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Xenopus sonic hedgehog as a potential morphogen during embryogenesis and thyroid hormone-dependent metamorphosis

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ABSTRACT

The hedgehog family of proteins have been implicated as important signaling molecules in establishing cell positional information and tissue patterning. Here we present the cloning and characterization of a hedgehog homologue from *Xenopus laevis* similar to the sonic class of vertebrate hedgehog genes. We isolated *Xenopus* hedgehog (Xhh) from a subtractive hybridization screen designed to identify genes induced by thyroid hormone during metamorphosis of the *X.laevis* gastrointestinal tract. In the intestine, Xhh mRNA expression was up-regulated at the climax of metamorphosis (stage 62) when intestinal epithelium underwent morphogenesis. Treatment of pre-metamorphic tadpoles with exogenous thyroid hormone (TH) resulted in a similar pattern of Xhh induction. Furthermore, TH induction was resistant to inhibitors of protein synthesis suggesting that Xhh is a direct thyroid hormone response gene. The expression and TH regulation of Xhh was not limited to the intestine, but was also observed in the limb and a mixture of pancreas and stomach. Throughout development, Xhh mRNA was present at varying levels with the earliest expression being detected at neurula stage. The highest levels of Xhh were observed between stages 33 and 40 shortly before tadpole feeding begins. Whole mount *in situ* hybridization analysis of Xhh expression in pre-hatching, stage 32 tadpoles demonstrated staining in the notochord and floor plate similar to that observed for other vertebrate hedgehog genes. Together, these data suggest a putative role for Xhh in organ development during both amphibian embryogenesis and metamorphosis.

INTRODUCTION

Amphibian metamorphosis is a complex process that results in the degeneration of tadpole tissues by cell death and the development of frog organs through extensive cell proliferation, migration and differentiation. During this transformation, many organs/tissues undergo systematic but very different changes, such as the early development of the hind limb and late resorption of the tail (1,2). However, the entire metamorphic process is directly initiated by a single molecule, thyroid hormone (TH). TH is presumed to bind to its nuclear receptors to induce a molecular cascade of events responsible for this transition. These thyroid hormone receptors (TR) have been shown to be transcription factors suggesting that TH controls metamorphosis by regulating gene expression (3–7). Many of these target genes have been isolated but the identities and functions of these genes during metamorphosis remain largely unknown (8).

To study the remodeling of an organ/tissue at the molecular level, we have chosen the *Xenopus laevis* gastrointestinal tract as a model system. The larval (or tadpole) intestine is a long tube-like structure with one longitudinal fold called the typhlosole (Fig. 1). The intestine contains a single layer of mitotically active but differentiated primary epithelial cells with very little surrounding connective tissue or muscle (9–12). During metamorphosis, the primary epithelium undergoes apoptosis. At the same time, the secondary (adult) epithelial cells, whose origin remains to be determined, proliferate and differentiate to form the adult epithelium. In addition, there is a dramatic increase in the connective tissue as well as the longitudinal and circular muscle layers. These complex morphogenic changes eventually produce the frog intestine with a multiple-folded epithelium. The proliferating cells are located at the troughs of the folds and mitotically inactive but differentiated epithelial cells at the crests of the folds, an organization remarkably similar to that in other adult vertebrate intestines.

This metamorphic process, like other organs and tissues, is under the control of TH (1). Such regulation offers unique advantages to studying the mechanism responsible for the degeneration of tadpole epithelium and the morphogenesis of the adult organ. First of all, it has been shown that isolated tadpole intestine can be induced in organ culture to undergo remodeling in response to TH similar to that observed in intact tadpoles (13,14). In addition, by isolating genes which are regulated by TH in the intestine, we have previously identified >20 distinct genes which may participate in intestinal remodeling (15). This group consists of a variety of genes, such as those encoding the matrix metalloproteinase stromelysin-3 and transcription factors (8,15).

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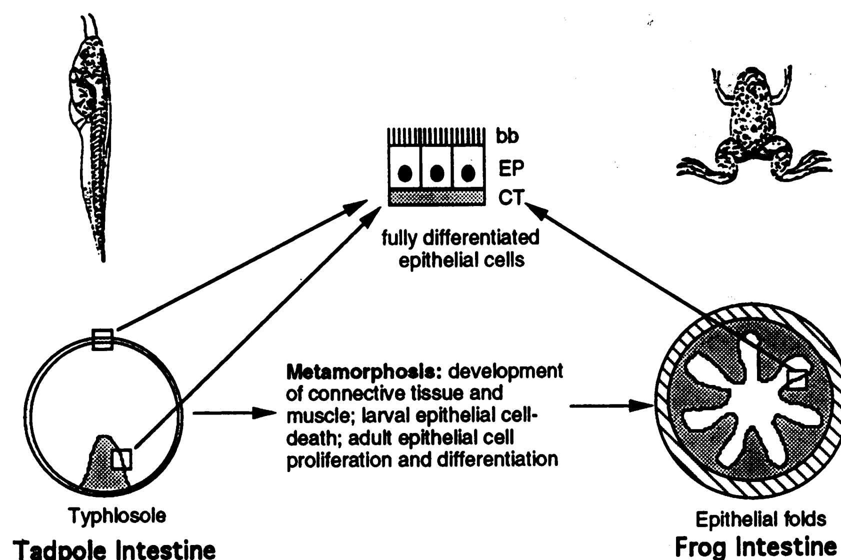

Figure 1. Schematic of intestinal changes in *X. laevis* during metamorphosis. The larval (tadpole) intestine is comprised of a single layer of fully differentiated epithelial cells (55,56). There is a single fold in the intestine called the typhlosole. During metamorphosis extensive remodeling occurs to create the adult frog intestine (9,10). The first stage of this process is the death of larval epithelial cells followed by adult epithelial cell proliferation and differentiation. In addition, there is an increase in the connective and muscle tissue. By the end of metamorphosis, the adult frog intestine consists of multiple epithelial folds, highly resembling the intestines of other vertebrates. bb, brush border of EP. EP, epithelial cells; CT, connective tissue.

