Patterning of the neural ectoderm of *Xenopus laevis* by the amino-terminal product of hedgehog autoproteolytic cleavage

Cheng-Jung Lai\(^1\), Stephen C. Ekker\(^2\), Philip A. Beachy\(^2\) and Randall T. Moon\(^1, *\)

\(^1\)Department of Pharmacology, University of Washington School of Medicine and Howard Hughes Medical Institute, Seattle, WA 98195, USA
\(^2\)Johns Hopkins University School of Medicine and Howard Hughes Medical Institute, Department of Molecular Biology and Genetics, Baltimore, MD 21205, USA

*Author for correspondence (e-mail: rtmoon@u.washington.edu)

SUMMARY

The patterns of embryonic expression and the activities of *Xenopus* members of the *hedgehog* gene family are suggestive of roles in neural induction and patterning. We report that these hedgehog polypeptides undergo autoproteolytic cleavage. Injection into embryos of mRNAs encoding *Xenopus banded-hedgehog* (*X-bhh*) or the amino-terminal domain (N) demonstrates that the direct inductive activities of *X-bhh* are encoded by N. In addition, both N and *X-bhh* pattern neural tissue by elevating expression of anterior neural genes. Unexpectedly, an internal deletion of *X-bhh* (ΔN-C) was found to block the activity of *X-bhh* and N in explants and to reduce dorsoanterior structures in embryos. As elevated *hedgehog* activity increases the expression of anterior neural genes, and as ΔN-C reduces dorsoanterior structures, these complementary data support a role for *hedgehog* in neural induction and antero-posterior patterning.

Key words: hedgehog, neural induction, protein processing, *Drosophila*, spatial patterning

INTRODUCTION

The *Drosophila* segment polarity gene *hedgehog* (*hh*) encodes a secreted polypeptide involved in the control of spatial patterning during segmentation, and during development of adult structures including the appendages and the eye (Mohler, 1988; Ingham et al., 1991; Lee et al., 1992, 1994; Ma et al., 1993; Heberlein et al., 1993; Basler and Struhl, 1994; Heemskerk and DiNardo, 1994). The recent cloning of members of the vertebrate *hh* family has provided an opportunity to study the involvement of *hh* in pattern formation in vertebrates (reviewed by Smith, 1994). One of the vertebrate *hh* family members, *sonic hedgehog* (*shh*), is expressed in the notochord, the floorplate of the neural tube and the zone of polarizing activity in limbs, all of which are embryonic structures considered to act as sources of patterning influence on neighboring structures. Moreover, misexpression of *shh* in vertebrate embryos supports the potential involvement of *shh* in patterning in the neural tube, the limb, and somites (Echelard et al., 1993; Krauss et al., 1993; Riddle et al., 1993; Chang et al., 1994; Fan and Tessier-Lavigne, 1994; Johnson et al., 1994; Laufer et al., 1994; Niswander et al., 1994; Roelink et al., 1994).

A recent study has revealed that endogenous *hh* protein in *Drosophila* is found predominantly as amino- (N) and carboxy- (C) terminal fragments derived by an internal autoproteolytic cleavage of a precursor (U) (Lee et al., 1994). This autoproteolytic cleavage has also been observed with zebrafish *shh* and *twhh* (Lee et al., 1994) as well as mouse *shh* (Chang et al., 1994; Bumcrot et al., 1995; Porter et al., 1995). The existence of these *hh*-derived polypeptides in many species and the observation that the N and C domains differ in their spatial localization with respect to the site of synthesis raise the possibility that each domain has a distinct function (Lee et al., 1994).

In the accompanying paper (Ekker et al., 1995) we describe the isolation of *Xenopus hh* genes and present evidence that *banded hedgehog* (*X-bhh*), *cephalic hedgehog* (*X-chh*), and *sonic hedgehog* (*X-shh*) are expressed in anterior and neural ectoderm as well as other tissue layers, consistent with potential roles in induction and patterning. To test for inductive activities, members of the *X-hh* gene family were overexpressed in embryos, which resulted in the induction of ectopic cement glands and the enhancement of dorso-anterior structures (Ekker et al., 1995). To investigate whether *X-hhs* can directly divert prospective ectoderm to another fate, *X-hhs* were overexpressed in animal caps, where they were found to strongly induce cement gland. Thus *hh* joins noggin (Lamb et al., 1993) and follistatin (Hemmati-Brivanlou et al., 1994) as putative signalling factors capable of inducing an extreme anterior ectodermal structure, the cement gland.

In the present study we establish that *X-bhh* protein is processed to generate N and C domains, and we investigate whether the resulting *X-bhh*-derived polypeptides have biological activity in animal cap explants and in embryos. We report that N, like *X-bhh*, has the ability to directly induce cement gland. Since both *follistatin* and *noggin* induce anterior
neural gene expression in addition to cement gland, we next investigated whether X-bhh or X-bhh-derived polypeptides could induce neural genes. We found that both X-bhh and N have a very weak ability to directly induce extreme anterior neural markers, and that both synergistically elevate the expression of these neural markers in explants of embryos which are under the influence of other neural inducers. These results reveal an unexpected activity of hh as an anteroposterior neural patterning agent. Surprisingly, an internal deletion of X-bhh, ΔN-C, is an inhibitor of X-bhh activity in animal caps. When injected into embryos, ΔN-C reduces drososteatior structures, consistent with the hypothesis that it is interfering with the activity of endogenous Xhhs. Together with the results of X-bhh overexpression, the loss-of-function analysis represented by ΔN-C activity supports a role for hedgehog in neural induction and patterning along the anteroposterior axis.

