Post-translational Processing and Renal Expression of Mouse Indian Hedgehog*

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The full-length mouse Indian hedgehog (Ihh) cDNA was cloned from an embryonic 17.5-day kidney library and was used to study the post-translational processing of the peptide and temporal and spatial expression of the transcript. Sequence analysis predicted two putative translation initiation sites. Ihh translation was initiated at both initiation sites when expressed in an in vitro transcription/translation system. Expression of an Ihh mutant demonstrated that the internal translation initiation site was sufficient to produce the mature forms of Ihh. Ihh post-translational processing proceeded in a fashion similar to Sonic and Drosophila hedgehog; the unprocessed form underwent signal peptide cleavage as well as internal proteolytic processing to form a 19-kDa amino-terminal peptide and a 26-kDa carboxyl-terminal peptide. This processing required His313 present in a conserved serine protease motif. Ihh transcript was detected by in situ RNA hybridization as early as 14.5 days postcoitum (dpc) in the cartilage primordium, and in the developing urogenital sinus. In semiquantitative reverse transcription-polymerase chain reaction experiments, Indian hedgehog transcript was first detected in the mouse metanephros at 14.5 dpc; transcript abundance increased with gestational age, becoming maximal in adulthood. In adult kidney, Ihh transcript was detected only in the proximal convoluted tubule and proximal straight tubule.

Mouse Indian hedgehog (Ihh) is a member of a multigene family that includes Hedgehog (Drosophila) and its vertebrate homologue, Sonic hedgehog (1, 2). The Hedgehog proteins are secreted extracellular signals that communicate with neighboring cells to regulate the production of patterning molecules such as Wingless and Decapentaplegic (3, 4). Hedgehog (Hh) regulates the anterior-posterior patterning of the imaginal disc structures in Drosophila, while Sonic hedgehog (Shh) carries out a similar function in the vertebrate limb in concert with the fibroblast growth factor-4 (5–11). In addition, Shh affects the dorsoventral patterning of the mouse neural tube and somites leading to the induction of floor plate cells, motor neurons, and sclerome (12–15). Both Hh and Shh undergo autoproteolytic cleavage to generate a functional amino-terminal peptide, with inducing activity, and a carboxyl-terminal peptide that can tether the precursor protein to the cell membrane (14–20).

Two additional vertebrate hedgehog genes, Desert hedgehog (Dhh) and Ihh have also been identified (2). For Ihh, partial cDNA sequences are available for human and mouse with expression reported in the embryonic lung of human, the developing gut and cartilage of chick, and the adult kidney in both mouse and human (2, 21–23). In the developing cartilage, Ihh is produced by proliferating chondrocytes of the prehypertrophic growth zone and signals to the surrounding perichondrium to induce the release of parathyroid hormone-related protein (PTHrP) (24, 25). Once bound to its receptor in the undifferentiated chondrocytes, PTHRPr blocks cells from entering the hypertrophic pathway. Thus, this Ihh/PTHrP feedback loop can regulate chondrocyte differentiation to balance the growth and ossification of long bones. While the complete chick Ihh cDNA sequence has been reported recently, the post-translational processing of the peptide has not been described.

In this report the entire coding region of the mouse Ihh cDNA was identified, its protein products analyzed, and the embryonic expression pattern determined. The mouse Ihh cDNA contains two putative translation initiation sites, unique to vertebrate hedgehog family members. Ihh undergoes proteolytic processing like Hh and Shh. Ihh transcript expression in the developing gut and in the growth zone of cartilage of developing long bones was confirmed by in situ RNA hybridization. In addition, Ihh transcript was detected as early as 14.5 dpc in the developing mouse kidney and its abundance increased with gestational age. Furthermore, the Ihh transcript localized to the proximal convoluted and proximal straight tubule in the adult kidney.

MATERIALS AND METHODS

Animals—CD-1 mice (Charles River Laboratories) were bred to obtain mouse embryos. Detection of a vaginal plug was used to define the first day of gestation. At the appropriate time point, pregnant females were sacrificed and kidneys or other organs were dissected from isolated embryos and rapidly frozen in liquid nitrogen.

Northern Analysis—A nylon membrane containing 2 μg/lane of poly(A)+ RNA extracted from multiple adult mouse tissues (Clontech, Palo Alto, CA) was hybridized in a buffer containing 50% formamide, 5× SSPE (0.75 M NaCl, 0.05 M NaH2PO4, 5 mM EDTA, pH 7.4), 10 × Denhardt’s, 2% SDS, and 100 μg/ml denatured salmon sperm DNA at 42 °C overnight with a 32P-labeled 1.1-kb polymerase chain reaction (PCR) amplified Ihh cDNA fragment (see below). The blot was washed with 0.1× SSC, 0.1% SDS at 55 °C for 40 min and was autoradiographed.