Here, we report the further characterization of one of these up-regulated genes, IU27 (15). We have identified IU27 as the *Xenopus* homologue of the vertebrate sonic hedgehog genes and thus have renamed it as *Xenopus* hedgehog (Xhh). The hedgehog family of genes has been isolated from a variety of organisms including mouse, rat, zebrafish, chicken and the fruit fly, *Drosophila melanogaster* (16–24). Recent studies indicate that both the *Drosophila* and vertebrate hedgehog genes act as signaling molecules to provide cells with positional information during development (25). In *Drosophila*, hedgehog was originally identified as a segment polarity gene responsible for marking the boundaries of developing parasegments in the fly embryo (26–29). In addition to its role as a segment polarity gene, hedgehog is implicated in patterning of the wing, leg and eye of the adult fly (30–33). Experiments with the vertebrate homologues of hedgehog suggest its involvement in limb formation, patterning of the central nervous system and ventral somite differentiation (16–20,34–37).

This paper describes the cloning of the full-length *Xenopus* hedgehog and its regulation by TH in metamorphosing tadpoles. In the intestine, the expression of Xhh mRNA peaked at the climax of metamorphosis and then sharply declined. Up-regulation of Xhh was also observed in the intestine when premetamorphic tadpoles were precociously induced to metamorphose by simply adding TH to their rearing water. In addition, Northern blot and whole mount *in situ* hybridization suggested that Xhh is also involved during embryogenesis, especially neural induction.

MATERIALS AND METHODS

Isolation and characterization of Xhh cDNA clones

The small IU27 cDNA fragment isolated from the subtractive hybridization libraries was used as a probe to screen for full length IU27 cDNA clones from libraries made from intestinal mRNA (15). Several clones were isolated and the largest, 2462 bp in length, was chosen for sequence analysis using the Sequenase v.2.0 kit (US Biochemicals) and determined to be the *Xenopus* sonic hedgehog gene. Compressions were resolved by using 7-deaza guanosine (US Biochemicals). The GenBank accession number for the *Xenopus* hedgehog sequence reported in this paper is L39213.

In vitro translation and DNA constructs

In vitro translations were performed with the use of the TNT coupled transcription/translation system (Promega). [^{35}S]methionine (Amersham) and Autofluor (National Diagnostics) was used for detection by autoradiography. DNA plasmids used for *in vitro* translation were constructed using standard cloning techniques (38). The plasmid, XHH, was constructed by inserting the coding region of the *Xenopus* hedgehog cDNA into pSP64polyA (Promega). The coding region was amplified by PCR using oligos to the 5′ and 3′ ends of the sequence (5′ oligo-1 GATCAAGCTTATGCTGGTTGCGACTCAATC; 3′ oligo-1 GATCAAGCTTTCAACTGGATTTCGTTGCCA). Both oligos, 5′ oligo-1 and 3′ oligo-1 contain *Hin*dIII restriction enzyme sites at their 5′ ends. The PCR product was digested with *Hin*dIII and subcloned into the *Hin*dIII site of pSP64polyA.

The plasmid, T7-XHH, was constructed by inserting the coding region of the *Xenopus* hedgehog cDNA into pET28a (Novagen). The coding region was amplified by PCR using oligos to the 5′ and 3′ ends of the sequence (5′ oligo-2, GATCGAATTCATGCTGGTTGC GACTCAATC, 3′ oligo-1, GATCAAGCTTTCAACTGGATTTCGTTGCCA). Both oligos contain restriction enzyme sites at their 5′ ends; 5′ oligo-2, *Eco*RI and 3′ oligo-1, *Hin*dIII. The PCR product was digested with *Eco*RI and *Hin*dIII and subcloned into the *Eco*RI and *Hin*dIII sites of pET28a. This

insertion created an N-terminal fusion protein containing a histidine tag and a T7 gene 10 tag followed by the coding region of Xhh. Both XHH and T7-XHH were fully sequenced to ensure that no mutations had occurred due to PCR amplification.

Treatment of *X.laevis* tadpoles and RNA isolation

About 20–40 tadpoles at stage 52–56 (39) were treated in 1 l of dechlorinated tap water with 5 nM 3,5,3′-L-triiodothyronine (T3) unless otherwise indicated. The solution was changed daily. Whenever a treatment lasted >2 days, the tadpoles were fed continuously. The protein synthesis inhibitors cyclohexamide and anisomycin (CHX) were added at 20 and 25 μg/ml, respectively, for 13 h beginning 1 h before the addition of TH. This treatment inhibits protein synthesis in tadpole tissues by 99% (40). RNA was isolated from different tissues using the guanidinium thiocyanate method (41). The entire tadpole intestinal tract except the stomach region was isolated for analysis.