MATERIALS AND METHODS

Expression constructs

All hh constructs were prepared in the vector pT7TS, which adds globin untranslated regions for RNA stability (a gift from A. Johnson and P. Krieg, University of Texas, Austin). Preparation of full-length X-bhh in this vector is described in the accompanying paper (Ekker et al., 1995). The N, ΔN-C, and UHA constructs (Fig. 1B) were made by in vitro mutagenesis of expression construct pT7TS-X-bhh using the method of RPCR (Jones and Winistorfer, 1992). The sequence of all constructs was confirmed by dideoxy sequencing. Amino acids 1-23 of X-bhh are predicted to encode a signal sequence. As N contains amino acids 1-197 of X-bhh, cleavage of the signal sequence would result in a polypeptide of amino acids 24-197. ΔN-C deletes amino acids 28-194 of X-bhh. The primary translation product is predicted to undergo signal sequence cleavage removing amino acids 1-23, and to undergo autoproteolysis. Based on the cleavage site in Drosophila hh (Porter et al., 1995) autoproteolysis would generate a C domain of X-bhh amino acids 198-409, as well as a predicted seven amino acid polypeptide, representing amino acids 24-27, and 195-197. In the construct UHA, the histidine at position 270 has been converted to an alanine. Construct pT7TS-X-shh-N was similarly constructed and encodes the first 198 amino acids of the X-shh open reading frame (Ekker et al., 1995). The frame shift construct, X-shhΔ530, is described in Ekker et al. (1995).

In vitro translations

In vitro translations were performed according to manufacturer’s instructions (Promega) using [35S]methionine with pT7TS-X-bhh and derivatives, pT7TS-X-shh, and pBS-X30 (X-chh cDNA construct in pBluescript) as templates.

Immunoprecipitation of X-bhh polypeptides

Embryos were co-injected with [35S]methionine and synthetic X-bhh or UHA RNA as described by Giebelhaus et al. (1987). Whole embryo extracts were made according to the method of Moon and Christian (1989) and subsequently used in the immunoprecipitation protocol (method B) of Blikstad et al. (1983) using the antibody X-bhhC2A. This polyclonal antibody was made in rabbits (Babco, Richmond, CA) to the following peptide sequence: GTQPSQQMGI (residues 370-379 of X-bhh), which was coupled to keyhole limpet hemocyanin.

Embryological methods

Manipulation of eggs and embryos for microinjection, and culturing of embryos, was accomplished as previously described (Moon and Christian, 1989). For experiments involving blastula cap explants, up to 1.5 ng of synthetic mRNAs were injected into the animal pole of each blastomere at the 2 cell stage, and blastula caps representing the upper one quarter to one eighth of the embryo were isolated at stage 8, and then cultured in vitro until control embryos reached stage 25. For experiments involving Keller sandwiches, which were prepared according to the method of Doniach et al. (1992), the mRNAs were injected into the animal pole of each blastomere at the 2 cell stage. Where indicated, blastula caps were incubated in recombinant human activin A, as previously described (Christian and Moon, 1993).

In situ hybridization

Blastula caps and intact embryos were processed for in situ hybridization as described by Harland (1991) using probes for Otx-A (a gift from R. Harland, University of California at Berkeley), the pan neural RNP clone pNPG152 (a gift from P. Good and I. Dawid, NIH), the cement gland marker XAG-1 (a gift from H. Sive, Whitehead Institute, Cambridge, MA), and the pituitary probe XANF-2 (a gift from P. Mathers and M. Jamrich, FD, Rockville, MD).

Analysis of RNA by RT-PCR

RNA was extracted from blastula caps and from embryos according to the method of Chomczynski and Sacchi (1987). RNA was then treated with RQ-1 DNase (Promega). In each group of experiments, approximately 60-70 animal caps or 5 embryos were prepared and 3 μg of RNA was used to generate cDNA using first strand synthesis kits from Life Sciences (St. Petersburg, Florida), in the presence or absence of reverse transcriptase as noted in the figure legends. cDNA was then used for RT-PCR using previously described primers and conditions for X-bra, gsc, Xwnt-8, En-2, Krox-20, Xlbox-6, N-CAM, and muscle actin (Hemmati-Brivanlou et al., 1994), and control experiments confirm the use of these published conditions to approximate exponential amplification. For detection of XAG-1, the primers were 5’ CTGACTGTCCGATCAGAC 3’ and 5’ GAG TTGCTTCTGCGCAT 3’ (amplified for 19 cycles), and for detection of XANF-2, the primers were 5’ AGTCATCATTTGCAGAC 3’ and 5’ AGTCGATCATTTGCAGAC 3’ (amplified for 29 cycles, at 58°C for annealing temperature). For detection of Otx-A, the primers were 5’ CATCGGACATAAAGCACGCCTATC 3’ and 5’ CTTCCCCTCTCCTGTTCCTGG 3’ (amplified for 24 cycles, at 58°C for annealing temperature). PCR products of previously unpublished primer sets were isolated from gels and sequenced. The use of PCR in our analyses is neither quantitative nor quantitative, comparisons, and if non-exponential amplification were to have occurred in some experiments it would have led to an underestimate of the magnitude of the described phenomena. X-ray films were routinely exposed until bands were evident in control lanes to ensure that we did not underestimate relative levels of PCR product by underexposure, or through inadequate amplification.

