Distinct expression and shared activities of members of the hedgehog gene family of Xenopus laevis

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SUMMARY

The hedgehog family of signaling proteins is associated with a variety of spatial patterning activities in insects and vertebrates. Here we show that new members of this family isolated from Xenopus laevis are expressed embryonically in patterns suggestive of roles in patterning in the ectoderm, nervous system and somites. Banded hedgehog is expressed throughout the neural plate and subsequently in both the nervous system and in the dermatome of somites. Cephalic hedgehog is expressed in anterior ectoderm and endodermal structures, and sonic hedgehog is expressed in patterns which parallel those in other species. Injection of RNAs encoding Xenopus hedgehogs induces ectopic cement gland formation in embryos. Similar to reported activities of noggin and follistatin, Xenopus hedgehogs share a common ability to induce cement glands in animal cap explants. However, hedgehog activities in naive ectoderm appear capable of acting independently of noggin and follistatin since, although all three are induced by activin in animal cap explants, X-hh expression does not induce noggin or follistatin.

Key words: hedgehog, neural induction, cement gland, follistatin, noggin, Xenopus laevis

INTRODUCTION

The hedgehog family of secreted signaling proteins represents a recent addition to the known repertoire of molecules employed by vertebrates in the establishment of embryonic pattern. Originally identified by Nüsslein-Volhard and Wieschaus (1980), the hedgehog gene functions in Drosophila to help coordinate the identities of cells within embryonic segments (reviewed by Hooper and Scott, 1992; see also Heemskerk and DiNardo, 1994), and later in the patterning of adult precursors including the appendages and the eye (Mohler, 1988; Ma et al., 1993; Heberlein et al., 1993; Tabata and Kornberg, 1994; Basler and Struhl, 1994). Whereas only single hedgehog genes have been found in Drosophila and several other invertebrate species (Chang et al., 1994), hedgehog genes in vertebrates constitute a multi-gene family (Echelard et al., 1993; Krauss et al., 1993; Chang et al., 1994), with the sonic hedgehog class (shh; also referred to as vhh-1 or Hhg-1) receiving the greatest degree of experimental attention. Studies of embryonic expression and function suggest that shh plays a role in dorsoventral patterning of the neural tube and somites (Echelard et al. 1993; Krauss et al., 1993; Roelink et al., 1994; Fan and Tessier-Levigne, 1994; Johnson et al., 1994) and in anteroposterior patterning of the developing limb (Riddle et al., 1993; Chang et al., 1994; Niswander et al., 1994; Laufer et al., 1994). Since the shh class of hedgehog genes has been the major focus of experimental effort in vertebrates, little is known regarding the expression and functions of other vertebrate hedgehog family members. In particular, it is unclear whether distinct hedgehog genes have related activities and whether hedgehog genes play early roles in such processes as mesodermal or neural induction and patterning.

Since Xenopus remains the vertebrate most amenable to examination of embryonic signaling processes that are involved in mesodermal and neural induction (reviewed by Harland, 1994), analyses of Xenopus hh genes (X-hh) provide the opportunity to place this newly described class of signaling molecules within the context of well studied signaling pathways. We report here the isolation of four X-hh family members, which constitute three distinct classes of X-hh proteins. One of these represents the Xenopus homologue of the previously reported sonic hedgehog (X-shh) class. The other two, banded hedgehog (X-bhh) and cephalic hedgehog (X-chh), display novel patterns of hh expression. Since high level expression of all four family members induces ectopic cement gland formation in animal cap explants, suggesting qualitatively similar activities for these proteins, we focused on the activities of X-bhh as representative of X-hh activities.
in general. High level expression in whole embryos and in animal cap explants leads to direct induction of cement gland, which is also a reported activity of the neural inducers noggin (Lamb et al., 1993) and follistatin (Hemmati-Brivanlou et al., 1994). X-hh inducing activities can act independently of noggin and follistatin since, although all three are induced by activin, X-bhh expression in animal cap explants is neither induced by nor induces noggin and follistatin.

MATERIALS AND METHODS

Isolation of X-hh cDNAs

Fragments of X-hh sequences were isolated from genomic DNA using degenerate primers in PCR reactions as described (Chang et al., 1994). Using these fragments as probes, four distinct full-length cDNAs were obtained from a Xenopus laevis stage 24 developmental library (Richter et al., 1988). One of the initial X-hh fragments isolated by PCR amplification did not yield a cDNA in this screen. The regions encoding the predicted open reading frames (Fig. 1) were sequenced on both strands as recommended (US Biochemicals). All four open reading frames are closed upstream of the initiating methionine (data not shown). Part of the X-shh open reading frame was confirmed through the isolation of the corresponding region from genomic DNA (data not shown).