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In a separate experiment, 10 μg of total RNA extracted from 16.5 dpc mouse embryonic tissues was hybridized with the same random-labeled 32P-Ihh cDNA probe as described above. Ethidium bromide staining of the gel was used to assess the equivalence of RNA loaded in each lane.

**Molecular Cloning—**Using synthetic oligonucleotide primers complementary to partial cDNA sequence of mouse Indian hedgehog (GenBank™ accession number X76291), the PCR was used to amplify an Ihh cDNA fragment from a 17.5 dpc mouse kidney cDNA library. The amplification product was DNA sequenced to assure Taq DNA polymerase fidelity to the previously published partial Ihh cDNA sequence. The PCR-amplified 1.1-kb Ihh cDNA fragment was radiolabeled and used to screen a 10% filter approximately 500 rpm. The oligo(dT)-primed ZAP II mouse embryonic 17.5-day kidney cDNA library. Filters were hybridized and washed as described previously (26). Six positive clones were identified, purified to homogeneity, in vivo excised according to the protocol of the manufacturer (Stratagene), and restriction-mapped. The two longest clones were sequenced along both strands over their entire length. DNA sequencing was performed using the dideoxy nucleotide termination method. Sequences 77 DNA polymerase (U. S. Biochemical Corp.), its reagent kit, and synthetic oligonucleotides were employed according to the directions of the manufacturer. Gel compressions were resolved with 7-deaza-dGTP and/or formamide. DNA sequence was assembled and analyzed using Sequence Analysis Software Package of the Genetics Computer Group (version 8.1).

**Eukaryotic Expression Constructs—**The full-length Ihh cDNA (2103 bp) was excised from pBluescript (pBS-Ihh) and subcloned into a cytomegalovirus promoter-based eukaryotic expression vector pcDNA3 (Invitrogen, San Diego, CA) and designated pcDNA3-Ihh. Next, an expression plasmid Ihh cDNA plasmid was prepared that deleted the domain NH2-terminal to MetL30 and added a COOH-terminal His-epitope tag for the antisense primer. Site-directed mutagenesis was performed using the unique site elimination method (U.S. mutagenesis kit, BRL). A synthetic oligonucleotide, 5'-[CTCAGGCTCCGGGCTGTCTCTAT-3'] , that substitutes for the pcDNA3-Ihh Met site. Synthetic oligonucleotides used included 5'-[ACACAGGTTTACCCGGCCATGTCTCCC]-3' for the sense primer and 5'-[GTCTGAGTAAGACACTCTCTCTCGAGAACATATTTGGTCGCTCCTGCGGCCGACGTCCAGCTTA]-3' for the antisense primer. The amplified cDNA product was prepared and ligated directly into the eukaryotic TA cloning vector pcR 3.1-Uni (Invitrogen) according to the manufacturer's instructions. The subcloned DNA amplification product was bidirectionally sequenced.

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In Vitro Expression of Ihh cDNA—Using an in vitro transcription-translation reticulocyte lysate assay (TNT-Promega Corp., Madison, WI), 1.0 μg of pcDNA3 alone or the indicated Ihh expression constructs were transcribed in vitro with T7 RNA polymerase and translated in a reaction mix containing 12.5 μl of reticulocyte lysate and 20 μl of [35S]methionine (Amersham Corp., Arlington Heights, IL) and 5 μl of conditioned medium (Amersham). Reverse Transcription-PCR—Adult rat kidneys were harvested, collage-nase-treated, and the nephrons were microdissected as described previously (27). These nephrons were further sectioned by morphology into separate segments consisting of the distal convoluted tubule, outer medullary collecting duct, proximal straight tubule, inner medullary collecting duct, proximal convoluted tubule, thick ascending limb, macula densa segment, and glomerulus. Total RNA from these segments was isolated in a commercially prepared phenol:4% guanidine isothiocyanate reagent (TRI-Reagent, Molecular Research Center, Cincinnati, OH) and was reverse-transcribed using an oligo(dT) primer. Samples were incubated at 65 °C for 5 min, then ramped to 37 °C over 5 min. After 5 min at 37 °C, 100 units of Maloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.) was added to each reaction mix, except the reverse transcription negative control, and samples were incubated for an additional 55 min at 37 °C. The cDNA generated by reverse transcription was used as a template for the PCR as described (27).

