

## SHORT COMMUNICATION

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## Characterization and developmental expression of *AmphiNk2-2*, an NK2 class homeobox gene from amphioxus (Phylum Chordata; Subphylum Cephalochordata)

Received: 24 November 1997 / Accepted: 2 February 1998

**Abstract** The genome of amphioxus includes *AmphiNk2-2*, the first gene of the NK2 homeobox class to be demonstrated in any invertebrate deuterostome. *AmphiNk2-2* encodes a protein with a TN domain, homeodomain, and NK2-specific domain; on the basis of amino acid identities in these conserved regions, *AmphiNk2-2* is a homolog of *Drosophila vnd* and vertebrate *Nkx2-2*. During amphioxus development, expression of *AmphiNk2-2* is first detected ventrally in the endoderm of late gastrulae. In neurulae, endodermal expression divides into three domains (the pharynx, midgut, and hindgut), and neural expression commences in two longitudinal bands of cells in the anterior neural tube. These neural tube cells occupy a ventrolateral position on either side of the cerebral vesicle (the probable homolog of the vertebrate diencephalic forebrain). The dynamic expression patterns of *AmphiNkx2-2* suggest successive roles, first in regionalizing the endoderm and nervous system and later during differentiation of specific cell types in the gut (possibly peptide endocrine cells) and brain (possibly including axon outgrowth and guidance).

**Key words** NK2 homeobox · Amphioxus · Brain · Gut

### Introduction

Genes of the NK2 class have a distinctive homeobox (Bürglin 1994) and are transcribed in the developing gut, nervous system and heart of insects and vertebrates. Expression patterns of some NK2 genes have recently suggested possible homologies between invertebrate and vertebrate hearts and between regions of invertebrate and

vertebrate nervous systems. Specifically, *Drosophila tinman* (*NK4*) and vertebrate *Nkx2-5* initiate heart differentiation on the dorsal and ventral side of the embryo, respectively (Bodmer 1993, 1995), while *Drosophila vnd* (*ventral nervous system defective*) (*NK2*) and vertebrate *Nkx2-2* appear to function similarly to regionalize the nervous system on the ventral and dorsal sides of the embryo, respectively (Bier 1997). Such inverse relationships between body parts expressing homologous developmental genes are consistent with a reversal of the dorsoventral axis between *Drosophila* and vertebrates (François and Bier 1995; Jones and Smith 1995; De Robertis and Sasai 1996) and have revived evolutionary scenarios deriving the vertebrates from ancestors with annelid-like or arthropod-like body plans.

NK2 class genes encode proteins that have a tyrosine at homeodomain position 54 and are often further characterized by two highly conserved motifs in addition to the homeodomain. The first of these motifs (the TN domain) comprises 12 amino acids near the amino terminus, and the second (the NK2-specific domain) is a 19-amino acid sequence not far downstream from the homeodomain (Harvey 1996). Genes of the NK2 class, although best studied for *Drosophila* and for vertebrates, have also been found in sponges (Seimiya et al. 1994), cnidarians (Grens et al. 1996), flatworms (Garcia-Fernández et al. 1991), nematodes (Okkema and Fire 1994), and annelids (Nardelli-Haeffliger and Shankland 1993).

To date, no gene of the NK2 class has been described from any invertebrate deuterostome, a group comprising the echinoderms, hemichordates, tunicates, and amphioxus. Studies of NK2 class genes in amphioxus are of special interest, because amphioxus is widely believed to be the closest living invertebrate relative of the vertebrates (Wada and Satoh 1994) and is a useful proxy for the immediate ancestor of the vertebrates. The present paper is the first demonstration of any NK2 class gene from amphioxus. On the basis of its structure, this gene (*AmphiNk2-2*) is homologous to *Drosophila vnd* (Jiménez et al. 1995; Nierenberg et al. 1995) and vertebrate *Nkx2-2* (Price et al. 1992; Saha et al. 1993; Rud-

Edited by D. Tautz

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nick et al. 1994; Barth and Wilson 1995; Shimamura et al. 1995; Hartigan and Rubenstein 1996). Like its homologs in *Drosophila* and vertebrates, amphioxus *AmphiNkx2-2* is expressed in the developing gut and central nervous system. The results suggest that *AmphiNkx2-2* may function early in development for regionalization of the gut and central nervous system and might later be involved in the differentiation of specific cell types.