Expression of X-hh RNAs during development and RT-PCR analyses

Northern analyses were performed using poly(A)-containing RNA isolated from various developmental stages (McGrew et al., 1992). Aliquots of total RNA were digested with RNAse-free DNase (Promega) and approximately 5 µm was used as template to generate first strand cDNA according to manufacturer’s instructions (Life Sciences, Inc.); 1/20th of this cDNA was used as template in subsequent RT-PCR analyses. Control tubes contained everything except reverse transcriptase and were run in parallel with the experimental reactions, and were negative in each case. Detection of X-bhh was done using primers C1 (5'-TACGGATCTGGCTAGG-3') and C2 (5'-CCCAGTGTGTTGCTCAGC-3') in standard RT-PCR conditions with the following reaction profile: 94°C for 1 minute, 58°C for 1 minute, and 72°C for 1 minute, for 30 cycles. The resulting product was sequenced directly to confirm its identity. Detection of histone H4 was as described by Niehrs et al. (1994).

Relative levels of transcripts encoding EF-1α and XAG-1 were assayed by RT-PCR as described in the accompanying paper (Lai et al., 1995), and noggin and follistatin transcripts were monitored by RT-PCR as described (Hemmati-Brivanlou et al., 1994).

Injection constructs

The expression construct pT7TS-X-bhh was made by insertion of the open reading frame into the SpeI site of the vector pT7TS (gift from A. Johnson and P. Krieg, University of Texas at Austin). The open reading frame segment was generated by PCR using cDNA as template with primers bhh-U (5'-GGAGATCTCCATCAGCGATTGCGTGAGATCGTGGCAAGGT-3') and bhh-D (5'-AGCATCAATAGGCTCAGTGGCTTTCCACATT-3'). Similarly, expression construct pT7TS-X-shh was made by insertion of the X-shh open reading frame into the BglII site of pT7TS using primers shh-U (5'-ATATGGATCCCGAGATGCGTGAGTGGCAGT-3') and shh-D (5'-GGCGGATCTTCTTCCACTGTTCAGGTCAGG-3'). The resulting construct was sequenced after digestion with BglII. Expression constructs pSP64-X-chh and pSP64-X-bhh were made by insertion of the open reading frames into BglII site of plasmid pSP64T. Open reading frames were generated using primers chh-U (5'-ATATGGATCCCGAGATGCGTGAGTGGCAGT-3') and chh-D (5'-GGCGGATCTTCTTCCACTGTTCAGGTCAGG-3') for X-chh and primers hh4-U (5'-ATATGGATCCCGAGATGCGTGAGTGGCAGT-3') and hh4-D (5'-GGCGGATCTTCTTCCACTGTTCAGGTCAGG-3') for X-hh4 in PCR reactions and subsequent BamHI digestion. A noggin expression construct in the vector pSP64T was generated by PCR of the coding region of the reported noggin sequence, and was also provided by Richard Harland (University of California, Berkeley). A follistatin expression construct in the vector pT7TS was generated by PCR of the coding region of the reported Xenopus follistatin coding sequence (Hemmati-Brivanlou et al., 1994), using the forward primer 5'-GAAGATTCTCCCCAGCATGGGATG-3' and the reverse primer 5'-GGACTGTTCACTTACAGTGTTGAGATC-3'. The resulting construct was sequenced.

In situ hybridization and immunohistochemistry

Whole-mount in situ hybridizations were performed according to Harland (1991). For histological examination embryos were embedded in paraffin and 8-10 µm sections were prepared (Kelly et al., 1991). Detection of the monoclonal antibody 12/101 (Kintner and Brockes, 1984) was according to the method of Klymkowsky and Hanken (1991). Detection of β-galactosidase activity after coinjection of lacZ and X-bhh RNAs was performed according to the method of Westerfield et al. (1992) using 3-chloro-4-indolyl-β-galactopyranoside (Biosynth AG, Switzerland) as substrate, followed by in situ hybridization for XAG-1.