**In Situ RNA Hybridization—**Metanephric kidney from timed pregnant CD-1 mice were obtained from 4-week intervals with 100 mCi of 3H]methylmethanesulfonate (Amersham), and 2 μl of each PCR reaction, except the reverse transcriptase negative control, and samples were serially diluted and used in PCR reactions. The cDNA reaction mix from reverse transcription minus reagents was also included in each PCR run; these samples were consistently negative. To assess product abundance, 12 μl of each PCR reaction was separated by electrophoresis in a 5%-acrylamide gel.

Metanephric kidney from timed pregnant CD-1 mice were obtained by dissection of embryos at 11.5, 14.5, and 17.5 dpc. Additionally, mouse kidney tissue was harvested from newborn and adult mice. Tissues were snap-frozen in liquid nitrogen in TRI-Reagent as described above. Isolated total RNA was reverse-transcribed and subjected to the PCR as described above. Oligonucleotides were selected to minimize sequence similarity with other hedgehog family members. In control experiments, unlabeled amplified product was sequenced to ensure sequence identity with Indian hedgehog.

In Situ RNA Hybridization—A BamHI digest of pBS-Ihh was performed to eliminate the 5'-untranslated region and the coding region of Ihh. Religation of this construct resulted in a 540-bp fragment from the 3'-untranslated region of Ihh within pBluescript and designated pBS-
Isolation and Molecular Cloning of Full-length Mouse Ihh cDNA—The complete Ihh coding region was assembled from a set of six overlapping cDNA clones isolated from an embryonic 17.5 dpc kidney cDNA library. The cDNA extended over 2103 bp and included a 183-bp 5′-untranslated region, a continuous open reading frame of 1347 bp, and a 3′-untranslated region of 573 bp. The initial 339 bp of the open reading frame as well as the 5′- and 3′-untranslated regions have not been reported previously. The 1008 bp overlapping the published partial cDNA sequence of Ihh contained a single base change resulting in an arginine to tryptophan substitution at amino acid 171 and a two-base substitution altering amino acid 421 from a serine to a tryptophan (2). Of note, both tryptophan residues are conserved in hh, Shh, and Desert hedgehog (2).

The Indian hedgehog primary peptide sequence is shown aligned with representative hedgehog family members in Fig. 1. The largest predicted Ihh open reading frame encodes a polypeptide sequence of 449 amino acids with a predicted relative molecular mass of 49 kDa. The Indian hedgehog cDNA contains an in frame stop codon followed by two AUGs near its 5′ end that are favorable for translation initiation as defined by Kozak (30). These AUGs begin at nucleotide 184 (Met1) and nucleotide 298 (Met39), respectively. If both putative translation initiation sites are utilized, the Ihh cDNA would encode a 49-kDa and a 45-kDa protein product. Immediately following Met39 is a 27-amino acid sequence which closely fits von Heijne's consensus for a signal peptide (31). Comparison with this consensus predicts that the signal peptide cleavage likely follows the (−3, −1) rule and occurs preceding Cys49. Cleavage of this signal peptide would yield a mature protein of 384 amino acids with a predicted relative molecular mass of 42 kDa. Primary sequence alignment reveals that mouse Ihh shows 89% identity and 96% similarity to mouse Shh over the NH2-terminal region, from the end of the signal peptide to the proteolytic processing site following Cys49.

Analysis of in Vitro Ihh Expression and Processing—Fig. 2A outlines the potential Ihh precursor proteins and their proteolytic cleavage products. To test whether both Met1 and Met39 could function as translation initiation sites, the 2103-bp full-length Ihh cDNA (pcDNA3-Ihh) was translated in a rabbit reticulocyte lysate. Two predominant protein products of 49 and 45 kDa were expressed (Fig. 2B, lane 3). Pancreatic microsomes were added to the reactions to study post-translational modifications such as signal peptide cleavage and core glycosylation. Expression of pcDNA3-Ihh in the presence of microsomes yielded protein products of 49, 45, and 42 kDa (Fig. 2B, lane 4) and additional smaller peptides (discussed below). To test whether the 45-kDa protein product initiated at Met39, the Ihh cDNA was deleted from its 5′ end to nucleotide 289, thereby eliminating Met1 but preserving the Kozak's consensus sequence surrounding Met39. In addition, this construct (plhh-Met39-Myc) encoded a Myc-epitope tag added immediately 5′ to the stop codon. When programmed into the reticulocyte lysate system, plhh-Met39-Myc yielded a 46-kDa protein (Fig. 2B, lane 7). In the presence of microsomes, a 43-kDa protein was also observed (Fig. 2B, lane 8). These proteins had slightly reduced electrophoretic mobility than predicted since the 10-amino acid Myc-epitope tag added 1 kDa to their relative molecular masses. When compared with the protein products observed with expression of the full-length Ihh cDNA, it was noted that the 49-kDa band was not observed. Taken together, these results suggested that both Met1 and Met39 function as translation initiation sites when expressed in vitro.