RNA and DNA blot analyses

Total RNA (2.5–10 μg) was electrophoresed through a 1% agarose–formaldehyde gel for RNA blots (38,42). After electrophoresis, the RNA was partially hydrolyzed using NaOH, transferred to a Genescreen nylon membrane (DuPont) and fixed onto the membrane by UV crosslinking. The membrane was stained with methylene blue to check the quantity and quality of the RNA (43). To further measure the quantity of RNA, blots were hybridized with the cDNA probe rpL8, whose expression is not changed by TH treatment and/or protein synthesis inhibitors and its mRNA is constant (within 3-fold) throughout development in different tissues (44).

To determine the tissue specific gene regulation by TH, Southern blots were performed using PCR amplified +cDNA (TH treated) and –cDNA (control) fragments prepared from limb, intestine, tail and brain (gifts of Dr D. D. Brown, Carnegie Institution of Washington; also see 15,45,46).

Both RNA and DNA blots were hybridized overnight in hybridization buffer (10% dextran sulfate, 50% formamide, 5× Denhardt's solution, 5× SSPE, 0.1% SDS and 100 mg/ml denatured salmon sperm DNA) with DNA fragments labeled with [^{32}P]dCTP using a random priming method (Amersham Corp.). After two washes at room temperature with 2× SSC and 0.2% SDS, the filters were washed twice with 0.25× SSC and 0.2% SDS at 65°C for 25 min each.

Whole mount *in situ* hybridization analysis

In situ hybridization analysis was performed following published methods (47,48). Antisense RNA probes were prepared using the Boehringer Mannheim RNA labeling kit with digoxigenin-UTP. A clone containing the entire Xhh cDNA was used as the template for transcription.

Embryo sections

Embryos that gave a positive signal with whole-mount *in situ* hybridization were incubated 2 h in 100% methanol, 2 h in xylene, 1 h in 1:1 xylene–paraffin (Paraplast-Plus) and finally, 6 h in paraplast. Incubations in paraffin were performed at 60°C. The paraffin was solidified by transferring it to room temperature, and 30 μm sections were cut and dried down on poly-lysine-coated slides. Paraffin was dissolved in xylene, and xylene was washed out in 100% ethyl alcohol. Sections were viewed and photographed under a coverslip in 100% alcohol.

RESULTS

Isolation and characterization of *Xenopus* hedgehog cDNA

In an attempt to study the earliest changes in gene expression in tadpole intestine during metamorphosis, we previously employed a PCR-based subtractive hybridization method to isolate genes that were regulated by thyroid hormone (TH) (15). This method utilized poly(A)$^+$ RNA purified from total intestinal RNA of control (–) tadpoles or tadpoles treated with TH for only 18 h (+). Such a short treatment ensured that the genes isolated would likely act in the earliest phase of the complex cascade of events involved in metamorphosis.

In this paper, we focus on one of the up-regulated genes, IU27. We obtained a full length cDNA clone of IU27 by screening a λ cDNA library made with intestine mRNA from TH-treated tadpoles. We used the short PCR cDNA fragment isolated from the subtraction as a probe. This clone was 2.5 kb in length with the longest open reading frame being 1335 bp (not shown; see GenBank sequence # L39213). The IU27 cDNA contained 200 bp of 5′ and 1127 bp of 3′ UTR sequences. In addition, an alternatively polyadenylated cDNA was isolated which is likely to be one of the smaller transcripts detected by Northern blot hybridization (data not shown and see below).

A sequence homology search revealed that the full length IU27 cDNA clone shared striking similarity to genes of the hedgehog family from *Drosophila* to human (17–20) (Fig. 2 and data not shown). Thus, we have renamed IU27 as *Xenopus* hedgehog (Xhh). Among the different hedgehog genes, Xhh is most homologous to the sonic class of vertebrate hedgehog genes, sharing 68–73% identity at the amino acid sequence level (Fig. 2). In contrast, Xhh is only 60 and 54% identical to the Indian and Desert hedgehog protein, respectively (17). Similarly, compared with *D.melanogaster* hedgehog, Xhh protein is only 46% identical (21,22). Thus, it is concluded that Xhh is the *Xenopus* homolog of vertebrate sonic hedgehog.

In general, the hedgehog family of proteins do not contain any distinguishing features other than a putative hydrophobic signal peptide at the N-terminus. However, in Xhh, there is a unique triple repeat of an eight amino acid sequence (QVDLQSHH) in the C-terminus of the protein (Fig. 2). This triplet repeat is not present in any other hedgehog gene in the database nor could it be detected in any other gene sequences in data banks. The functional significance, if any, of this repeat is unknown at this time.

The predicted full length Xhh protein is 445 amino acids long. The N-terminus of the protein contains a short hydrophobic stretch of amino acids, suggesting the presence of a putative signal peptide. The full length protein is predicted to be 49 kDa with an isoelectric point of 7.46. *In vitro* translation of full length XHH produced a protein which migrated at 45 kDa by SDS–PAGE (Fig. 3). In addition, one smaller peptide was also detected migrating at 30 kDa. No polypeptides were detected by *in vitro* translation of reticulocyte lysate alone (–) (Fig. 3). *In vitro* translation of T7-XHH, an N-terminal tagged fusion protein (see Materials and Methods), produced the full length fusion protein, 53 kDa, as well as a smaller 30 kDa polypeptide (Fig. 3).