RESULTS

Autoproteolytic cleavage of Xenopus hh homologs

Endogenous hh protein in Drosophila is found predominantly as amino- and carboxy-terminal fragments (N and C, respectively) derived by an internal autoproteolytic cleavage of a larger precursor (U) (Lee et al., 1994). This raises the question of whether the ability of X-bhh to induce cement gland (Ekker et al., 1995) involves the activity of the full-length polypeptide, or potential proteolytic derivatives. We first tested X-bhh, X-shh, and X-chh for proteolytic processing following in vitro translation in a rabbit reticulocyte lysate, and found multiple polypeptide species (arrowheads, Fig. 1C). The sizes of the three proteins generated in these reactions are consistent with the autoproteolytic cleavage of the largest polypeptides (top arrowhead in each lane of Fig. 1C) to generate two smaller
fragments (bottom arrowheads). If these polypeptide species arise through an autoproteolytic mechanism similar to that in
*Drosophila*, which requires a histidine at amino acid residue
329 (Lee et al., 1994), then mutation of this highly conserved
amino acid (Lee et al., 1994; Ekker et al., 1995) should prevent
formation of these polypeptide species. The mutation of this
histidine to an alanine in the construct UHA (Fig. 1B) blocks
proteolytic cleavage of X-bhh in vitro (compare lanes 4 and 5,
Fig. 1D), suggesting that this cleavage occurs by an autopro-
teolytic mechanism similar to that in *Drosophila* (Lee et al., 1994).

To investigate whether X-bhh is processed in embryos, and
whether it can be blocked by the UHA mutation, synthetic
mRNA was co-injected into fertilized eggs with [35S]methio-
nine. Extracts prepared from cleavage-stage embryos were
subjected to immunoprecipitation using an antibody against a
region of the C terminus of X-bhh (see Materials and methods).
Injection of X-bhh (Fig. 1E, lane 12) or UHA (lane 11) leads to
the synthesis of a polypeptide which migrates just below the
50x10^3 M_r marker (arrowhead upper gel, Fig. 1E) on SDS gels.
As this band co-migrates with the in vitro translation product of
X-bhh RNA (upper gel, lane 10), it represents X-bhh prior
to autoproteolysis. Two additional lower molecular mass
species were immunoprecipitated from X-bhh-injected
embryos, (Fig. 1E, arrowheads in bottom gel of lane 12), the
smaller of which co-migrates with C derived from in vitro
translation (lane 9; see below for a description of the construct
AN-C). No corresponding bands were detected following
injection of UHA RNA (lower gel, lane 11), suggesting that
Xbhh is processed in vivo.

A comparison of the sequences of the predicted N and C
domains of different members of the *Xenopus hh* family
reveals that the N domains are quite conserved, whereas the
C domains display considerable divergence (Fig. 1A). Con-
structs were generated to test the activities of polypeptides
derived from these predicted domains (Fig. 1B). The N
construct was generated to mimic the cleavage site in
*Drosophila* hh, which was defined by direct sequencing of the
C cleavage product (Porter et al., 1995). Another construct
(AN-C) removes most of N, leaving the signal sequence and
several residues amino-terminal to the predicted cleavage site
(see Materials and Methods). After translation and cleavage of
the signal sequence, AN-C would be expected to yield a
polypeptide which has not undergone autoproteolysis, and two
polypeptides which arise from autoproteolysis: a fully
processed C-terminal domain, and a predicted seven amino
acid peptide containing residues to the amino terminal side of
the autoproteolytic cleavage site (Fig. 1B; Materials and
Methods).

**Inductive activities of hh-derived polypeptides in
animal cap explants**

We next tested whether either N or ΔN-C, which encode
amo- and carboxy-terminal regions of X-bhh, respectively,
display the ability of full-length X-bhh to divert prospective
ectoderm to differentiate as cement gland (Ekker et al., 1995).
Animal caps from RNA-injected embryos were explanted at
the blastula stage, and processed for in situ hybridization for
the cement gland marker, *XAG-1* (Sive et al., 1989; Table 1).
N induces foci of *XAG-1* hybridization signal in over 90% of
explants, as reported for X-bhh (Ekker et al., 1995). In contrast,

<table>
<thead>
<tr>
<th>RNA</th>
<th>Dose injected</th>
<th>XAG-1 induction (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninjected</td>
<td>3 ng</td>
<td>0% (9)</td>
</tr>
<tr>
<td>X-bhh-N</td>
<td>3 ng</td>
<td>97% (35)</td>
</tr>
<tr>
<td>X-bhh-ΔN-C</td>
<td>3 ng</td>
<td>0% (33)</td>
</tr>
<tr>
<td>X-bhh-UHA</td>
<td>3 ng</td>
<td>92% (38)</td>
</tr>
<tr>
<td>X-shh-N</td>
<td>3 ng</td>
<td>89% (19)</td>
</tr>
</tbody>
</table>

ΔN-C shows no hybridization signal. From these results we
conclude that N, unlike ΔN-C, is sufficient for the induction of
cement gland. As the corresponding N construct from X-shh
was also able to induce XAG-1 in animal caps (Table 1), it is
likely that the inductive activity of N is conserved between
members of the hh family gene.

We then asked whether this inductive activity of N was
dependent upon autoproteolysis of X-bhh. Injection of the
autoprocessing-defective UHA and analysis of blastula caps
reveals that UHA induces cement gland, and thus autoproteol-
ysis is not an absolute requirement for the induction of this
structure (Table 1). It is worth noting that the UHA mutation in a
transgenic strain of *Drosophila* strongly reduces but does not
abolish hh activity (Lee et al., 1994), suggesting that
differences in activity may exist at physiological levels of the
polypeptides.