Sequence analysis showed that Ihh contains a conserved serine protease motif as well as a conserved proteolytic cleavage motif identified in other Hedgehog species, Gly-Cys-Phe (16, 18) (Fig. 1). In addition to the unprocessed Ihh proteins, pcDNA3-Ihh in vitro translation yielded protein products of 26, 23, and 22 kDa in the absence of microsomes (Fig. 2B, lane 3). This was consistent with the Ihh-N**; Ihh-C*, and Ihh-N* products predicted (Fig. 2A). When microsomes were added, some persistence of these products was seen. Additionally, a 25-kDa band and a less intense 19-kDa band were observed (Fig. 2B, lane 4). Upon addition of microsomes, core glycosylation of Ihh-C* should result in an increase in the molecular mass from 23 to 25 kDa; the appearance of the protein product designated Ihh-C (Fig. 2B, lane 4) was consistent with this prediction. Moreover, following addition of microsomes, signal peptide cleavage from Ihh-N** or Ihh-N* resulted in a protein of 19 kDa, predicted to be Ihh-N (Fig. 2B, lane 4). Expression of Ihh-Met39-Myc in vitro resulted in protein bands at 22, 24, and 46 kDa. These bands were consistent with Ihh-N*, Ihh-C*, and Ihh*, the size of the latter two products were increased slightly by the Myc-epitope tag. Also in the absence of microsomes, no protein was seen at 26 kDa which was consistent with the disappearance of Ihh-N**. In the presence of microsomes, the smaller products included the aforementioned 22- and 24-kDa proteins in addition to a band at 26 kDa and a less intense band at 19 kDa. These latter two protein bands were believed to represent Ihh-C and Ihh-N after post-translational modifications, respectively. Core glycosylation of Ihh-C* at Asn220 could explain a 2-kDa increase in molecular mass seen in Ihh-C. Likewise, signal peptide cleavage of Ihh-N* would explain the presence of a 19-kDa protein, Ihh-N.

Substitution of alanine for the conserved histidine residue (His313) within the serine protease motif of full-length Ihh (plhh-H313A) yielded protein products of 49 and 45 kDa (Fig. 2B, lane 5). The 42-kDa protein lacking its signal peptide was also observed with the addition of microsomes (Fig. 2B, lane 6). However, the more rapidly migrating peptide bands were not observed either in the presence or absence of microsomes. Therefore, the 26-, 23-, and 22-kDa proteins required His313 within the serine protease motif for their generation. Like Shh and Ihh, this suggests that Ihh undergoes internal proteolytic processing dependent upon the presence of an autoproteolytic serine protease domain (16, 17).

Generation of Ihh-N Polyclonal Antiserum—To better identify the Ihh protein fragments and characterize Ihh processing in mammalian cells, a rabbit polyclonal antisera was raised to a bacterially expressed NH2-terminal Ihh fusion protein. Ihh-N polyclonal antisera recognized glutathione S-transfer-
ase (GST only) and GST-Ihh-N-terminal peptide fusion protein (amino acids 83–140, GST-Ihh-N) by immunoblotting (Fig. 3). Preadsorption of the Ihh-N antiserum with a molar excess of GST essentially eliminated the detection of GST but failed to block the detection of GST-Ihh-N, demonstrating that this Ihh-N antiserum specifically recognized the Ihh-N peptide.