Embryo manipulations

RNA was synthesized from X-hh pT7TS-based constructs and injected by standard methods (Moon and Christian, 1989). Embryos were injected with the indicated amounts of synthetic mRNA in the animal pole of each blastomere at the 2-cell stage, then cultured (Moon and Christian, 1989) and analyzed as indicated. In experiments involving animal caps, the explants were made from stage 8 blastulas as in the accompanying paper (Lai et al., 1995). After further incubation to the stages indicated for sibling embryos, explants were subjected to in situ hybridization as described above or to RT-PCR reactions as described above.

RESULTS

Isolation of a family of Xenopus hedgehog homologues

Degenerate primers were used in PCR reactions with genomic DNA as template as described (Chang et al., 1994), and the resulting X-hh gene fragments were used as probes to screen a stage 24 cDNA library (Richter et al., 1988). Four distinct family members were isolated, and the predicted protein products are shown in Fig. 1A. Xenopus cephalic hh (X-chh) and X-hh4 are closely related (93% amino acid identity) while the other two X-hh family members are more divergent (Fig. 1B). The high degree of identity between X-chh and X-hh4 relative to the other X-hh family members suggests a recent gene duplication event in the Xenopus lineage and our analysis consequently focused primarily on one of these two, X-chh. Like all other known vertebrate hh genes, these X-hh genes contain an amino-terminal signal sequence indicating probable secretion of X-hh protein products in vivo. Comparison of X-hh genes with murine hh genes indicates that Xenopus banded hedgehog (X-bhh) is most similar to mouse Indian hh (70%
amino acid identity) and X-chh and X-hh4 are most similar to mouse Desert hh (64% and 63% amino acid identities, respectively). Based upon its expression pattern (see below) and high sequence identity to mouse sonic hh (78%), X-shh appears to represent the Xenopus homologue of sonic/vhh-1/Hhg-1. The spatial patterns of expression for X-bhh and X-chh (see below) are novel among reported vertebrate hh genes and thus define two new classes of vertebrate hh gene expression during development.