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        1                          ↓                        50
Xhh     ..MLVATQSL LLLSFICTLV TPPGLACGPG RGIGKRRH.. PKKLTPLAYK
M-Shh   ..M LLLARC F VILASS L VC           F     ..
C-Shh   MVEMLLLTRI   VG   A L VSS  T             ..
M-Ihh   .......... .......... .......... .......... ..........
M-Dhh   ....MALPAS   PLCCLA L ALSAQS       PVG  RYV R Q V  L

        51                                                 100
Xhh     QFIPNVAEKT LGASGRYEGK ITRNSDCFKE LTPNYNPDIM FKDEESTGAD
M-Shh                              ER             I      N
C-Shh                              ER             I      N
M-Ihh   .......... .......... .....ER             I      N
M-Dhh     V SMP R       PA  R V  G ER RD  V       I      NS

        101                                                150
Xhh     RLMTQRCKDK LNALAISVMN QWPGVKLRVT EGWDEDGHHL EESLHYEGRA
M-Shh                              R              S
C-Shh                              R
M-Ihh            R   S                     R      S
M-Dhh       E   E  V     A    M    R              A QD

        151                                                200
Xhh     VDITTSDRDR SKYGMLGRLA VEAGFDWVYY ESKAHIHCSV KAENSVAAKS
M-Shh                    A
C-Shh                    A
M-Ihh              N   L A                    V      S H A   T
M-Dhh   L          N   L A                 RN   V     D  L VRA

        201                                                250
Xhh     GGCFPAGARV MVEFGGTKAV KDLRPGDRVL SSDPQGNLLY SDFLMFIDQE
M-Shh         S T  HL Q    L             AA D  R         T L RD
C-Shh        GS T  HL H    L     S       AA AD R         T L RM
M-Ihh           Q  RL N ERV L SAVK       AMGED TPTF   V I L R
M-Dhh        GN T  RLRS ER GL RE HR   W   AA AA RVVP TPV L L RD

        251                                                300
Xhh     RDVKKLFYVI ETSQRKIR.. LTAAHLLFVA ....QTKVNG TRSFKSVFAS
M-Shh   EGA  V       LE RE LL            P.HNDSGPTP GP ..AL
C-Shh   DSSR         R PRA LL            PQHN SEAT  ST GQAL
M-Ihh   PNRLRA Q     QDPPR LA   P     I  DNHTEPAAH. ... RAT
M-Dhh   LQRRASFVAV ETERPPRKLL LTPWHLVFAA ....RGPAPA PGDFAPVFAR

        301                                                350
Xhh     NIQPGDLIYT ADPK..TMTL KAVKVEKVDL .EEDTGAYAP LTAHGTVVID
M-Shh   RVR  QRV V VAERGGDRR  LPAA HS T  R  EA            IL N
C-Shh    VK  QRV V LGE..GGQQ  LPAS HS S  R  AS         Q  IL N
M-Ihh   HV   QYVLV ....SGVPG  QPAR AA S. THVAL S      R   L VE
M-Dhh   RLRA  SVLA ....PGGDA  QPAR AR A. R  AV VF         LLVN

        351                                                400
Xhh     QVLASCYAVI EEHTWAHLAF APLRFGMSLS SYIYPRDSSP PSGLQPHHQV
M-Shh   R             S   R     F LAHA L AALA ARTDG GG .......
C-Shh   R             S   W     F LAQG L AALCP..... ..D.......
M-Ihh   D V   F AV AD HL Q    W   LFP  . .......... ..........
M-Dhh   D        L  S Q   R       LLHA G ALLP...... ..........

        401                                                450
Xhh     DLQSHHQVDL QSHHQVDLQS HHQLEGIHWY SQLLYQIGTW LLDSNSLHPL
M-Shh   ......GSIP AAQSATEARG AEPTA           H         ETM
C-Shh   ......GAIP TA........ ATTTT       R   R  S  V  GDA
M-Ihh   .......... .......AWG WTPS  V S  P M  RL RL   EESTF
M-Dhh   .......... .........G AV PT  M     R   RLAEE  MG*......

        451
Xhh     GMATKSS*
M-Shh      V   *
C-Shh     VAPA *
M-Ihh     SGAG *
M-Dhh   ........
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Figure 2. Comparison of *Xenopus*, mouse and chicken hedgehog sequences. Sequence comparison at the amino acid level is displayed for *Xenopus* hedgehog (Xhh), mouse sonic hedgehog (M-Shh) (17), chicken sonic hedgehog (C-Shh) (20), mouse Indian hedgehog (M-Ihh) (17) and mouse Desert hedgehog (M-Dhh) (17). Blanks in the sequence indicate identical amino acids as compared with Xhh. Gaps represented by dots were introduced to allow for the best alignment. Xhh is 71% identical to M-Shh, 73% to C-Shh, 60% to M-Ihh and 54% to M-Dhh. The termination codons are marked by asterisks (*). The predicted site of signal peptide cleavage is marked with an arrow (57). The triple repeat amino acid sequence unique to Xhh is underlined.