Expression of Ihh in Mammalian Cells—COS-7 cells were transiently transfected with the full-length Ihh construct (pcDNA3-Ihh), pIhh-Met39-Myc, pIhh-H313A, and vector controls, to determine if Ihh was processed in mammalian cells in vivo. Processing and Renal Expression of Mouse Ihh

**FIG. 1. Translated amino acid sequence of the full-length mouse Indian hedgehog cDNA (mIhh) aligned with the amino acid sequences of other hedgehog family members.** Underlined indicates the translation of the previously unpublished mouse Ihh cDNA sequence. Shown in *italics* is the putative signal peptide. *Bold* indicates: 1) the universally conserved Gly-Cys cleavage site, 2) the universally conserved His residue within the serine protease motif, and 3) the putative N-linked glycosylation site, Asn. *mShh*, *mDhh*, *Hh*, and *chIhh* represent mouse Sonic hedgehog, mouse Desert hedgehog, *Drosophila* hedgehog, and chick Indian hedgehog, respectively.
A reticulocyte lysate system containing \[35S\]methionine was programmed for translation. A transcription and translation system was used to study the effects on Ihh. The Ihh-C fragment represents an Ihh-C* fragment that has undergone glycosylation and should be 19 kDa in size. The predicted Ihh-C fragment (209 amino acids) of 23 kDa and an Ihh-N** peptide (240 amino acids) with a predicted molecular mass of 49 kDa (unprocessed 449-amino acid Ihh protein has a predicted relative molecular mass of 59 kDa) were used in this study.

A schematic diagram of proposed Ihh processing is shown as proposed from amino acid sequence. The unprocessed 449-amino acid Ihh protein has a predicted relative molecular mass of 49 kDa (Ihh**). Translation initiation beginning at an internal AUG, A***, should yield a 411-amino acid Ihh protein of 45 kDa (Ihh*). Signal peptide cleavage from either Ihh** or Ihh* results in a 42-kDa product (Ihh). Predicted internal cleavage at the conserved proteolytic cleavage site (Gly**-Cys*** of Ihh** should yield an Ihh-C* fragment (209 amino acids) of 23 kDa and an Ihh-N** peptide (240 amino acids) with a predicted molecular mass of 28 kDa. Cleavage of Ihh* at this same proteolytic cleavage site should produce the same Ihh-C* fragment with a smaller 22-kDa NH2-terminal fragment, Ihh-N*. The Ihh-N fragment represents Ihh-N** or Ihh-N* devoid of its signal peptide and should be 19 kDa in size. The predicted Ihh-C fragment represents an Ihh-C* fragment that has undergone glycosylation at Asn** and increased its apparent molecular mass to 28 kDa. Ab-N indicates the 58-amino acid epitope to which Ihh-N polyclonal antiserum is directed. 

B, Ihh processing in an in vitro transcription/translation reticulocyte lysate system. A transcription and translation reticulocyte lysate system containing \[^{35}S\]methionine was programmed with pcDNA3, pcDNA3-Ihh, plhh-Met39-Myc, or plhh-H313A. As indicated, lysates were programmed with or without Ihh transcript in the presence (lanes 2, 4, 6, and 8) or absence (lanes 1, 3, 5, and 7) of pancreatic microsomes. Products were resolved on a 12.5% SDS-PAGE under reducing conditions. Relative molecular mass standards are shown at left of each gel.

**Fig. 2.** **Proteolytic processing of Ihh.** A, schematic diagram of proposed Ihh processing as predicted from amino acid sequence. The unprocessed 449-amino acid Ihh protein has a predicted relative molecular mass of 49 kDa (Ihh**). Translation initiation beginning at an internal AUG, A***, should yield a 411-amino acid Ihh protein of 45 kDa (Ihh*). Signal peptide cleavage from either Ihh** or Ihh* results in a 42-kDa product (Ihh). Predicted internal cleavage at the conserved proteolytic cleavage site (Gly**-Cys*** of Ihh** should yield an Ihh-C* fragment (209 amino acids) of 23 kDa and an Ihh-N** peptide (240 amino acids) with a predicted molecular mass of 28 kDa. Cleavage of Ihh* at this same proteolytic cleavage site should produce the same Ihh-C* fragment with a smaller 22-kDa NH2-terminal fragment, Ihh-N*. The Ihh-N fragment represents Ihh-N** or Ihh-N* devoid of its signal peptide and should be 19 kDa in size. The predicted Ihh-C fragment represents an Ihh-C* fragment that has undergone glycosylation at Asn** and increased its apparent molecular mass to 28 kDa. Ab-N indicates the 58-amino acid epitope to which Ihh-N polyclonal antiserum is directed. B, Ihh processing in an in vitro transcription/translation reticulocyte lysate system. A transcription and translation reticulocyte lysate system containing \[^{35}S\]methionine was programmed with pcDNA3, pcDNA3-Ihh, plhh-Met39-Myc, or plhh-H313A. As indicated, lysates were programmed with or without Ihh transcript in the presence (lanes 2, 4, 6, and 8) or absence (lanes 1, 3, 5, and 7) of pancreatic microsomes. Products were resolved on a 12.5% SDS-PAGE under reducing conditions. Relative molecular mass standards are shown at left of each gel.

