possibility, because in cyclopamine-treated embryos, the pigment cells and PMCs do not differentiate (Egañà and Ernst, unpublished results).

Sphh Expression Is Not Altered by NiCl₂ Treatment

L. variegatus embryos treated with NiCl₂ develop a radialized phenotype. In normal embryos, the PMCs group together in two ventrolateral clusters where they begin the formation of spicules. In NiCl₂-treated embryos, the radialized phenotype is marked by the presence of PMC clusters around the entire perimeter of the embryo and correlates with altered expression of dorsal and ventral markers. Therefore, it has been proposed that nickel ventralizes the embryo by altering the commitment of ectodermal cells along the dorsal/ventral axis (Hardin et al., 1992).

An aspect of the NiCl₂ phenotype that was reported but not investi-
gated is the lack of pigment cell differentiation in NiCl₂-treated embryos. The cell fate maps of the blastula stage vegetal plate indicated that the cells located on the dorsal side of the secondary mesenchyme cell (SMC) territory are specified to become pigment cells (Ruffins and Ettensohn, 1996). If NiCl₂ ventralizes the embryo, then the lack of pigment cells in NiCl₂-treated embryos could be due to a failure in the specification of dorsally located cells to become pigment cells. Furthermore, the analysis of the developmental time in which NiCl₂ has its effect in relation to pigment cell differentiation presented here has shown that NiCl₂ acts between 17 and 24 hr, which corresponds to the transition period between the late cleavage and blastula stages. This is the period when the segregation of the SMC territory into a dorsal and ventral side is thought to occur (Ruf

Adult animals were maintained in sea water tanks at 15°C and fed algae 3× a week. Spawning, fertilization, and culturing of embryos was carried out as previously described (Pittman and Ernst, 1984).

Cloning and Sequencing

A seminested PCR approach was used to clone fragments that contained the sequence 5′ and 3′ from the previously reported Sphh fragment (Chang et al., 1994) from a 44 hr S. purpuratus random-primed ZAP library. The 5′ sequence was obtained in a two-step PCR screen using the bacteriophage arm primer in combination with Sphh-specific primers (Chang et al., 1994). In the first step, 1 μl of the phage library was used as a template in a PCR reaction with a combination of T7 and 5′ TGGTTGGTGATGTCTACTG 3′ primers. The PCR reactions were run for 40 cycles with the following cycle conditions: denaturing, 95°C for 2 min; annealing, 54°C for 1 min; extension, 72°C for 2 min with the addition of 10 sec of extension per cycle. The PCR reaction components were the following: 1× Taq polymerase buffer, 1.5 mM MgCl₂, 0.2 mM dNTPs, each primer at 1 μM, and 1.25 U of Taq polymerase (Promega). Fragments amplified in the first round of PCR were purified and used as a template in a second PCR reaction with a combination of T7 and 5′ ACTGGCACCAAAA 3′ (this primer is located upstream of the primer used in the first reactions) primers. The PCR conditions were the same as in the first set of cycles except that the annealing temperature was 36°C. The amplified fragments resulting from the second round PCR reactions were cloned into pGEM T-easy vector (Promega) and sequenced by the Tufts Sequencing Facility. For each clone, both strands were sequenced. The same seminested PCR approach used for the 5′ sequences was used to obtain the 3′ sequence. In the first round of PCR, the primers used were the T3 and the 5′ CAGTAGACATCACCACA 3′ primers. The PCR reaction was run with the same conditions as described above except that the annealing temperature was 54°C. In the second round of the PCR, the primers used were T3 and 5′ AATATGGCCGCTCTAGC 3′ (a primer located 3′ from the primer used in the first reactions). The PCR reaction was run with the same conditions as described above except that the annealing temperature was 48°C. The PCR containing the 3′ sequence was cloned and sequenced as described above. To check the quality of the PCR amplification, several fragments were sequenced. It was determined that under the PCR conditions used, the fidelity of the Taq polymerase was very good. The sequence obtained was stored in GenBank (accession no. AY602192).

Southern Blot

Eight micrograms of S. purpuratus genomic DNA were digested with MspI, EcoRI, or HindIII overnight at 37°C. The digestions were run in a 1% agarose gel at 40 volts. The DNA was blotted on a nitrocellulose membrane overnight by using 20× standard saline citrate (SSC, 3 M NaCl, and 3 M sodium citrate) as transfer buffer. DNA was ultraviolet cross-linked by using a ultraviolet (UV) Stratalinker. The membrane was hybridized overnight at 47°C or 65°C with hybridization buffer (5× SSC, 5× Denhardt’s solution, and 0.1% SDS) plus the probe. The probe was synthesized by PCR using the Sphh sequence encoding most of the N-terminal peptide cloned in a pGem 3 vector (Promega) as a template (100 ng). The PCR reaction components were the following: 1× Taq buffer, 1.5 mM MgCl₂, 0.2 mM digoxigenin-labeled dNTPs (Boehringer-Mannheim), 1 μM T3 primer, and 1 μM T7 primer 1.25 U of Taq polymerase (Promega). The PCR conditions were the following: denaturing, 95°C for 2 min; annealing, 50°C for 1 min; extension, 75°C for 2 min for 35 cycles. After washing, the membrane was blocked for 30 min in 5% skim milk in maleate buffer (100 mM maleic acid, 150 mM NaCl, and 0.1% Tween-20 (Sigma) pH 7.5) and then incubated with a 1:500 dilution of a digoxigenin antibody (Boehringer-Mannheim). After washing, the membrane was developed with nitroblue tetrazolium/5-bromo-4-chloro-3-indoly phosphate (NBT/BCIP, Promega).