## Materials and methods

Adult males and females of the Florida amphioxus (*Branchiostoma floridae*) were collected during the summer breeding season in Tampa Bay, Florida. Eggs obtained from electrically stimulated females were fertilized, and the developmental stages were raised in laboratory culture according to Holland and Holland (1993).

Polymerase chain reaction (PCR) was used to amplify the NK homeodomain sequence from genomic DNA pooled from 20 adults of *B. floridae*. The degenerate primers corresponded to the first and third helices of the NK homeodomain of *Drosophila* Nk4 and mouse Nkx2-5. The forward primer sequence, coding for AQFVFELEV, was CA(GA)GC(N)CA(GA)GT(N)TA(TC)GA(AG)CT(N)GA, and the reverse primer sequence, coding for TQVKIWFQN, was AG(CT)(CT)TG(AG)AACCA(AGT)AT(CT)TT(N)AC(CT)TG(N)GT. For PCR, 500 ng of genomic DNA from *B. floridae* was used in a 50 µl reaction with 1 mg primer, 200 nM dNTPs, 1.5 mM Mg<sup>++</sup>, and 1 unit of Taq polymerase. After a 4-min denaturation at 91°C, samples passed through 30 cycles, each consisting of 30 s at 90°C, 30 s at 40°C, and 30 s at 90°C. After cycling, samples were incubated for 7 min at 72°C to complete synthesis of PCR products. When run on an agarose gel, the reaction products included a single band of expected size (120 bp). DNA from this band was cloned (TA cloning kit, Invitrogen, Carlsbad, Calif.), and plasmid DNA from 10 clones was purified and sequenced.

**Fig. 1A–C** Amino acid sequences of homeodomain (A), TN domain (B), and NK2-specific domain (C) of amphioxus *AmphiNkx2-2* compared to corresponding domains encoded by related NK2 class genes of mouse (Nkx2-2 from Hartigan and Rubenstein 1996; Nkx2-1 from Guazzi et al. 1990), zebrafish (Znk2-2 from Barth and Wilson 1995), *Xenopus* (XeNk-2 from Harvey 1996), and *Drosophila* (vnd from Jiménez et al. 1995; Nierenberg et al. 1995). For each conserved domain, identical amino acids are indicated by *dashes*; percentages of identical amino acids are given on the *right*. Predicted helical stretches of homeodomain amino acids are *underlined*

<b>A</b>			
	helix I	helix II	helix III/IV
<i>AmphiNkx2-2</i>	<u>KRKRRLVFSKAOTYELERRFROQRVLSAPEREHLARLIRLTPTQVKIWFQNHRYKCKRAO</u>		
mNkx2-2	-----S-----	-----S-----	-----M---R 95%
Znk2-2	-----S-----	-----S-----	-----M---R 95%
XeNk-2	-----S-----	-----S-----	-----M---R 95%
vnd	-----T-----	-----S-----	-----T--- 95%
mNkx2-1	R-----Q--V-----K--K-----	SM-H-----	-----M--QA 85%

<b>B</b>		<b>C</b>	
<i>AmphiNkx2-2</i>	TSFTVKDILDMP	<i>AmphiNkx2-2</i>	LPSRRVAVPVLVRDGKPC
mNkx2-2	-G-S-----L- 75%	mNkx2-2	----- 100%
Znk2-2	-G-S-----L- 75%	Znk2-2	----- 100%
XeNk-2	-G-S-----L- 75%	XeNk-2	----- 100%
vnd	SG-HIS---NLE 33%	vnd	-----N---- 95%
mNkx2-1	-P-S-S---SPL 50%	mNkx2-1	QQ-----K----- 84%