**Fig. 3.** **Production and initial characterization of anti-NH2-terminal Ihh polyclonal antiserum.** A bacterially expressed NH2-terminal Ihh-fusion protein was produced, purified, and used to immunize rabbits. Glutathione S-transferase (GST) only or a GST-Ihh-N-terminal peptide fusion protein amino acids 83–140, GST-Ihh-N, were separated by SDS-PAGE under reducing conditions, transferred to nitrocellulose, and immunoblotted with either preimmune serum, antiserum raised to GST-Ihh-N (Ihh-N), or anti-Ihh-N preadsorbed with a molar excess of GST. Relative molecular mass standards are indicated.

A manner similar to that observed in the in vitro transcription/translation system. Following transfection with pcDNA3-Ihh, proteins of 42 and 19 kDa were detected in the cell layer by immunoblotting with the Ihh-N antiserum. The 42-kDa band corresponded to the intact Ihh lacking the signal peptide, similar to the in vitro translated product following addition of microsomes (Fig. 2B, lanes 4, 6, and 8). The 19-kDa protein corresponded to the Ihh-N after proteolytic cleavage, as observed in vitro (Fig. 2B, lanes 4 and 8). The 19-kDa NH2-terminal peptide was found associated only with the cell layer and was not detected in the conditioned medium. Consistent with observations made in the in vitro transcription/translation system, expression of plhh-H313A in COS-7 cells produced only a 42-kDa protein as detected by immunoblotting (Fig. 4B, lane 4). No 19-kDa protein was detected, consistent with failure of proteolysis of Ihh into its NH2- and COOH-terminal peptides.

COS-7 cells transiently transfected with plhh-Met39-Myc expressed Ihh products with electrophoretic mobility similar to those expressed by pcDNA3-Ihh transfected cells. When an aliquot of the cell layer of plhh-Met39-Myc-transfected cells was resolved by SDS-PAGE and immunoblotted with Ihh-N antiserum, 43- and 19-kDa peptides were detected (Fig. 4B, lane 3). The 43-kDa protein had a slightly reduced electrophoretic mobility relative to that derived from pcDNA3-Ihh transfected cells due to the presence of its Myc epitope tag. The Ihh-N protein (19 kDa) had identical mobility relative to that derived from pcDNA3-Ihh transfected cells. When an aliquot of the cell layer of pIhh-Met39-Myc-transfected cells was resolved by SDS-PAGE and immunoblotted with Ihh-N antiserum, 43- and 19-kDa peptides were detected (Fig. 4B, lane 3). The 43-kDa protein had a slightly reduced electrophoretic mobility relative to that derived from pcDNA3-Ihh transfected cells due to the presence of its Myc epitope tag. The Ihh-N protein (19 kDa) had identical mobility relative to that derived from pcDNA3-Ihh transfected cells.
script expression in the metanephric kidney at 16.5 dpc, its cDNA was cloned from a 17.5 dpc kidney library, and its transcript was highly expressed on Northern blots in the adult kidney. To better investigate the renal expression of Ihh, semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) was utilized to establish an embryonic time course of Ihh transcript expression in the metanephros. Total RNA was extracted from dissected mouse metanephric kidney at 11.5, 14.5, and 17.5 dpc in addition to newborn and adult kidney. Indian hedgehog transcript was first detected by amplification from the mouse metanephros at 14.5 dpc. Indian hedgehog transcript abundance increased with gestational age and was maximal in adulthood (Fig. 7).

**Ihh Transcript Localizes to the Proximal Tubular Structures of the Adult Kidney**—The RT-PCR was used to localize the Ihh transcript to a specific nephron segment in microdissected adult kidney. Nephrons from collagenase treated adult rat kidneys were microdissected by morphology into separate segments. Total RNA was isolated from these segments and subjected to RT-PCR. As seen in Fig. 8, Ihh transcript was detected only in the proximal convoluted tubule and proximal straight tubule and not in other portions of the adult nephron.