Recently, evidence was presented for the auto-proteolytic processing of *Drosophila* hedgehog both *in vivo* and *in vitro* (49). The 30 kDa species that we detected could be one of these proteolytic

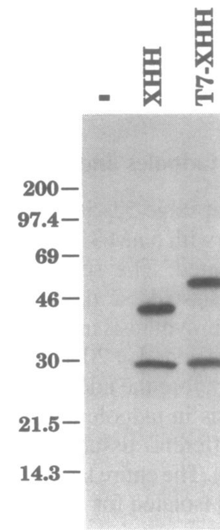

Figure 3. *In vitro* translation of Xhh cDNA. Full length Xhh cDNA (XHH) or full length Xhh cDNA fused to a T7 gene 10 tag (T7-XHH) were translated in reticulocyte lysate using radiolabeled methionine. *In vitro* translation of reticulocyte lysate alone is indicated by (–). Protein products were displayed by SDS–PAGE and visualized by autoradiography. Protein markers are indicated on the left. Note that the shorter polypeptide was most likely derived from the C-terminus as it had the same size from the intact and tagged Xhh clones.

products. If so, this polypeptide is presumably derived from the C-terminus of the protein because the processed product migrated at the same position by SDS–PAGE in the absence or presence of the T7 N-terminal tag.

Southern blotting analysis was used to determine the number of copies of Xhh in the *X.laevis* genome. The Xhh cDNA fragment isolated by subtractive hybridization (bases ~1170–1470) (GenBank # L39213) was hybridized to homozygous *Xenopus* diploid genomic DNA digested with three different restriction enzymes (not shown). Only a single band was detected for each enzyme digestion suggesting that Xhh is a single copy gene. However, this result does not rule out the possible existence of *Xenopus* homologs of other hedgehog proteins, such as Desert hedgehog, as our stringent hybridization conditions might have prevented cross-hybridization among these divergent members.

Xhh is highly expressed during intestinal morphogenesis

The expression of Xhh during metamorphosis was analyzed by Northern blot hybridization using total RNA from the intestine of tadpoles at different stages (Fig. 4). A major band of ~2.8 kb was detected and at least two small mRNA species could also be observed under better gel conditions (also see below). In premetamorphic tadpoles up to stage 58, a low level of Xhh mRNA was present in the intestine. The Xhh gene began to be up-regulated by stage 60, reaching its peak level of expression within a few days (stage 62) as intestine epithelial morphogenesis begins (9,12). Subsequently, the mRNA level dropped very quickly as the end of metamorphosis is reached at stage 66. These results suggest that Xhh is indeed involved in intestinal remodeling, as we had hoped for genes isolated from the subtractive screen.

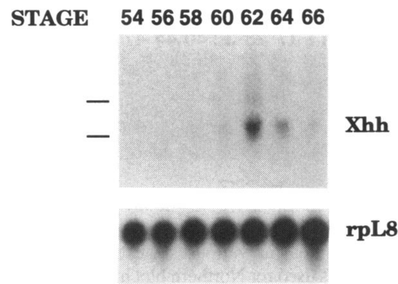

Figure 4. Xhh is activated during intestinal metamorphosis. Intestinal RNA was isolated from the stages indicated and analyzed by RNA blot hybridization using Xhh cDNA as a probe. The filter was also hybridized with rpL8 which served as a loading control. 28S and 18S ribosomal markers are indicated on the left.

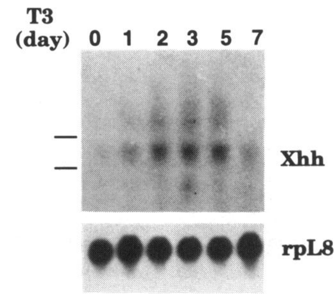

Figure 5. Xhh expression is also activated during TH induced intestinal metamorphosis. Stage 56 tadpoles were treated with 5 nM T3 for various times as indicated in days. RNA was isolated from the intestine for Northern blot analysis using either Xhh or rpL8 as a probe. 28S and 18S ribosomal markers are indicated on the left. It is interesting to note that in the absence of exogenous TH it normally takes 8 days for stage 56 tadpoles to reach stage 60 when Xhh mRNA levels are upregulated (Fig. 4), 11 days to reach the peak levels of Xhh mRNA (stage 62), and 3 weeks to reach stage 66 when Xhh expression is repressed again.

Induction of Xhh by thyroid hormone

During metamorphosis, the high levels of Xhh expression we observed at stage 62 in the intestine correspond to the period when the endogenous plasma TH concentration is at its peak (50). This correlation suggests that Xhh mRNA levels in the intestine should be controlled by TH. Intestinal remodeling can be precociously induced by the simple addition of exogenous TH to the water in which pre-metamorphic tadpoles are being raised (1). If Xhh plays a role in intestinal morphogenesis, one would expect that its expression should be regulated similarly during both precociously induced metamorphosis and normal development.

To test these possibilities, stage 56 premetamorphic tadpoles were treated with 5 nM thyroid hormone T_3, a concentration similar to that of endogenous T_3 at the metamorphic climax (50), for up to 7 days. RNA was isolated from the intestines of treated tadpoles and analyzed by Northern blot hybridization (Fig. 5). The Xhh mRNA levels were up-regulated after 1 day of TH treatment. Xhh mRNA continued to accumulate for >3 days. After ≥5 days of treatment the mRNA levels began to decrease. This expression profile mimicked that observed during normal development but in a much reduced time span (compared with the results in Fig. 4, when stage 56 tadpoles normally take 8 days to reach stage 60, 11 days to stages 62, and 20 days to stage 66). Under our treatment conditions, it is known that normal intestinal remodeling, including the shortening in length and epithelial folding, occurs (51). Thus, these results suggest that expression of Xhh during the treatment also corresponded to the period of intestinal morphogenesis.