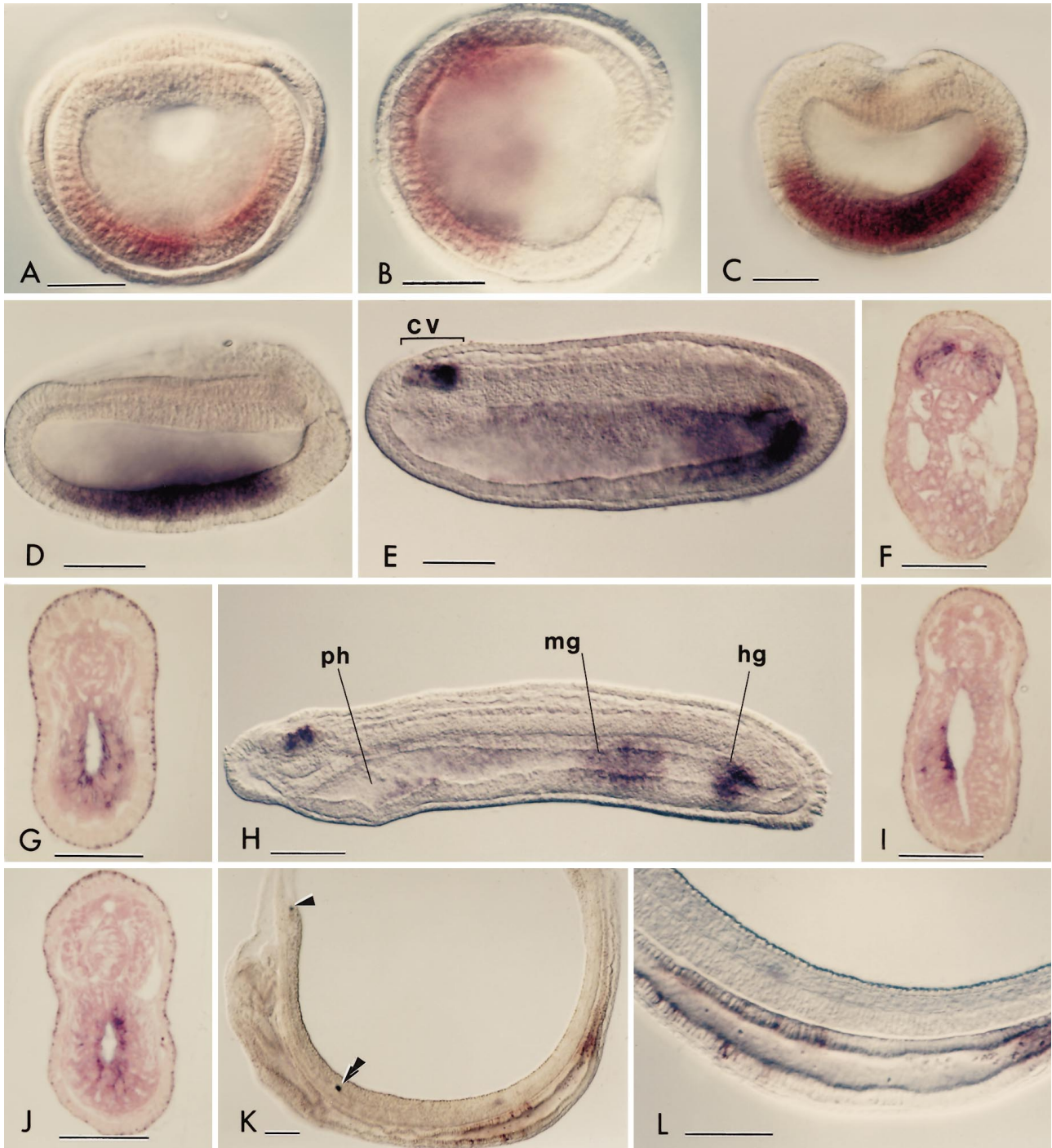
The PCR clone most closely matching the homeodomain of mouse Nkx 2.5 was used to probe a cDNA library in Lambda Zap II (Stratagene Inc., La Jolla, Calif.) made from pooled 2- to 4-day larvae of *B. floridae* (Holland et al. 1996). Approximately 4×10<sup>5</sup> plaques were screened at low stringency. Filters were hybridized at 37°C in 35% formamide, 6× SSPE (1M NaCl, 60 mM NaH<sub>2</sub>PO<sub>4</sub>, 6mM EDTA, pH 7.4), 0.1% sodium dodecyl sulfate (SDS), 1% dry milk powder and washed three times at room temperature in 0.2× SSPE with 0.1% SDS. The screen yielded one strongly positive and two weakly positive clones, which were sequenced with the AmpliTaq FS sequencing kit (Perkin Elmer, Branchburg, N.J.). One of the weakly positive clones (*AmphiNkx2-2*) had a homeobox sequence closely matching that of mouse *Nkx2-2*.