The secreted factors noggin (Lamb et al., 1993) and follis-
tatin (Hemmati-Brivanlou et al., 1994) have been reported to
induce cement gland in addition to inducing neural gene
expression. As we found that only X-bhh, N and UHA induced
cement gland which could be scored by histological and whole-
mount in situ hybridization assays, we focused on these con-
structs to ask whether they could directly induce neural markers
in animal cap explants. Consistent with the histology and in situ
hybridizations, RT-PCR analysis demonstrated that X-bhh (Fig.
2, lane 6), N (lane 8) and UHA (lane 10) induce the expression of
the cement gland marker *XAG-1*. When we examined the
expression of the pituitary marker, XANF-2 (Mathers et al.,
1995), and the forebrain marker, Otx-A (Lamb et al., 1993), we
found that X-bhh (Fig. 2, lane 6), N (lane 8), and UHA (lane 10)
induce these genes, albeit to much lower levels than the neural
inducer, noggin (Lamb et al., 1993) (lane 14). Neither X-bhh,
N, nor UHA induces expression of the general neural marker,
N-CAM (Kintner and Melton, 1987), or muscle actin (Gurdon
et al., 1985) (Fig. 2), nor does X-bhh induce early mesodermal
markers in animal caps (Fig. 4A). Our analysis shows that the
ability of X-bhh to strongly induce cement gland in the absence
of mesoderm and to weakly induce anterior neural markers can
be fully explained by the activity of the N-terminal hh-derived
polypeptide.

**X-bhh modifies the anteroposterior pattern of neural
gene expression in explants under the influence of
endogenous neural inducers**

The results described above show that full-length X-bhh and N
directly induce formation of the cement gland and display a
limited ability to induce anterior neural genes in naive
ectoderm. To address the question of whether X-bhh can
cooperate with additional signals normally present during
neural induction, we asked whether X-bhh can alter neural gene
expression in explants from gastrula embryos which are in the process of forming neural tissues. The explants we used were Keller sandwiches, consisting of planar explants of dorsal ectoderm and mesoderm that are made from early gastrulae before the mesoderm has come into vertical contact with the ectoderm (Fig. 3A). These explants constitute an in vitro experimental system which has been used for studies of neural induction and patterning (Keller and Danilchik, 1988; Doniach, 1993). Data suggest that an extensive number of neural genes, representing discrete positions along the anteroposterior axis, can be induced in these explants by planar signaling in the absence of vertical signaling from dorsal mesoderm (Doniach, 1993; Mathers et al., 1995).

To assess the ability of X-bhh to modify the anteroposterior pattern of neural gene expression in Keller sandwiches, we examined a range of neural markers in explants from uninjected controls and from X-bhh-injected embryos. The expression of the pituitary gland marker XANF-2 and the forebrain marker Otx-A are higher in the Keller sandwiches from embryos injected with X-bhh (Fig. 3B lane 4) than in the control Keller sandwiches (lane 2). In contrast, the expression of En-2 (a midbrain hindbrain boundary marker; Hemmati-Brivanlou et al., 1991), Krox-20 (a hindbrain marker; Bradley et al., 1993) and XlHbox-6 (a spinal cord marker; Wright et al., 1990) decrease in the X-bhh sandwiches, when compared with uninjected control explants. We observed negligible effects of X-bhh on levels of N-CAM induction in this assay system, suggesting that X-bhh does not globally affect neural gene expression. Instead, X-bhh is able to induce higher levels of anterior neural markers such as Otx-A and XANF-2 to an extent greatly exceeding its capacity to directly induce these anterior genes in animal cap assays, suggesting that X-bhh acts as a neural patterning agent.

**X-bhh modifies the anteroposterior pattern of neural gene expression in animal caps treated with the indirect neural inducer, activin**

The results of the experiments with Keller sandwiches show
that X-bhh can enhance the expression of anterior neural genes and can reduce the expression of posterior neural genes in tissues undergoing neural induction. This raises the question of whether this effect on neural patterning involves changes in the mesoderm or exclusively in the neural ectoderm. To address this and to corroborate the neural patterning activity in an independent assay, we examined the effects of X-bhh on animal caps treated with the mesoderm inducing factor activin (Smith et al., 1990). Activin directly induces both dorsal and ventral mesoderm and secondarily promotes the formation of neural tissue in animal cap explants, allowing one to investigate the expression of neural genes spatially expressed along the anteroposterior embryonic axis (reviewed by Harland, 1994). Embryos were injected at the 2 cell stage either with X-bhh mRNA or, as a control for expression of a secreted protein, with RNA encoding bovine prolactin. The embryos were allowed to develop to the blastula stage, and animal caps were dissected and cultured with or without activin. A portion of the explants were analyzed for the expression of early mesodermal markers, demonstrating that the activin-induced expression of a dorsal mesoderm marker, goosecoid (Cho et al., 1991), and a ventral mesoderm marker, Xwnt-8 (Christian et al., 1991), were not influenced by X-bhh (Fig. 4A compare lane 8 to lane 6), whereas the general mesodermal marker Xbra (Smith et al., 1991) was slightly reduced. As described below, the remaining animal caps were assayed at a later stage for the effects of X-bhh on neural gene expression.