To study further Xhh gene regulation by TH, the kinetics of Xhh activation were analyzed in more detail (Fig. 6). The up-regulation of Xhh mRNA levels was evident after 4 h of TH treatment in the intestine of stage 52–54 tadpoles and this level continued to increase within the next 2 days of treatment (Fig. 6A). In addition, even when cyclohexamide and anisomycin (CHX) were added to the rearing water of the tadpoles 1 h before and throughout the TH treatment to block protein synthesis, the up-regulation of Xhh mRNA levels was also observed (Fig. 6B). In fact, all three major Xhh mRNA species were super-induced in the presence of TH and CHX due to the stabilization of the mRNA by the presence of CHX. Although the mechanism of the stabilization of the Xhh mRNA by these protein synthesis inhibitors is still unknown, such a phenomenon has been observed

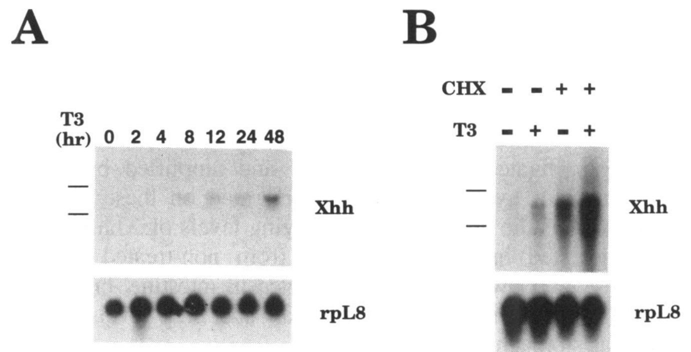

Figure 6. Xhh is a direct TH response gene. (**A**) Kinetics of T3 induction. Stage 52–54 tadpoles were treated with 5 nM T3 for various times as indicated in hours. RNA was isolated from intestine for RNA blot hybridization using full length Xhh or rpL8 as a probe. (**B**) TH activation of the Xhh gene is independent of new protein synthesis. Protein synthesis inhibitors cyclohexamide and anisomycin (CHX) were added to stage 52–54 tadpoles 1 h before the addition of 50 nM T3. The treatment was continued for another 12 h before RNA was isolated from intestine for RNA blot analysis using Xhh or rpL8 as a probe. Ribosomal markers, 28S and 18S, are indicated on the left.

for a number of TH response genes isolated from different tadpole tissues (15,45,46). It is likely that the inhibitors prevented the synthesis of a protein factor(s) that is involved in Xhh mRNA turn over. In any case, these data suggest that Xhh is an early response gene that is up-regulated directly by TH.

Tissue-specific regulation of Xhh

Metamorphosis transforms every organ of a tadpole (1,2). It is possible that Xhh is also involved in the remodeling of other tadpole organs. To begin to address this possibility, we asked whether the TH-dependent induction of Xhh occurred in other organs or tissues. Thus, tadpoles at stage 52–54 were maintained in the presence or absence of TH for 18–24 h followed by RNA isolation from different tissues including brain, limb, tail, intestine and a mixture of pancreas and stomach. RNA was copied

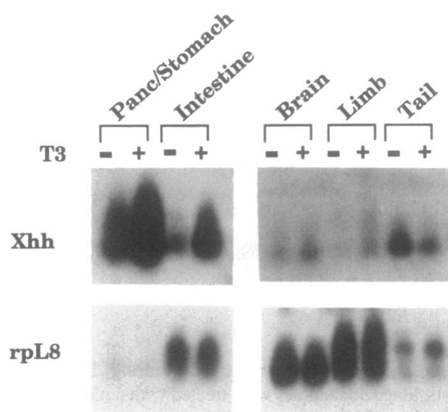

Figure 7. Tissue specific induction of Xhh by TH. Tadpoles at stage 52–54 were treated in the presence (+) or absence (–) of T3 for up to 24 h. After treatment, poly(A)$^+$ RNA was isolated from brain, hindlimb (limb), tail and intestine; total RNA was isolated from a mixture of pancreas and stomach (panc/stomach). RNA was copied into cDNA using reverse transcriptase, restricted to small fragments, ligated to a PCR-linker, and amplified by PCR. Southern blot hybridization was performed on these cDNAs using Xhh as the probe. Filters were also hybridized with rpL8 (see Materials and Methods) to assay for variation in loading and PCR amplification.