**Liver in the 16.5 dpc Mouse Embryo**—To better localize the Ihh transcript during embryonic mouse development, *in situ* RNA hybridization was performed using 35S-labeled riboprobes transcribed from an Ihh cDNA template. Serial section of 10, 12, 14.5, and 16.5 dpc mouse embryos were hybridized with Ihh sense or antisense probes. The control 35S-labeled Ihh sense riboprobe produced no detectable signal. However, the anti-sense Ihh riboprobe could detect expression in the gut at all embryonic time points investigated (Fig. 5, light and dark fields, top left). Higher power examination of the light field demonstrated localization in the luminal portion of the gut (Fig. 5, top right). Ihh transcript was also detected in the cartilage primordium of the cervical and tail vertebrae at 14.5 dpc (Fig. 5, top left). Closer inspection of the cartilaginous structures of the tail and cervical vertebrae suggested that Ihh is expressed in chondrocytes of these developing bones (Fig. 5, bottom left and right). Less prominent Ihh transcript expression existed in the region of the developing urogenital sinus at 14.5 dpc (Fig. 5, top left). It should be noted that no expression of Ihh was detected in the developing mouse kidney at any embryonic time point, at least at the level of sensitivity of *in situ* hybridization.

Northern blot of total RNA extracted from multiple 16.5 dpc embryonic mouse organs was probed with a 1.1-kb Ihh cDNA probe. Ihh transcript expression was detected in the developing stomach and gut with lower levels of expression in the kidney and liver (Fig. 6).

**Ihh Transcript in Kidney Increases with Developmental Maturation**—Although Ihh transcript was undetectable in the embryonic kidney by *in situ* hybridization, data presented above clearly indicated its renal expression at various time points of renal development. Northern analysis demonstrated Ihh transcript expression in the metanephric kidney at 16.5 dpc, its cDNA was cloned from a 17.5 dpc kidney library, and its transcript was highly expressed on Northern blots in the adult kidney. To better investigate the renal expression of Ihh, semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) was utilized to establish an embryonic time course of Ihh transcript expression in the metanephros. Total RNA was extracted from dissected mouse metanephric kidney at 11.5, 14.5, and 17.5 dpc in addition to newborn and adult kidney. Indian hedgehog transcript was first detected by amplification from the mouse metanephros at 14.5 dpc. Indian hedgehog transcript abundance increased with gestational age and was maximal in adulthood (Fig. 7).

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that Hedgehog may undergo an additional cleavage step at its COOH-terminal end when expressed in vivo but not in vitro. The increased molecular weight of the Myc-tagged Ihh translated in vitro would be consistent with absence of further proteolytic processing of the COOH terminus in this system. Terminal cleavage of Ihh-C in vivo could explain our inability to detect this fragment with the anti-Myc antibody in transfected mammalian cells.

In this study in situ RNA hybridization detected Ihh transcript in the developing gut epithelium, cartilage, and urogenital sinus in good agreement with previous reports (22). Ihh is expressed in the columnar epithelial cells lining the length of the intestine, including the rectum. In the duodenum at 14.5 dpc, Ihh is expressed in the more differentiated epithelial cells of the villi, whereas Sonic hedgehog is expressed in the more
undifferentiated cells remaining in the crypts (22). Abundant Ihh mRNA has also been detected in cartilage as early as 11.5 dpc. This expression is highest in chondrocytes in the growth zone regions of the cartilage with a lower level of expression in the hypertrophic zone (22). In developing chick bone, Ihh is produced by cells making a transition to hypertrophic or differentiated chondrocytes and appears to signal to neighboring perichondrial fibroblasts (24). Via a negative feedback loop that involves the induction of PTHrP in the perichondrium, Ihh secretion results in the inhibition of premature differentiation by chondroblasts in the bone growth plate (24, 25).

In the kidney, Northern blotting confirmed Ihh transcript expression at 16.5 dpc and semiquantitative RT-PCR could detect Ihh as early as 14.5 dpc. Yet, Ihh transcript could not be localized in the developing kidney by in situ hybridization. RT-PCR allowed the localization of Ihh transcript to a specific terminally differentiated tubular epithelia in the adult kidney. Given that other hedgehog species function in early inductive events, it was surprising to find that the abundance of metanephric Ihh transcript increased with gestational age. This temporal pattern of Ihh expression suggested that Ihh is expressed in more differentiated metanephric cell phenotypes. This expression pattern is similar to Ihh expression in the duodenum, where Ihh transcript is found in more differentiated epithelium of the villi (22). That Ihh expression in differentiated tubular epithelia can signal adjacent, proliferating cells and maintain their undifferentiated state is a testable hypothesis that will require further investigation.