Animal caps treated with activin express neural genes that normally are expressed in discrete domains along the anteroposterior axis of embryos (Fig. 4B, lane 6, with the most anterior spatially restricted markers at the top). When animal caps from embryos injected with X-bhh were treated with activin (Fig. 4B, lane 8), dramatic increases in expression of the anterior markers XAG-1, XANF-2 and Otx-A were observed in comparison to activin-treated explants from uninjected (lane 6) and prolactin-injected (lane 10) embryos. Comparing X-bhh-injected animal caps either with or without activin (Fig. 4B, lanes 8 and 4, respectively), Otx-A and XANF-2 are reproducibly induced to greater levels by the combined treatment, whereas the cement gland marker, XAG-1 is reduced. Regarding the effects on XAG-1, it is worth noting that since cement gland forms only from ectoderm, and activin induces mesoderm and ultimately neural ectoderm, less ectoderm would remain available in the animal cap to respond to the effects of hh. Notably, the cooperation of X-bhh and neural inducing factors within the activin-treated animal caps to elevate the relative levels of these transcripts was specific to anterior markers as the expression of the more posterior markers, En-2, Krox-20, and XlHbox-6, either decreased slightly or did not change (Fig. 4B lane 8) relative to the control activin-treated animal caps (lanes 6 and 10). A similar trend was observed in the Keller sandwiches (Fig. 3).

Collectively, the results demonstrate that X-bhh increases the expression of the anterior neural markers XANF-2 and Otx-A in activin-treated explants, and does not increase the expression of mesodermal or general neural markers. Given that the relative induction of XANF-2 and Otx-A is greatest in the animal caps with combined X-bhh and activin treatment compared to either treatment alone, these results corroborate the Keller sandwich experiments and support the hypothesis that X-bhh promotes anterior neural cell fates in ectodermal tissue that is under the influence of neural inducers. Thus, in addition to its ability to directly induce cement gland in naive ectoderm (Ekker et al., 1995), X-bhh displays the characteristics of a neural patterning agent.
Opposite effects of N and ΔN-C in activin-treated animal caps

We next investigated whether the neural patterning activity of X-bhh was inherent to N, which fully mimics X-bhh in the direct induction of cement gland and anterior neural markers in naive ectoderm. As shown in Fig. 4C, N behaves like X-bhh in that it induces elevated levels of XANF-2 and Otx-A (lane 6) relative to control activin-treated animal caps (lane 4). Moreover, N also leads to a decrease in the expression of more posterior markers, such as krox-20 and Xlhbox-6, as observed following injection of X-bhh.

As described in the Discussion, ΔN-C is expected to generate multiple polypeptide species, including a mature C-terminal domain and an approximately seven residue peptide product of autoproteolysis that is derived from the N domain, and which normally would not exist in embryos (Fig. 1B). Although ΔN-C does not induce XAG-1 in animal caps (Table 1), we tested its effects in activin-treated explants to assay for activity in the context of ongoing neural induction. In contrast to the activity of N (Fig. 4C, lane 6), ΔN-C decreases the expression of the anterior neural genes XANF-2 or Otx-A (Fig. 4C, lane 8) in activin-treated animal caps when compared to uninjected controls (lane 4). Moreover, ΔN-C also leads to an increase in the expression of more posterior markers, such as En-2 and Xlhbox-6.

ΔN-C interferes with X-bhh and N activity in animal cap explants

To pursue this further, embryos were injected with X-bhh or N, both of which had been mixed with equivalent amounts of either prolactin or ΔN-C RNA to normalize the total amount of RNA injected. Animal cap explants were prepared and analyzed by RT-PCR for the expression of XAG-1, a marker strongly induced by N. Interestingly, we found that ΔN-C reduces the induction of XAG-1 by X-bhh (Fig. 5, compare lanes 4 and 6), and is also effective in blocking the ability of N to induce XAG-1 (compare lanes 8 and 10). Whether this effect of ΔN-C is attributable to the primary translation product or to products arising from autoproteolysis (Fig. 1B) is unknown.

Distinct effects of expression of N and ΔN-C in Xenopus embryos

In the experiments described above, we establish that (a) N-inductive activities of X-bhh-derived polypeptides in animal cap explants.

Animal caps from embryos injected with various RNAs were cultured until sibling embryos had reached stage 25, at which time samples were processed for RT-PCR for the markers shown. Minus (−) lanes are controls omitting reverse transcriptase in the first strand synthesis, and plus (+) lanes contain reverse transcriptase. Lanes 1, 2: stage 25 embryos as positive controls. Lanes 3, 4: animal caps from uninjected embryos as negative controls. Lanes 5, 6: animal caps from embryos injected with X-bhh RNA. Lanes 7, 8: animal caps from embryos injected with N. Lanes 9, 10: animal caps from embryos injected with UHA. Lanes 11, 12: animal caps from embryos injected with a frame-shifted version of X-shh (X-shhfs) as a negative control. Lanes 13, 14: Animal caps from embryos injected with noggin RNA for comparison of induced neural markers. Note the high-level induction by active constructs (X-bhh, N, and UHA) of the cement gland marker XAG-1 and the relatively lower level induction of the anterior neural markers XANF-2 and Otx-A, without induction of the general neural marker, N-CAM.
mimics $X\text{-}bhh$ in all explant assays and (b) $\Delta N\text{-}C$ interferes with the activity of both injected $N$ and $X\text{-}bhh$ in animal caps. Two hypotheses could be tested by injection of these RNAs into embryos. First, injection of $N$ would complete the analysis of whether this $hh$-derived polypeptide encodes most apparent inductive and patterning activities of full-length $X\text{-}bhh$. Second, as $\Delta N\text{-}C$ interferes with the activity of $hh$ in explants, injection of this construct might be expected to interfere with endogenous $hh$, thereby testing the importance of $N$-mediated signaling in embryos. We therefore injected $N$ and $\Delta N\text{-}C$ into 2-cell embryos and analyzed the morphology of tadpoles (Fig. 6A) and the expression patterns of a pan-neural RNP marker (Fig. 6B), and a forebrain marker, $Otx-A$ (Fig. 6C). Interestingly, while $N$ has the ability to expand expression of the cement gland in 79% of embryos ($n=61$) (Fig. 6A) and to induce the formation of ectopic cement glands (data not shown), as also reported for full-length $X\text{-}bhh$ (Ekker et al., 1995), $\Delta N\text{-}C$ reduces the size of dorsoanterior structures including the cement gland in 97% of injected embryos ($n=92$) (Fig. 6A). This effect is also apparent in embryos stained with a pan-neural RNP marker or with a forebrain marker, $Otx-A$. Specifically, 72% of embryos ($n=29$) injected with $N$ show expanded neural structures anterior to the otic vesicle, while 94% of embryos ($n=47$) injected with