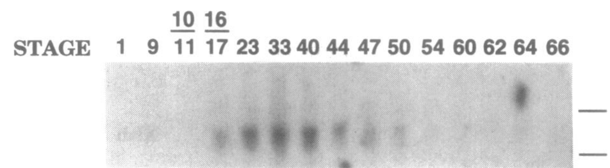

Figure 8. Xhh is highly expressed during embryogenesis. Total RNA was isolated from whole animal at various stages throughout development as indicated. This RNA was used for Northern blot hybridization with Xhh cDNA as the probe. 28S and 18S ribosomal markers are indicated on the right. An equal amount of RNA was present in each lane as confirmed by staining the membrane with methylene blue (43). Note that high levels of Xhh mRNA were present in the animal from neurula stages (stages 16/17) to the onset of tadpole feeding (stages 44/45).

into cDNA using reverse transcriptase, restricted to small fragments, ligated to a PCR-linker and amplified by PCR. Southern blot hybridization was performed on these cDNAs using Xhh as the probe (Fig. 7). Varying levels of Xhh mRNA were detected in different tissues from non-treated control tadpoles (–). Low levels were observed in intestine, brain and hind limb, moderate levels in the tail and high levels in the pancreas/stomach.

Treatment of tadpoles with TH (+) induced the expression of Xhh in some but not all tissues. Most striking was the level of induction in the pancreas/stomach and intestine. Low levels of TH induction were present in the limb. No difference in mRNA levels were detected in the brain and only a slight decrease in signal was seen in the tail. Differences in the regulation of the mRNA levels by TH were not due to loading variation or PCR amplification artifact because filters hybridized with a control probe rpL8 (see Materials and Methods) indicated that the two samples of each organ were equivalent. These data suggest that not all tissues expressing Xhh mRNA are competent to respond to thyroid hormone treatment. Thus, induction of Xhh mRNA by TH occurs in a tissue-specific manner. Furthermore, Xhh is likely to play a role in the development of many different organs.

Developmental pattern of Xhh expression

The widespread expression of Xhh in different tadpole organs, although at varying levels, also suggests a potential role of Xhh during embryogenesis. To examine this further, especially to determine the spatio/temporal pattern of Xhh expression during the entire developmental period of *X.laevis*, Northern blot analysis was performed using total RNA isolated from whole animals at various stages throughout development (Fig. 8). The earliest Xhh expression was detected at stage 16/17 (neurula stage). Xhh mRNA levels continued to increase with peak expression around tadpole hatching (stage 35/36). Xhh mRNA subsequently declined to very low levels between stages 54 and 66, the period of metamorphosis. These low levels of Xhh expression in whole tadpoles during metamorphosis contrasts sharply with those present in the intestine, again indicating that Xhh expression is highly tissue-dependent.

To examine the tissue localization of Xhh mRNA during embryogenesis, whole mount *in situ* hybridization analysis was performed on stage 32 *X.laevis* embryos with a digoxigenin labeled Xhh cRNA (47,48). Cross-section analysis of the hybridized embryo revealed the strong Xhh mRNA expression in the notochord and neural floor plate (Fig. 9). These hybridization patterns are similar to that observed for the sonic class of vertebrate hedgehog genes in mouse, rat, chicken and zebrafish (16–20). Thus, the tissue distribution pattern of Xhh observed in stage 32 embryos suggests that Xhh may play a similar role as its vertebrate homologues in the patterning of the central nervous system.

DISCUSSION

The remodeling of the amphibian intestine during metamorphosis is under the control of a complex cascade of genes regulated by thyroid hormone (15). Here we describe the cloning and initial characterization of one of the immediate early response genes involved in this process, *Xenopus* hedgehog (formerly IU27). *Xenopus* hedgehog (Xhh) is a member of a growing family of signaling molecules conserved from flies to mammals (25,52). It most resembles the sonic class of vertebrate hedgehog genes. Toward our goal of understanding the biological function of Xhh, we have demonstrated the up-regulation of Xhh mRNA by thyroid hormone in a tissue specific manner, examined the developmental pattern of mRNA expression, and localized mRNA by *in situ* hybridization to the developing tadpole nervous system.

Functional studies on the hedgehog family of genes have suggested a role for these proteins in relaying positional information necessary for patterning of a variety of tissues including limb, wing, eye and the central nervous system (16–20,23,29–32,34,35). During development, all of these organ systems require cell–cell communication to establish polarity within the tissue. Hedgehog is the proposed signaling molecule responsible for this communication. This type of cellular signaling could potentially exist in other tissues as well. In this study, we present data suggesting that in the intestine Xhh may be playing a similar role.

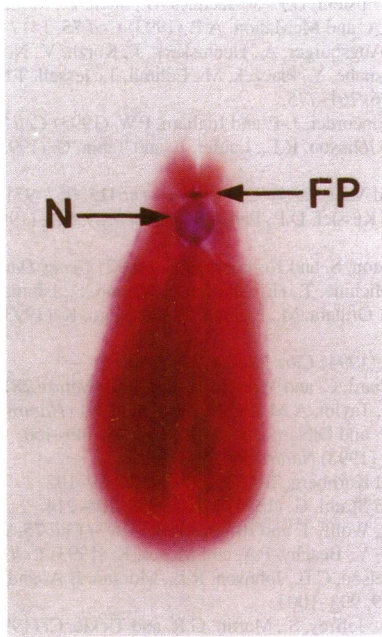

Figure 9. *In situ* hybridization localized Xhh mRNA in the notochord and floor plate. *In situ* hybridization was performed on stage 32 *X.laevis* embryos with a digoxygenin labeled Xhh cRNA and the expression of Xhh mRNA was analyzed after cross-sectioning the hybridized embryos. The most intense hybridization was detected in the notochord (N) and the neural floor plate (FP). The background signal below the notochord is likely due to non-specific binding by the yolk proteins present in early embryos.