Expression of X-hh transcripts is temporally and spatially restricted

Steady-state transcript levels for X-bhh, X-shh and X-chh (Fig. 1C-E) indicate a peak of gene expression during neural induction and early organogenesis. Expression of X-bhh was monitored by RT-PCR since it is expressed at lower levels than the other two genes. Additional cycles of PCR begin to show X-bhh bands in the minus RT control, but longer exposure of films and independent experiments detect specific amplification of X-bhh transcript by stage 8 (data not shown). Transcript localization by in situ hybridization showed that transcripts from all three of these genes are localized to the sub-epithelial layer of the marginal zone with no dorsal or ventral bias at early gastrulation (Fig. 2A,E,I). Differences in X-hh expression become apparent at or before stage 14, with X-bhh expression observed in peripheral regions of the neural plate (arrows, Fig. 2B), but not in the presumptive midline (arrowhead, Fig. 2B; see Hausen and Riebesel, 1991 for description of early embryogenesis). At neural tube closure, a prominent anterodorsal patch of X-bhh expression is observed (Fig. 2C, arrow) with more diffuse expression apparent in the somitic and pre-somatic mesoderm. By the early tadpole (stages 28-30), widespread expression is observed throughout anterior structures with highest levels in the otic vesicle, the eye, and the branchial arches (Fig. 2D; ov, e, ba). Also by this stage, mesodermal expression of X-bhh occurs as an array of
Fig. 2. Localization of X-hh transcripts during early development. Localizations of X-hh transcripts during development are revealed by in situ hybridization to embryos with antisense probes for X-bhh (A-D), X-shh (E-H), and X-chh (I-K). (A) Side view of an early gastrula embryo (stage 10), with animal hemisphere up. X-bhh transcripts are localized to the sub-epithelial layer of the entire marginal zone (arrow). (B) Anterior view of a neurula embryo (stage 14). X-bhh expression occurs throughout most of the neural plate, with strongest expression along the lateral border (arrows) and no expression along the midline (arrowhead). (C) Lateral view of a late neurula embryo, after neural tube closure (stage 19/20). The most prominent domain of X-bhh expression is in the anterior of the embryo (arrow), with more diffuse expression observed posteriorly in the mesoderm. (D) Lateral view of a tailbud embryo (stage 28) with anterior toward the left. High levels of X-bhh transcripts are seen in specific structures of the head including the eye (e), otic vesicle (ov) and branchial arches (ba). X-bhh transcripts are also localized in a chevron-shaped band within each somite, predominantly within the dermatome (see text; data not shown). (E) Side view of an early gastrula embryo (stage 10), with animal hemisphere up. X-shh transcripts are most prominent in the sub-epithelial layer of the entire marginal zone (the lower and upper arrows mark the dorsal and ventral sides of the embryo, respectively). With more extended incubation during the color reaction, lower level expression can be seen to extend over the animal hemisphere (not shown). (F) Anterior view of a neurula embryo (stage 14). X-shh expression is restricted to the presumptive midline (arrowhead) and is absent from the rest of the neural plate (arrows). (G) Lateral view of a late neurula embryo (stage 19/20) with anterior to the left. X-shh expression is in the notochord and ventral neural tube with the most prominent expression observed in an anterior domain (arrow). (H) Lateral view of a tailbud embryo (stage 29/30) with anterior toward the left. X-shh is expressed exclusively in cells of the notochord (n) and floorplate (fp) throughout the axis. Strong expression in the brain and other anterior structures persists. (I) Side view of an early gastrula embryo (stage 10), with animal hemisphere up. X-chh expression extensively overlaps that of X-bhh and X-shh and is most prominent in a band of cells within the marginal zone (arrow). (J) Top view of a mid-neurula embryo (stage 15/16) with anterior to the left. X-chh transcripts are most abundant in an extreme anterior domain of the embryo; the anterior portion of the neural plate is indicated by the arrows. (K) Lateral view of a tailbud embryo (stage 29/30) with anterior to the left. Low levels of X-chh transcripts are present in the pharyngeal cavity. (L) Side view of a tailbud embryo hybridized with a X-bhh sense probe as a control.
Fig. 3. Expression of X-bhh in mesoderm and ectodermal derivatives. Whole embryos, hybridized in situ with a probe specific for X-bhh transcripts, were embedded in paraffin and sectioned (see Materials and methods). (A) Sagittal section of a stage 14 neurula with anterior to the left. X-bhh transcripts are most prominent in the sub-epithelial and epithelial layers of the neuroectoderm but are also present at lower levels in the underlying chordamesoderm (see arrows in the insert). (np) neural plate, (a) archenteron. (B) Parasagittal section through the head of a stage 28 tailbud embryo with anterior to the left. X-bhh-expressing cells are seen in the sub-epithelial layer of the ectoderm (arrows), just dorsal to the cement gland (cg); (e) eye. (C) Transverse section of a stage 28 embryo at the level of the eye (e). X-bhh expression is found in both the superficial and deep layers throughout the roof of the mesencephalon (me). Expression is also observed prominently in the prospective retinal layer of the eye vesicle (e). (D) Transverse section of a stage 28 embryo at the level of the otic vesicle (ov). X-bhh expression is seen in the dorsal roof of the rhombencephalon (rh), and in the epithelial layer of the ectoderm. Expression is also prominent in the otic vesicle (ov), (n), notochord. (E) Para-sagittal section through the head of a stage 28 embryo with anterior to the left. X-bhh transcripts are detected in the dorsal mesencephalon and rhombencephalon (arrows) and in the branchial arches (arrowheads). (F) Dorsal view of a whole tailbud embryo (stage 23) with anterior to the left. The embryo has been double-labeled by in situ hybridization with a probe specific for X-bhh transcripts prior to immunostaining with monoclonal antibody 12/101, specific for the myotomal portion of the somite (Kintner and Brockes, 1984). A block of two adjacent somites (s) is delimited by the arrows. (G) Lateral view of the embryo in F, at higher magnification, and with anterior to the left. The brown 12/101 stain highlights the myotome of each somite (area between two arrows); the dark blue X-bhh stain is localized to a single central portion of the dermatome of each somite (see text; data not shown). (H) Glancing section in the plane of the dermatome of an embryo similar to that in F with anterior to the left. The arrows demarcate the boundaries of a single somite and the arrowhead denotes X-bhh staining.
Expression of X-shh in the early gastrula is also localized to the sub-epithelial layer of the marginal zone (Fig. 2E, arrows), but differs from X-bhh in showing a low level of expression throughout the animal pole. Expression in the early neurula is largely restricted to the midline (Fig. 2F, arrowhead), in marked contrast to the broad distribution of X-bhh transcripts throughout the neural plate during the same period (Fig. 2B). By stage 20, after neural tube closure, X-shh expression is localized to the axial mesoderm including the prechordal plate as well as the entire ventral midline of the neural tube (Fig. 2G; histology not shown) and, as noted with X-bhh, expression is elevated in anterior structures. A similar pattern of expression occurs throughout later development in the notochord and floorplate (Fig. 2H; n, fp) which parallels expression in other species.