Developmental expression of *Nkx2-2* was studied by in situ hybridizations of amphioxus embryos and larvae. The template for antisense riboprobe synthesis, which averaged about 1,600 bp, was a full-length cDNA of *Nkx2-2* linearized at the 5' end. Methods for riboprobe synthesis, whole-mount in situ hybridization, and histological preparation were according to Holland et al. (1996).

## Results and discussion

The *AmphiNkx2-2* cDNA sequence from *B. floridae* is recorded in GenBank (accession number AF032999). The 2312-bp sequence includes the 5' UTR, the coding region, and a long 3' untranslated region (UTR) not ending in a polyadenylation signal. If one assumes that translation starts at the first ATG downstream from the in-frame stop codon, the longest open reading frame encodes 312 amino acids and ends at a stop codon. The protein includes a TN domain, a homeodomain, and an NK2-specific domain, which closely resemble the corresponding domains of vertebrate Nkx2-2 and *Drosophila* vnd (Fig. 1). Conserved domains encoded by other genes of the NK2 class (e.g. mouse Nkx2-1 in Fig. 1) share markedly fewer identical amino acids with the conserved domains of *AmphiNkx2-2*.

During amphioxus development, expression of *AmphiNkx2-2* is first detected by in situ hybridization in late gastrulae (8 h after fertilization) throughout the anteroventral endoderm of the hypoblast (Fig. 2A, B). At this and subsequent stages, *AmphiNkx2-2* is not expressed in the most posterior hypoblast. In early neurulae (10 h), as the epidermis is overgrowing the neural plate, expression remains strong throughout much of the ventral endoderm



**Fig. 2A–L** Expression of *AmphiNk2-2* in amphioxus embryos and larvae in whole mounts (anterior toward *left*) and cross sections (counterstained *pink*; *dark material* in epidermal cells comprises pigment granules and not gene expression; *scale lines* 50  $\mu\text{m}$  for whole mounts, 25  $\mu\text{m}$  for sections). **A** Late gastrula viewed from posterior (=vegetal) pole showing expression in ventral endoderm. **B** Preceding specimen viewed from dorsal side showing expression in anterior and ventral (out of focus) endoderm. **C** Early neurula viewed from posterior pole showing expression in ventral endoderm. **D** Preceding specimen in lateral view showing expression in ventral endoderm, excepting its most anterior and posterior regions. **E** Mid neurula in lateral view with neural expression in cerebral vesicle (anteroposterior extent indicated by *bracket labeled cv*) and endodermal expression that is strongest posteriorly (right center). **F** Cross section of preceding specimen through level of neural expression; ventrolateral cells on either side of cerebral vesicle contain conspicuous transcripts of *AmphiNk2-2*. **G** Cross section of mid

neurula through level of strong endodermal expression; transcripts conspicuous in cells all around perimeter of gut lumen. **H** Lateral view of late neurula showing neural expression in cerebral vesicle at *top left* and endodermal expression in three zones (weak in the pharynx, *ph*; intermediate in the midgut, *mg*; and strong in the hindgut, *hg*). **I** Cross section of preceding specimen through the level of pharyngeal expression; only cells on left side of the gut lumen contain detectable transcripts. **J** Cross section of late neurula through level of midgut; expression is visible in cells all around perimeter of gut lumen. **K** Lateral view of three-gill slit larva; neural expression no longer detectable (dark spots in nervous system are primary and anterior pigment spots, indicated by *tandem arrowheads* and *single arrowhead*, respectively); in gut, pharyngeal expression is no longer visible, but transcripts are detectable in scattered midgut cells and more generally in the hindgut epithelium. **L** Lateral view of posterior region of preceding specimen with midgut *on the left* and hindgut *on the right*

(Fig. 2C), although not in its most anterior and posterior regions (Fig. 2D).

By the mid neurula stage (14 h), the embryo is elongating and the neural plate has rolled up into a dorsal hollow nerve cord (Fig. 2E). Endodermal expression of *AmphiNk2-2* continues and neural expression has commenced in the anteriorly dilated portion of the nerve cord, which is called the cerebral vesicle (cv in Fig. 2E). Detectable expression extends along the middle 75% of anteroposterior axis of the cerebral vesicle and is most intense posteriorly. A cross section at any level along the anteroposterior axis of the cerebral vesicle shows transcripts of *AmphiNk2-2* in ventrolateral cells on either side (Fig. 2F). Serial sections show that the stripes of neural expression on either side stop short of the anterior end of the nerve cord and do not become confluent there. In mid neurulae, endodermal expression of *AmphiNk2-2* is most intense posteriorly in the gut (Fig. 2E); cross sections show that this expression is no longer exclusively ventral, but occurs in epithelial cells all around the perimeter of the gut lumen (Fig. 2G).