Throughout metamorphosis in the intestine, extensive changes in tissue architecture are required to transform an herbivorous tadpole into a carnivorous frog (53,54). In order to make this transition, many types of processes must be initiated in this organ. These include apoptosis of the primary epithelium, proliferation and differentiation of mesenchymal and muscle tissue, and development of the secondary epithelium. Xhh could be involved in any or all of these processes.

One very likely possibility for Xhh function is the establishment of polarity along the intestinal epithelial folds or villi, a common feature of vertebrate intestinal development. At the base of the folds, or troughs, lie the precursor stem cells for the secondary (adult) epithelium. As these stem cells divide, daughter cells migrate towards the tips or crests of the folds. During this migration process, cells gradually undergo differentiation. By the time a cell reaches the crest, it is fully differentiated. The establishment of the epithelial fold and/or this differentiation gradient could be influenced or initiated by Xhh similar to the way that sonic hedgehog establishes the polarity of digit formation in the chick limb bud (20,34,35).

Alternatively, Xhh could function in the establishment of cell types such as the precursor stem cells of the secondary epithelium. To date, the origin of these stem cells is unknown. Just as sonic hedgehog in the central nervous system (CNS) of mouse induces neural tube cells to differentiate into motor neurons (17), so could Xhh induce some unknown cells to become secondary epithelium stem cells.

Another possible role for Xhh could be in the establishment of boundaries between epithelium and underlying mesenchymal cells. The strict spatial organization of these cell types is responsible for the maintenance of the villi structures. If Xhh were acting in this capacity it would be similar to the role presented for *Drosophila* hedgehog in establishing and maintaining the parasegment boundaries of the developing fly embryo (22,23,27–30). Together, these are just a few possible functions of Xhh in the intestine. Other unknown functions may certainly exist. Further studies would be required to determine the exact role of Xhh in this tissue. However, the high levels of Xhh expression in the intestine at the metamorphic climax and low levels before and after metamorphosis would favor the possibility of Xhh as a morphogen during secondary epithelial morphogenesis.

The finding that Xhh is expressed in the intestine is in agreement with previous reports on the expression of the gene in mouse, rat, zebrafish and fly (16–19,21–24). In these organisms, hedgehog homologues were found to be expressed in gut tissues. Unique to this study, however, is the finding that the transcription of Xhh in the intestine of frogs is regulated by thyroid hormone. Thyroid hormone has long been known to be the active agent in initiating amphibian metamorphosis (1). It initiates this process by binding to its nuclear receptors which subsequently activate transcription of specific target genes (3–7). Here, we have shown Xhh to one of these direct targets.

Induction of Xhh by thyroid hormone was not limited to the intestine but was also observed in the limb and in a mixture of pancreas and stomach. Expression of Xhh in the limb is not unexpected considering the importance of the sonic class of hedgehog genes in chick limb development (16,20,34,35). Both chicken and mouse sonic hedgehog genes have been demonstrated to function as the signaling molecule arising from the zone of polarizing activity (16,20). It is not surprising then that Xhh would also be expressed in this tissue. In chicken, sonic hedgehog expression can be induced in the developing limb bud upon the addition of retinoic acid (20). Likewise, Xhh can be induced in the frog hindlimb by thyroid hormone. Thus, the expression of hedgehog genes in the limbs of these two organisms are controlled by seemingly different molecules; yet, both retinoic acid and thyroid hormone are the molecules which control limb development.

In addition to studying Xhh in the intestine, we have also examined Xhh expression in the whole animal during development. From developmental Northern analysis, we observed two interesting features of Xhh gene expression. First, despite the fact that Xhh is highly expressed in intestine at stage 62, the climax of metamorphosis, very low levels of Xhh mRNA were detected in the whole animal throughout the metamorphosis period (stages 54–66). These data suggest that high levels of Xhh expression are restricted to a subset of tissues or organs during metamorphosis, consistent with the fact that different tissues undergo vastly different transformation. Thus, it is not surprising to see very low levels of Xhh expression in the tail during metamorphosis as it is being resorbed (not shown) while high levels are present during intestinal morphogenesis.

A second feature that we observed was in regard to the high levels of Xhh mRNA between stages 16/17 and stage 50. During these stages of development no endogenous thyroid hormone can be detected, yet Xhh mRNA is still being expressed. This suggests that Xhh is regulated under either thyroid hormone-dependent (e.g. metamorphosis), or thyroid hormone-independent (e.g. early development) transcriptional control.

As mentioned above, the earliest expression of Xhh was detected around neurula stage, the stage when the notochord and

neural tube (precursor tissues of the CNS) are beginning to form. The expression of Xhh at this stage is consistent with the role of hedgehog acting as a signaling molecule to establish patterning in the nervous system (17,18). Further evidence suggesting that Xhh plays a similar role as sonic hedgehog in the CNS was obtained from whole mount *in situ* hybridization analysis of stage 32 tadpoles. Here, we observed the strongest hybridization signals in the notochord and neural floor plate. This pattern is similar to that seen in mouse, rat, chicken and zebrafish (16–20). Together, these data suggest that in frogs Xhh not only functions in late phases of development (e.g. metamorphosis), but also in early patterning processes as well. Currently, we are focusing on the role of Xhh in later development to gain a better understanding of this gene's function in tissue remodeling and in the orchestration of gene regulation cascades during metamorphosis.

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