The expression of X-chh in the marginal zone is similar to that of both X-bhh and X-shh during early gastrulation (Fig. 2I). In the neurula X-chh displays a novel pattern of expression that is restricted exclusively to anterior structures, encompassing both neural plate and endodermal cells and providing the basis for the name cephalic (Fig. 2J). Thereafter, expression is observed on the inner surface of the pharynx in the early tadpole stage (Fig. 2K), and the overall decline in signal corresponds to the observed decline in steady-state levels of transcript (Fig. 1E).

Expression of X-bhh was further examined by embedding and sectioning of whole embryos after in situ hybridization. A sagittal section through a stage 14 neurula demonstrates a high level of X-bhh expression in both layers of the neuroectoderm with lower levels of expression in the underlying chordamesoderm (Fig. 3A and insert; see arrows). Later in embryonic development X-bhh continues to be expressed in both neural and non-neural tissues. For example, a sagittal section through a tailbud embryo (arrows, Fig. 3B) shows expression of X-bhh in sub-epithelial ectodermal cells that border the cement gland but not within the adjacent neuroepithelium in the prosencephalon. A transverse section through a similar stage embryo at the level of the eyes (Fig. 3C) demonstrates strong expression in both the roof of the mesencephalon and in the lateral wall of the eye vesicle, but also lower levels of expression in the sub-epithelial layer of the epidermis. More posteriorly at the level of the rhombencephalon (Fig. 3D), X-bhh-expressing cells are still localized to the dorsal portion of the neural tube with particularly abundant expression also observed in the otic vesicle that invaginates from the sub-epithelial layer of the epidermis. Fig. 3E shows a parasagittal section that highlights the diffuse signal along the dorsal mesencephalon and rhombencephalon (arrows) as well as expression in the branchial arches (arrowheads, Fig. 3E).

To further characterize X-bhh expression in somitic mesoderm, early tailbud stage embryos were double-labeled (Fig. 3F) by in situ hybridization to detect X-bhh and with the antibody 12/101 (Kintner and Brockes, 1984) to highlight the myotomal portion of the somite. A lateral view of the same embryo (Fig. 3G) indicates that X-bhh expression is localized to the midline of each somite (arrows demarcate somite boundaries). This is seen more clearly in a parasagittal section of a similarly staged embryo (Fig. 3H; arrowhead denotes X-bhh expression between somite boundaries, marked by arrows).

Transverse sections through the trunk of a slightly later stage embryo demonstrate that X-bhh-expressing cells are on the perimeter of the myotome, with greatest expression in the dermatome (data not shown), at a location reminiscent of that reported for expression of Xwnt-11 (Ku and Melton, 1993). Embryos doubly stained for the expression of Xwnt-11, by in situ hybridization, and for the expression of X-bhh, by whole-mount immunohistochemistry with an anti-peptide antibody (Lai et al., 1995), provide the suggestion that Xwnt-11 and X-
bhh may be expressed in many of the same cells of the dermatome (data not shown).

**Overexpression of X-bhh induces enlargement or ectopic formation of cement gland and anterior pituitary gland in embryos**

We began investigating the activities of the X-hh family of genes by examining the effects of high level expression of one family member on embryonic development. Embryos at the 2-cell stage were injected at the animal pole in both blastomeres with synthetic X-bhh or control (lacZ) RNA. In several independent experiments, 57% of X-bhh-injected embryos (n=68) showed enlarged cement glands (Fig. 4B,D, white arrows) as compared to control embryos (Fig. 4A,C, white arrows). In situ hybridization using the cement gland marker XAG-1 (Sive et al., 1989; Lamb et al., 1993) confirmed the enlargement of the cement gland and further showed that ectopic cement glands were present in 10% of the injected embryos (n=95), usually clustered near the primary cement gland (Fig. 4B, arrowheads). We also used the anterior pituitary marker XANF-2 (Mathers et al., 1995) to inquire whether structures arising from ectoderm, yet posterior to the cement gland, can be influenced by X-bhh RNA injection. The anterior pituitary gland is indeed enlarged (Fig. 4D, black arrow) in 34% of X-bhh-injected embryos (n=35) relative to control embryos (Fig. 4C, black arrow).