REFERENCES
1. Lee, J. J., von Kessler, D. P., Parks, S., and Beachy, P. A. (1992) Cell 71, 33–50
2. Echelard, Y., Epstein, D. J., St-Jacques, B., Shen, L., Mehler, J., McMahon, J. A., and McMahon, A. P. (1993) Cell 75, 1417–1430
3. Ingham, P. W. (1993) Nature 366, 560–562
4. Heberlein, U., Wolff, T., and Ruhn, G. M. (1993) Cell 75, 913–926
5. Ma, C., Zhou, Y., Beachy, P. A., and Moses, K. (1993) Cell 75, 927–938
6. Riddle, R. D., Johnson, R. L., Laufer, E., and Tabin, C. (1993) Cell 75, 1401–1416
7. Kraus, S., Concordet, J.-P., and Ingham, P. W. (1993) Cell 75, 1431–1444
8. Basler, K., and Struhl, G. (1994) Nature 368, 208–214
9. Tabata, T., and Kornberg, T. B. (1994) Cell 76, 89–102
10. Heemskerk, J., and D’Nardo, S. (1994) Cell 76, 449–460
11. Laufer, E., Nelson, C. E., Johnson, R. L., Morgan, B. A., and Tabin, C. (1994) Cell 79, 993–1003
12. Fan, C.-M., and Tessier-Lavigne, M. (1994) Cell 79, 1175–1186
13. Johnson, R. L., Laufer, E., Riddle, R. D., and Tabin, C. (1994) Cell 79, 1165–1173
14. Roelink, H., Porter, J. A., Chiang, C., Tanabe, Y., Chang, D. T., Beachy, P. A., and Jessell, T. M. (1995) Cell 81, 445–455
15. Fan, C.-M., Porter, J. A., Chiang, C., Chang, D. T., Beachy, P. A., and Tessier-Lavigne, M. (1995) Cell 81, 457–465
16. Lee, J. J., Ekker, S. C., von Kessler, D. P., Porter, J. A., Sun, B. I., and Beachy, P. A. (1994) Science 266, 1528–1537
17. Bumerot, D. A., Takada, R., and McMahon, A. P. (1995) Mol. Cell. Biol. 15, 2294–2303
18. Porter, J. A., von Kessler, D. P., Ekker, S. C., Young, K. E., Lee, J. J., Moses, K., and Beachy, P. A. (1995) Nature 374, 363–366
19. Marti, E., Bumerot, D. A., Takada, R., and McMahon, A. P. (1995) Nature 375, 322–325
20. Porter, J. A., Ekker, S. C., Park, W. J., von Kessler, D. P., Young, K. E., Chen, C. H., Ma, Y., Woods, A. S., Cotter, R. J., Koonin, E. V., and Beachy, P. A. (1996) Cell 86, 21–34
21. Marti, E., Roberts, D. J., Lee, S. M. K., Tsukurukov, O., Levi, T., Gastier, J. M., Epstein, D. J., Gilbert, D. J., Copeland, N. G., Seidman, C. E., Jenkins, N. A., Seidman, J. G., McMahon, A. P., and Tabin, C. (1996) Genomics 28, 1–11
22. Bitgood, M. J., and McMahon, A. P. (1995) Dev. Biol. 172, 126–138
23. Roberts, D. J., Johnson, R. L., Burke, A. C., Nelson, C. E., Morgan, B. A., and Tabin, C. (1996) Development (Camb.) 121, 3163–3174
24. Vortkamp, A., Lee, K., Lacalle, B., Segre, G. V., Kronenberg, H. M., and Tabin, C. J. (1996) Science 273, 613–622
25. Lanskje, B., Karaplis, A. C., Lee, K., Luz, A., Vortkamp, A., Pirro, A., Rasmussen, M., Defize, L. H. K., Ho, C., Mulligan, R. C., Abou-Samra, A. B., Jopphner, H., Segre, G. V., and Kronenberg, H. M. (1996) Science 273, 663–666
26. Holzman, L. B., Merritt, S. E., and Fan, G. (1994) J. Biol. Chem. 269, 30808–30817
27. Yang, T., Hassan, S. A., Singh, I., Smart, A., Brosius, F. C., Holzman, L. B., Schnermann, J. B., and Briggs, J. P. (1996) Hypertension 27, 541–551
28. Kretzler, M., Fan, G., Rose, D., Arend, L. J., Briggs, J. P., and Holzman, L. B. (1996) Am. J. Physiol. 271, F770–F777
29. Dressler, G. R., Deutsch, U., Chowdhury, K., Nornes, H. O., and Gruss, P. (1990) Development (Camb.) 108, 787–795
30. Kuak, M. (1987) Mol. Cell. Biol. 106, 947–50
31. von Heijne, G. (1985) J. Mol. Biol. 184, 99–105