---

Fig. 3. $X\text{-}bhh$ modifies the anteroposterior pattern of neural gene expression in explants under the influence of endogenous neural inducers. (A) Isolation of dorsal explants from injected embryos for the preparation of Keller sandwiches (Keller and Danilchik, 1988; Doniach et al., 1992; redrawn from Doniach, 1993). (B) Keller sandwiches were made from un.injected (lanes 1 and 2) and $X\text{-}bhh$-injected (lanes 3 and 4) embryos, total RNA was isolated when control embryos reached stage 20, and RT-PCR was used to analyze the expression of XAG-1 and neural markers. XAG-1 is a cement gland marker, XANF-2 is an anterior pituitary marker, $Otx-A$ is a forebrain marker, En-2 demarcates the midbrain-hindbrain boundary, Krox-20 marks rhombomeres 3 and 5 of the hindbrain, and $Xlhbox-6$ is a spinal cord marker. N-CAM is a general neural marker whose expression is not restricted along the anteroposterior axis. The EF-1\alpha control demonstrates that a comparable amount of RNA was assayed in each set. Note that expression of XAG-1 and anterior neural markers is stimulated by $X\text{-}bhh$ treatment, whereas expression of posterior neural markers is suppressed.
Fig. 4. X-bhh and derived polypeptides modify the anteroposterior pattern of neural gene expression in activin-treated animal caps. Embryos were injected with X-bhh or prolactin RNA and animal cap explants were isolated from blastulae and incubated in the presence or absence of activin. (A) Explants were processed when the sibling embryos reached stage 11, and total RNA was assayed by RT-PCR for the mesodermal markers brachyury (Xbra), goosecoid, and Xwnt-8 and the control EF-1a. Lanes designated plus and minus refer to the presence or absence of reverse transcriptase in the first strand cDNA synthesis. Lanes 1, 2, 5, 6: control animal caps from uninjected embryos. Lanes 3, 4, 7, 8: animal caps from embryos injected with X-bhh. Lanes 9, 10: animal caps from embryos injected with bovine prolactin RNA. Animal caps in lanes 5-10 were treated with activin A. Note that no mesodermal markers are induced by X-bhh. (B) A second group of explants from the same experiment and in the same order as in A, were cultured until tailbud (stage 25) and assayed for the expression of anterior-posterior neural markers. The actin acted as a control for induction of mesoderm. Expression of anterior neural markers was enhanced by combined treatment with activin and X-bhh relative to either treatment alone; note also the reduction in expression of posterior neural markers by X-bhh in activin-treated explants. (C) In independent experiments, embryos were injected with N or ΔN-C, and some animal cap explants were treated with activin before culturing until sibling embryos reached tailbud stage. Lanes 1, 2: control animal caps from uninjected embryos. Lanes 3, 4: control animal caps from uninjected embryos, treated with activin. Lanes 5, 6: animal caps from embryos injected with N and treated with activin. Lanes 7, 8: animal caps from embryos injected with ΔN-C and treated with activin. Whereas N displays activities in activin-treated explants similar to those of X-bhh (see B) ΔN-C produces the opposite effect, decreasing anterior and increasing posterior neural marker expression.
ΔN-C develop with reduced anterior neural structures as compared with the wild-type control embryos (Fig. 6B). When we analyzed the development of the forebrain in these embryos, we found that 56% of embryos (n=45) injected with N show an overall pattern compared with the wild-type control embryos (Fig. 6C). In contrast, embryos injected with ΔN-C have a similar overall pattern compared to controls, but the total amount of staining was reduced in all injected embryos (n=45) (Fig. 6C).

**DISCUSSION**

X-bhh, X-chh, and X-shh are expressed in embryos in spatial patterns that are consistent with roles including neural induction and patterning (Ekker et al., 1995). In explant assays, the X-hh family members display a shared activity of directly inducing an anterior ectodermal structure, the cement gland (Ekker et al., 1995), which is also induced by the previously described neural inducers noggin (Lamb et al., 1993) and follistatin (Hemmati-Brivanlou et al., 1994). The X-hh family members thus meet the criteria of being expressed in the appropriate tissues and with measurable activities that are consistent with an involvement in anteroposterior neural induction and patterning, though additional functions are likely based on the distinct patterns of Xhh expression (Ekker et al., 1995). In this study, we first demonstrated that members of the Xhh family undergo autoproteolysis as reported for Drosophila hh (Lee et al., 1994) and mouse shh (Porter et al., 1995), and that the amino-terminal domain (N) accounts for the ability of Xhh to directly induce cement gland. By focusing on X-bhh as a representative member of a gene family that displays a common activity, we then demonstrate that both X-bhh and N pattern ectoderm undergoing neural induction, by further increasing the expression of the same anterior neural genes which they directly induce to low levels. Finally, we demonstrate that ΔN-C, a form of X-bhh that deletes all of the N domain except for seven residues, interferes with the activity of X-bhh and N in explants, and reduces dorsoanterior structures in embryos, consistent with the hypothesized role of endogenous X-hh.