The formation of foci for ectopic cement glands in response to injection of X-bhh RNA (Fig. 4B) suggested that not all cells expressing X-bhh participate in the formation of cement glands. To provide a marker to indicate which cells inherit injected mRNAs, we injected 32 cell stage embryos with a mixture of lacZ and X-bhh RNAs. After staining for β-galactosidase activity and for XAG-1 expression, we found that only a subset of the cells expressing β-galactosidase also express XAG-1 (Fig. 4E).

**Expression of all four X-hh genes can induce cement gland formation in animal caps**

These initial experiments, which sought to identify developmental events and embryonic structures that are sensitive to
high levels of hh, revealed that X-bhh can induce the formation of ectopic cement glands in whole embryos. This observation raised the question of whether the other three members of the X-hh family have a similar activity and whether this activity is able to directly divert embryonic ectoderm from epidermal to cement gland fates. To address these questions embryos were injected with synthetic RNAs from each of the four X-hh genes, or with lacZ and a frameshifted version of X-shh (X-shhfs) as controls, they were then reared to the blastula stage and animal caps dissected and incubated until sibling embryos reached stage 25. Animal caps were assayed for the expression of the cement gland marker XAG-1 by whole-mount in situ hybridization, and other animal caps were embedded and sectioned for light microscopy. Multiple foci of XAG-1 expression are apparent in animal caps that were injected with X-chh, X-shh, and X-hh4 (see Table 1). Animal caps from uninjected (Fig. 5B) and lacZ-injected embryos (Fig. 5C), in contrast, do not express XAG-1 and instead differentiate as atypical epidermis (see also Table 1). Histological analyses of X-bhh-injected animal caps revealed that surface cells differentiate into densely packed columnar cells (Fig. 5F, bracket), which are characteristic of the secretory cells that comprise the pigmented, mucous-secreting cement gland (Lyerla and Pellizari, 1974; Picard, 1975). Moreover, these histological analyses revealed no mesodermal cell types (data not shown), nor do these explants show induction of early or late mesodermal markers by RT-PCR (Lai et al., 1995). These results demonstrate that X-hhs induce the differentiation of cement gland in ectodermal tissue in the absence of mesoderm.

**X-bhh is induced by activin but can act independently of noggin and follistatin**

Since both noggin and follistatin (Lamb et al., 1993; Hemmati-Brivanlou et al., 1994) are able to induce cement gland and anterior neural markers in animal caps, we tested the possibility that X-hh activities can induce the expression of either of these factors, and also whether noggin and follistatin activities can directly increase the expression of X-bhh. In addition, since activin is capable of inducing the expression of noggin and follistatin (Thomsen and Melton, 1993; Hemmati-Brivanlou et al., 1994), we tested the ability of activin treatment to induce expression of X-bhh. Two-cell embryos were injected separately with X-bhh, noggin, or follistatin RNAs and animal caps were analyzed by RT-PCR for the expression of the other two genes. Fig. 6A demonstrates that, in contrast to activin (lane 1), X-bhh does not induce either noggin or follistatin (lane 2). Conversely, in explants cultured to the equivalent of stage 11 (data not shown) or stage 25 (Fig. 6B), it is apparent that neither noggin nor follistatin induce X-bhh to levels obtained by treatment with activin. These data are most consistent with the interpretation that X-bhh is neither directly upstream nor downstream of previously described anterior neural inducers XAG-1 and instead differentiate as atypical epidermis (see also Table 1). Histological analyses of X-bhh-injected animal caps revealed that surface cells differentiate into densely packed columnar cells (Fig. 5F, bracket), which are characteristic of the secretory cells that comprise the pigmented, mucous-secreting cement gland (Lyerla and Pellizari, 1974; Picard, 1975). Moreover, these histological analyses revealed no mesodermal cell types (data not shown), nor do these explants show induction of early or late mesodermal markers by RT-PCR (Lai et al., 1995). These results demonstrate that X-hhs induce the differentiation of cement gland in ectodermal tissue in the absence of mesoderm.