In the late neurula (17 h), the expression domains of *AmphiNk2-2* remain conspicuous in the cerebral vesicle and endoderm (Fig. 2H). There are now three zones of endodermal expression, which are, from anterior to posterior: the pharynx, midgut and hindgut. The pharyngeal expression appears least intense in whole mounts, because the domain is chiefly limited to a few cells on the left side of the gut (Fig. 2I). In contrast, cross sections of the midgut and hindgut show that transcripts occur in cells all around the perimeter of the lumen (Fig. 2J).

During the next few days of development, about the time when the mouth and the first two gill slits open, transcripts of *AmphiNk2-2* are still detectable in the cerebral vesicle and in the gut cells of the pharynx, midgut and hindgut. However, in the three-gill slit larva (5 days), expression is no longer seen in the cerebral vesicle and pharynx, but is still detectable in cells of the midgut and hindgut (Fig. 2K, L). At this stage, only a few midgut cells express *AmphiNk2-2*, although at high levels. In contrast, most of the cells in the anterior half of the hindgut express the gene, but at considerably lower levels. In larvae more than 1 week old, no further expression can be detected by whole-mount in situ hybridization.

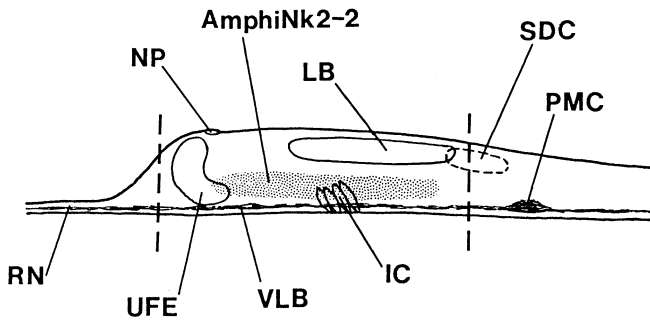
In the developing gut of amphioxus, the early pattern of *AmphiNk2-2* expression during the neurula stage indicates that the gene may be involved in regionalization of the digestive tract along the anteroposterior axis. The non-expressing region between the midgut and hindgut evidently corresponds to the ileo-colonic ring that separates those regions in the adult amphioxus. In the developing pharynx, detectable expression of *AmphiNk2-2* is largely limited to the left side. However, *AmphiNk2-2* does not appear to be one of the more up-stream genes determining the left-right axis, because the asymmetric expression appears only after the gut shows morphological evidence of left-right asymmetry. For example, rudiments of the endostyle (a thyroid homolog) and the mouth, respectively, are already present on the right and left sides of the pharynx (Conklin 1932).

In early larvae of amphioxus, detectable transcription of *AmphiNk2-2* in the midgut epithelium becomes limited to a relatively few cells that express the gene intensely. This pattern suggests that *AmphiNk2-2* may function later in amphioxus gut development for establishing and maintaining the differentiated state in specific cell types. Vertebrate *Nkx2-2* is expressed in the pancreas and in pancreatic islet alpha- and beta-cell lines (Rudnick et al. 1994). In vertebrate pancreatic islets, alpha-cells produce glucagon and beta-cells produce insulin. In adult amphioxus, there are scattered cells in the midgut epithelium that produce either glucagon or insulin (Reinecke 1981). Although it is not known whether amphioxus midgut cells expressing *AmphiNk2-2* are the same ones containing glucagon and insulin, it may be that *Nkx2-2* homologs have an ancient and conserved role in the differentiation of cells producing these hormonal peptides.