**Patterning activities of Xenopus hedgehogs**

To test the possibility that X-hh interacts with other neural inducing factors to pattern neural ectoderm, we prepared Keller sandwiches from the dorsal side of embryos injected with X-bhh. This allowed X-bhh the opportunity to interact with endogenous neural inducing signals. In Keller sandwiches, which undergo planar neural induction, X-bhh increases the expression of the pituitary marker XANF-2 and the forebrain marker Otx-A over that observed with control Keller sandwiches. X-bhh also reduced the expression of a range of more posterior neural markers. These data suggest that X-hh has the capacity to synergize with other signals in differentiating neural ectoderm and thus enhance anterior neural cell fate. Whether these other signals include basic fibroblast growth factor, recently implicated in neural induction (Kengaku and Okamoto, 1993), or other neural inducing agents such as noggin (Lamb et al., 1993) and follistatin (Hemmati-Brivanlou et al., 1994), which are co-expressed in both activin-treated animal caps and in Keller sandwiches, is a tenable but largely untested hypothesis. However, the data are consistent with the idea of Nieuwkoop that multiple signals are likely to be involved in neural induction and patterning (Nieuwkoop, 1985; reviewed by Doniach, 1993, Ruiz i Altaba, 1994).

As the Keller sandwiches contain ectoderm, neural ectoderm, and mesoderm, the experiments described above did not address the issue of whether changes in mesodermal gene expression accompany the elevation of expression of the anterior markers XANF-2 and Otx-A. We therefore compared the expression of mesodermal and neural genes in animal caps treated with activin, which were first isolated from un.injected embryos, and from embryos injected with X-bhh. Neural induction in activin-treated explants is likely to occur in response to signals from the dorsal mesoderm which is induced by activin (reviewed by Harland, 1994). We found that X-bhh had no effect on dorsal mesodermal gene expression in the activin-treated animal caps, while elevating the expression of the anterior markers XANF-2 and Otx-A to greater levels than was achieved by X-bhh or activin alone. Taking into account these results and the comparable results from the Keller sandwiches, X-bhh is likely to act directly on neural ectoderm, and in conjunction with other neural inducing factors, to divert cell fate towards anterior cell types along the anteroposterior continuum. We propose that X-bhh is able to directly induce ectoderm to differentiate as cement gland, but that its greatest effect on anterior neural gene expression, as monitored by XANF-2 and Otx-A, is primarily as a neural patterning agent.
The N-terminal X-bhh-derived polypeptide has inductive and patterning properties

Given that all hh proteins identified to date in both Drosophila and in vertebrates appear to undergo autoproteolytic cleavage (Lee et al., 1994; Chang et al., 1994), it is likely that the cleavage products possess biological activity. In this study, we asked whether hh-derived polypeptides have activities that would account for the observed activities of the full-length X-bhh. We found that the N-terminal polypeptide is as active as the full-length X-bhh in terms of cement gland induction, in direct low-level neural induction as monitored by the markers XANF-2 and Otx-A, and in synergy with activin treatment of explants to express elevated levels of these markers. N thus displays the complete inductive and patterning activity of the full-length X-bhh in animal caps. Importantly, when N is over-expressed in embryos, they develop with enlarged dorsoanterior structures including enlarged and ectopic cement glands, as we observed with X-bhh (Ekker et al., 1995). Interestingly, N appears to be functional in the context of the unprocessed protein, as a mutation which blocks autoproteolytic cleavage did not block the ability of the protein to induce cement gland.

\( \Delta N\text{-}C \) reduces the induction of cement gland by N and X-bhh in explants, and reduces dorsoanterior structures in embryos

Following translation and cleavage of the signal sequence, \( \Delta N\text{-}C \) is predicted to generate three polypeptides (Fig. 1B). The largest species would consist of an internal deletion of the N domain, along with all of the C domain. The remaining two polypeptides would arise following autoproteolysis, and would consist of the C domain carboxy-terminal to the proteolytic cleavage site, and a predicted seven residue peptide amino terminal to the cleavage site (Fig. 1B, and note lowest molecular mass species following in vitro translation and processing, Fig. 1D, lane 7). Based on comparison with the cleavage site in Drosophila (Porter et al., 1995), the predicted C domain would begin at Cys\(_{598}\) after autoproteolysis. To investigate the specific activities of the C domain in the absence of any amino acids from the N domain would require preparation of a construct which generates a polypeptide that undergoes signal sequence cleavage just prior to this residue. Our construct \( \Delta N\text{-}C \) comes close to meeting this goal, but clearly generates a small polypeptide from the N domain following autoproteolysis (Fig. 1B). Because of this caveat, the effects of injecting \( \Delta N\text{-}C \) cannot be considered as a strict assay of the C domain. Despite this qualification, expression of \( \Delta N\text{-}C \) has interesting effects in explants and embryos that support the importance of X-hhs and N in embryogenesis, and which raise questions for further investigation regarding potential activities of the polypeptides arising from \( \Delta N\text{-}C \).