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**Table 1. Induction of the cement gland marker XAG-1 by X-hh in Xenopus animal caps**

<table>
<thead>
<tr>
<th>RNA</th>
<th>Dose injected</th>
<th>XAG-1% induction (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>X-bhh</td>
<td>60 pg</td>
<td>32 (34)</td>
</tr>
<tr>
<td>X-bhh</td>
<td>1.5 ng</td>
<td>75 (39)</td>
</tr>
<tr>
<td>X-bhh</td>
<td>3 ng</td>
<td>79 (28)</td>
</tr>
<tr>
<td>X-chh</td>
<td>3 ng</td>
<td>59 (32)</td>
</tr>
<tr>
<td>X-hh4</td>
<td>3 ng</td>
<td>58 (19)</td>
</tr>
<tr>
<td>X-shh</td>
<td>3 ng</td>
<td>75 (8)</td>
</tr>
<tr>
<td>X-shhfs</td>
<td>3 ng</td>
<td>0 (10)</td>
</tr>
<tr>
<td>lacZ</td>
<td>3 ng</td>
<td>0 (19)</td>
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</tbody>
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**Fig. 6. X-bhh is not induced by nor induces the expression of noggin or follistatin.**

(A) Activin-treated (lanes 1, 4), X-bhh-injected (lanes 2, 5), and uninjected (lanes 3, 6) animal caps were assayed by RT-PCR for the expression of noggin, EF-1α, and follistatin when sibling embryos had reached stage 11. To confirm the effectiveness of X-bhh mRNA injection, remaining animal caps were assayed for expression of the cement gland marker, XAG-1 (lower panel) when sibling embryos reached stage 25. Note that activin but not X-bhh treatments induce noggin and follistatin.

(B) Uninjected animal cap explants were cultured in the presence (lanes 2, 7) or absence (lanes 5, 10) of activin, and explants were also prepared from embryos injected with noggin (lanes 3, 8) and follistatin (lanes 4, 9) RNAs. Explants were cultured until siblings reached stage 25, and were then assayed for the expression of X-bhh (upper panel), EF-1α, or XAG-1 (lower panels). Note that X-bhh expression is induced by activin but not by noggin or follistatin.
in *Xenopus* and thus represents an independent pathway for induction of anterior ectodermal fates.

**DISCUSSION**

**Expression of Xenopus hedgehog genes**

In the normal embryo, progressive induction and patterning of dorsal ectoderm to differentiate into cement gland and neural ectoderm begins during gastrulation (Kintner and Melton, 1987; Sive et al., 1989; Sharpe and Gurdon, 1990; reviewed by Saha and Grainger 1992, by Doniach 1993, and by Harland 1994). It is therefore noteworthy that expression of *X-hh* genes is detectable by whole-mount in situ hybridization within the early gastrula (Fig. 2A,E,I), although the general expression observed throughout sub-epithelial layers of the marginal zone is not immediately suggestive of dorsoanterior specification.

By the neurula stage, *X-hh* genes are expressed in distinct patterns which correlate well with a role for *X-hh* genes in promoting dorsoanterior fates. *X-bhh* (Fig. 2B,C) and *X-shh* (Fig. 2F,G) are expressed along most of the anterior-posterior extent of the body axis by the late neurula stage but with the most prominent expression observed in anterior structures of the head and brain. This anterior bias is most pronounced for *X-chh*, whose expression is restricted exclusively to the anterior-most portion of the embryo from the neurula stage onward (Fig. 2J). Thus, all three of these genes fulfill the criteria of being expressed in cells that are candidates for providing endogenous signals which are involved in the early induction and patterning of anterodorsal ectodermal and neural structures. Since all four *X-hh* genes display essentially indistinguishable activities in direct induction assays (Table 1), the overall distribution of *X-hh* activity in the embryo is most reasonably considered as the summed expression of all genes. Given the anterior emphasis in expression of individual genes at the neurula stage, this overall sum is strongly biased toward anterior expression, supporting our proposal that *X-hh* activities function in the induction and patterning of anterior structures in the normal embryo. With subsequent development, *X-bhh* and *X-shh* are expressed in distinct patterns suggestive of additional roles in both the somites and the nervous system, respectively.