RNase Protection

An Sphh antisense probe was constructed. The Sphh genomic fragment (Chang et al., 1991) was cloned in a pBlueScriptII KS plasmid (Stratagene). The plasmid was cut with XhoI and the Sphh fragment was transcribed with the T7 polymerase. RNase protection assays were carried out as specified by the manufacturer of the Ambion RPAII kit.
**Immunocytochemistry**

Embryos at different developmental stages were fixed in 2% paraformaldehyde for 2 hr at room temperature. Embryos were hydrated and stored at 4°C in 1× phosphate-buffered saline (PBS). Rehydrated material was blocked for 1 hr at room temperature in 5% bovine serum albumin in PBS. Slides were washed in PBS 4× for 15 min and incubated in 1 M hexylene glycol + 0.15% Triton X-100 for 35 min. After a brief wash in PBS, slides were incubated with a 1:200 dilution of Ihh goat polyclonal antibody (Santa Cruz Biotechnology, Inc.) or a 1:50 dilution of the Shh monoclonal antibody (provided by P. Maye and L. Grabel) or a 1:200 dilution of the SP-1/20.3.1 monoclonal antibody for 1 hr at room temperature. Slides were washed 3× for 20 min at room temperature in PBS, incubated with a 1:200 dilution of FITC-labeled anti-mouse (Sigma) or Cy3-labeled anti-goat IgG (Jackson ImmunoResearch Laboratories, Inc.), and washed overnight at 4°C in PBS. For the double-label experiments, the slides were then incubated with a 1:50 dilution of Shh monoclonal antibody or a 1:200 dilution of the SP-1/20.3.1 monoclonal antibody for 1 hr at room temperature, washed for 2 hr (6× for 20 min) at room temperature, incubated with a 1:200 dilution of FITC-labeled anti-mouse IgG (Sigma), and washed overnight at 4°C in PBS. The slides were mounted by using the Slowfade Light Antifade Kit (Molecular Probes) and viewed by using an Olympus fluorescent microscope. To preabsorb a sample of the Ihh antibody with the Ihh peptide, the antibody was incubated with a 10-fold (by weight) excess of the peptide in PBS overnight at 4°C (as recommended by Santa Cruz Biotechnolog- y, Inc.). To control for possible cross-reactivity between the fluorochrome-labeled secondary antibodies used in the double-labeling experiments, embryos were treated with only one primary antibody and both secondary antibodies in the same sequence as described above. In all cases, only the staining corresponding to the primary antibody and its secondary antibody was observed.

**Drug Treatments and RNA Isolation**

**NiCl₂ treatment.**

Embryos were fertilized and cultured in artificial sea water. At 5 hr, 17 hr, or 24 hr postfertilization, NiCl₂ was added to the culture to a final concentration of 1 mM or 2 mM and the embryos were maintained in the NiCl₂ until they were harvested for RNA. RNA was isolated from embryos using the RNAzol procedure (Tel-test).

**RT-PCR**

Forty micrograms of RNA were digested with RNasefree RNase (Promega) for 30 min at 37°C. The RNA was purified by using the Wizard mini PREP (Promega). Ten micrograms of RNA from the RNasefree RNase-digested samples were reverse transcribed by using an RT-PCR kit (Stratagene). The reactions were carried out by using random primers in the first-strand cDNA synthesis and following the protocol recommended by the manufacturer. The presence of the gene of interest was tested using the primer pairs and annealing temperatures and numbers of cycles described below. The general PCR conditions for all reactions were: 1× Taq polymerase buffer, 1.5 mM MgCl₂, 10 μM dNTPs, 20 μM of each primer, 1.25 U of Taq polymerase (Promega), and 1–3 μl of template. Amount of template used and cycles was determined through a titration series as to guarantee that the amount of product obtained from the reaction was the result of exponential amplification. To amplify Shh, the following were used: primers, 5’ GTCCTATCGCATGACAC 3’/5’ TCTAGGTCGTCTGGCT 3’; annealing temperature, 58°C; number of cycles, 35. To amplify 18S, the following were used: primers, 5’ GGTGCGATCCGTAGGG 3’/5’ CGCAATAGAACCGAGGTC 3’; annealing temperature, 55°C; number of cycles, 15. The PCR reactions were run in 2% agarose gels for 15 min at 110 volts. Pictures of the gels were taken and scanned into a PC. Densitometry analysis of the bands present in the PCR reactions was done by using the ImageQuant program (Molecular Dynamics).

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