Although \( \Delta N\text{-}C \) does not induce the cement gland marker XAG-1, it decreases the expression of anterior ectodermal and neural markers in activin-treated animal caps. Thus, \( \Delta N\text{-}C \) has the capacity to affect neural patterning. As we also observed that \( \Delta N\text{-}C \) promotes an increase in posterior neural markers in activin-treated animal caps, our data do not distinguish between the possibilities that \( \Delta N\text{-}C \) might have a direct signaling role which promotes posterior neural patterning, or that this effect on posterior neural gene expression is an indirect consequence of suppression of anterior inducing activity.

After observing that \( \Delta N\text{-}C \) reduces the expression of anterior neural genes in animal caps treated with activin, we postulated that \( \Delta N\text{-}C \) might interfere with X-bhh that had been induced by activin. To test this hypothesis, we asked whether co-expression of \( \Delta N\text{-}C \) with N, or with the full length X-bhh, would alter the inductive activities of N or X-bhh. We found that mixing \( \Delta N\text{-}C \) with the other two mRNAs at a 1:1 ratio leads to a dramatic inhibition of the induction of cement gland in animal cap assays, supporting the hypothesis. Taking this reasoning further, if there were normally any role for an

![Image](https://via.placeholder.com/150)

**Fig. 6.** Distinct effects of expression of N and \( \Delta N\text{-}C \) in Xenopus embryos. Uninjected embryos (WT), and embryos injected with N, or \( \Delta N\text{-}C \) were photographed at tadpole stage (A) or analyzed by in situ hybridization (B, C). (A) Cement glands (arrow) and other anterior structures are enlarged in N-injected embryos. In contrast, embryos injected with \( \Delta N\text{-}C \) display a smaller cement gland, reduced anterior structures, and enhanced posterior structures. (B) In situ hybridization was performed with a pan neural RNP marker. Arrows indicate the otic vesicle. The neural tissue anterior to the otic vesicle is enlarged in N-injected embryos, and reduced in \( \Delta N\text{-}C \)-injected embryos as compared to control embryos. (C) In situ hybridization was performed with the forebrain marker Otx-A. The pattern of Otx-A expression (arrows) is expanded in N-injected embryos while expression in \( \Delta N\text{-}C \)-injected embryos is similar, but reduced in comparison to that of control embryos.
endogenous X-hh in the formation of cement gland then one prediction is that embryos injected with ΔN-C should exhibit reduced or no cement glands. An examination of the ΔN-C embryos in Fig. 6 supports this hypothesis. However, the general pattern of expression of the forebrain marker, Otx-A, was recognizable though reduced in staining after injection of ΔN-C, suggesting that ΔN-C does not completely disrupt anterior neural patterning. We note that additional defects may exist in these embryos, but it seems most logical to pursue the specific mechanisms underlying the activity of ΔN-C prior to further use of this tool for perturbing hh activity. At present, we speculate that both in activin-treated animal caps and in embryos, ΔN-C acts to reduce the activity of N derived from endogenous X-hh. If endogenous C has a similar capacity to modulate the function of N in vivo, it is worth noting that current data suggest that C may be more diffusible than N from the site of hh synthesis (Lee et al., 1994; Bumcrot et al., 1995; Porter et al., 1995). Thus, at varying distances from the site of hh synthesis cells might be exposed to varying ratios of N and C which could provide a mechanism for a graded effect on gene expression. Consistent with this possibility, in preliminary experiments we have varied the ratio of ectopic N to ΔN-C in blastula cap explants and observed differences in the level of induction of XAG-1 (data not shown).

Although genetic ablation is necessary to demonstrate the embryonic requirements for any vertebrate hh, and considerable research is necessary to understand the mechanisms of action of hh-derived polypeptides, the overexpression of N in embryos and in explants represents a gain-of-function analysis, and we propose that the expression of ΔN-C represents the reduction of N function. These supporting and reciprocal analyses both strongly implicate X-bhh and the N domain derived from autoproteolytic cleavage as signaling factors involved in the specification and patterning of neural and ectodermal structures along the anteroposterior axis. Given that X-bhh, X-chh, and X-shh are normally expressed in anterior regions of the embryo during the process of neural induction and patterning, that they share a common activity in animal cap explants (Ekker et al., 1995), and that they all appear to undergo autoproteolytic processing, it is likely that multiple members of the X-hh family and their products of autoproteolysis are involved in both direct neural induction and in neural patterning.

We wish to thank the members of our labs for helpful discussions, Anne Ungar, Monica Torres and Shao Jun Du for comments on the manuscript, Janine Ptak and Carol Davenport for oligonucleotide synthesis and DNA sequencing, John Kuo and Hazel Sive for the XAG-1 primer sequences and cDNA, Peter Mathers and Milan Jamrich for providing the XANF-2 clone and sequence prior to publication, Peter Good and Igor Dawid for the pan-neural RNP clone pNPG152, Richard Harland for noggin constructs, Andy Johnson and Paul Krieg for the expression vector pT7TS, and Genentech for the recombinant human activin. A. P. A. B. and R. T. M. are investigators of the Howard Hughes Medical Institute. C. J. L. was supported by Public Health Service Award HD27525 to R. T. M.

REFERENCES

of convergence and extension during gastrulation of *Xenopus laevis*. 


(Accepted 5 May 1995)