**Induction of cement gland by *X-hh* activities**

On the basis of histology and expression of a specific marker gene, cement gland induction from naive animal cap ectoderm occurs through the activity of products from all four *X-hh* genes. Upon titration through a 50-fold concentration range of injected *X-bhh* mRNA, this inductive activity appears to occur in response to a critical threshold of *X-hh* activity. Above this threshold, foci for cement glands increase in number and size; below this threshold the foci for cement glands decrease in frequency, with unaffected caps forming atypical epidermis that is histologically indistinguishable from that of uninjected controls. Since multiple foci for cement glands are detected, this suggests that only a subset of cells expressing *X-hh* participate in formation of cement glands. Consistent with this idea, injection of embryos with a mixture of lacZ and *X-bhh* RNAs reveals that many cells expressing β-galactosidase do not express a cement gland marker, XAG-1. Further histological analyses and antibody staining for the muscle marker 12/101 and a neural-specific N-CAM marker detect no additional organized tissues that are induced by *X-hh* activities in either animal cap explants or in embryos (data not shown).

Since cement gland formation can be induced by dorsal mesoderm (Cooke et al., 1987; Sive et al., 1989) it is notable that animal caps from *X-bhh*-injected embryos form cement glands without induction of the mesodermal markers *Xwnt-8*, *goosecoid*, or *Xbra*, as monitored by RT-PCR (Lai et al., 1995), consistent with the idea of direct induction of cement glands from ectoderm. It remains possible that *X-hh* activities exert influences on mesoderm not evident from the expression of these marker genes since, in amniotes, *sonic hedgehog* expression appears to impose pattern upon the mesoderm of limbs and somites (Riddle et al., 1993; Chang et al., 1994; Niswander et al., 1994; Laufer et al., 1994; Fan and Tessier-Levigne, 1994; Johnson et al., 1994).

**Cement gland induction by *X-bhh* is independent of induction of noggin and follistatin**

*noggin* (Lamb et al., 1993) and *follistatin* (Hemmaiti-Brivanlou et al., 1994) have been shown previously to be expressed in cell types and to display activities consistent with potential roles in neural induction. Like *X-bhh*, both *noggin* and *follistatin* can divert animal cap ectoderm to differentiate as cement gland, an extreme anterior cell fate. However, there are significant differences in the activities of these factors, since *X-bhh* has a very limited ability to directly induce neural genes (Lai et al., 1995), whereas both *noggin* and *follistatin* directly induce general neural markers such as N-CAM, and at least in the case of *follistatin*, markers such as *En-2* that are indicative of more posterior neural fates. Thus, the direct inducing activity of *X-bhh* is primarily the cement gland, which is a subset of the reported activities of *noggin* and *follistatin*.

Given this overlap in the ability of *X-bhh*, *noggin*, and *follistatin* to induce the cement gland, it seemed plausible that either *noggin* or *follistatin* might be able to induce the expression of *X-bhh* in animal cap explants or, alternatively, that *X-bhh* activities involve the activation of *noggin* or *follistatin*. We therefore undertook experiments with animal cap explants prepared from embryos previously injected with *X-bhh* mRNA and demonstrated that neither *follistatin* nor *noggin* expression were induced, indicating that the direct cement gland inducing activity of *X-bhh* in naive ectoderm can act independently of *noggin* and *follistatin*. In like manner, animal caps from embryos injected with synthetic *follistatin* or *noggin* mRNA failed to show induction of *X-bhh*. We cannot rule out the possibility that an unidentified *X-hh* is induced by either factor, but since *X-bhh*, *noggin* and *follistatin* are all induced in animal caps by activin, the simplest interpretation is that *X-bhh* is neither directly upstream nor downstream of previously described neural inducers in *Xenopus* and thus represents an independent pathway for induction of the cement gland.

The distinct patterns of expression of *X-bhh*, *X-chh*, and *X-shh* and their common activity in induction of the cement gland in animal cap explants have implications for the mechanisms of action and normal functions of these genes. First, as reviewed above, during neurulation these genes are enriched in overlapping patterns in anterior structures of the embryo and *X-bhh* is expressed in cells adjacent to the cement gland (Fig. 3B). Given these patterns of expression and their common
ability to directly induce cement glands, Xhhs meet the criteria of being expressed in spatial patterns and with demonstrable activities consistent with roles in specifying differentiation of the cement gland and other extreme anterior ectodermal fates. Xhh-expressing cells of axial structures and of the somites are not likely to be involved in the induction of cement gland. In these structures Xhhs may affect patterning through modulation of a common signalling pathway; activation of this pathway in naive ectoderm leads to induction of the cement gland, whereas activation of this pathway in other cell types may have other effects. The ability of distinct members of the Xhh family to induce the cement gland may provide a useful system for further dissecting cellular responses to hh signals.

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REFERENCES


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