Amphioxus *AmphiNk2-2* is transcribed only at the anterior end of the neural tube, unlike its *Drosophila* and vertebrate homologs, which are transcribed along the entire neuraxis. For *Drosophila vnd*, although the more posterior neural expression has been much studied (Jiménez et al. 1995; Mellerick and Nierenberg 1995; Nierenberg et al. 1995; Bier 1997), little attention has been given to the most anterior (procephalic) region. For vertebrate *Nkx2-2*, however, expression has been well studied for all parts of the central nervous system (Price et al. 1992; Saha et al. 1993; Barth and Wilson 1995; Shimamura et al. 1995; Ericson et al. 1997), and these data can more readily be compared with the neural expression of *AmphiNk2-2*. Vertebrate *Nkx2-2* is expressed in two bilateral stripes through all the brain regions and spinal cord. In the vertebrate hindbrain and spinal cord, these stripes run ventrally, on either side of the floorplate (Shimamura et al. 1995), and the gene specifies a ventral class of motor neurons (Ericson et al. 1997). By contrast, in the vertebrate fore- and midbrain, the expression stripes run along the boundary apparently dividing the more dorsal alar region from the more ventral basal region (Shimamura et al. 1995), and it has been suggested that the gene may function in determining positional or boundary information (Saha et al. 1993) and in regulating neuronal differentiation (Barth and Wilson 1995).

In contrast to vertebrate *Nkx2-2*, the neural expression of amphioxus *AmphiNk2-2* is never detected in the developing nerve cord posterior to the cerebral vesicle. These posterior regions of the amphioxus nerve cord, which are thought to be homologs of the vertebrate hindbrain and spinal cord (Holland et al. 1994), are unusual in having ventral motor neurons that never send axons to the body wall muscles; instead the muscles send cytoplasmic extensions to neuromuscular junctions located on the surface of the nerve cord (Flood 1966). It is possible that the absence of detectable *AmphiNk2-2* expression in the ventral motor neurons of amphioxus is correlated with their lack of axons extending beyond the bounds of the central nervous system.

The neural expression of amphioxus *AmphiNk2-2* is detected only in the cerebral vesicle, most of which is probably homologous to the diencephalic forebrain of



**Fig. 3** Diagrammatic side view of anterior part of neural tube of larval amphioxus. Anterior toward left; cerebral vesicle limits indicated by vertical dashed lines. Stippling indicates position of basal ends of the neural cells expressing *AmphiNk2-2*. Landmark structures are based on microanatomical studies of Lacalli et al. (1994) and Lacalli (1996). In the following list, presumed homologies within the vertebrate diencephalon are indicated in parentheses after each structure: *IC* infundibular cells (infundibulum of vertebrate pituitary), *LB* lamellar body (vertebrate epiphysis), *NP* neuropore, *PMC* primary motor center, *RN* rostral nerve, *SDC* small dorsal cells, *UFE* unpaired frontal eye (vertebrate paired lateral eyes), *VLB* ventrolateral bundle (vertebrate tract of the post-optic commissure)

vertebrates on the basis of computer assisted, 3D reconstructions of serial electron micrographs of Lacalli et al. (1994) and Lacalli (1996); the presumed homologous structures are diagrammed in Fig. 3, which also includes the neural expression domain of *AmphiNk2-2*. Limitation of neural expression to the cerebral vesicle suggests that the gene defines positional information along the antero-posterior axis of the nervous system. The gene might also define positional information along the dorsoventral axis of the nervous system, because *AmphiNk2-2* transcripts are limited to a ventrolateral band of cells on either side of the midline; a similar pattern is seen for *Nkx2-2* in the vertebrate diencephalon (Shimamura et al. 1995). In amphioxus, there is no obvious anatomical boundary separating an alar and a basal region along the dorsoventral axis of the cerebral vesicle. Nevertheless, it is possible that the expression domain of *AmphiNk2-2* runs along a boundary homologous to that of *Nkx2-2* in the vertebrate forebrain.

During development of the amphioxus central nervous system, the earliest axon tracts appear on either side of the amphioxus cerebral vesicle. Lacalli et al. (1994) proposed that these tracts are homologous to the tract of the postoptic commissure (TPOC), which runs anteroposteriorly on either side of the developing vertebrate forebrain (Wilson et al. 1990). In the vertebrates, some of the axons in these tracts apparently arise from cells within the zone of *Nkx2-2* expression and trace a course parallel to it; therefore, *Nkx2-2* may help establish and maintain the differentiated identity of neurons giving rise to the TPOC and might also play a role in axon guidance (Barth and Wilson 1995). In amphioxus, the earliest axon tracts also trace a course along the ventral edge of the domain of *AmphiNk2-2*, which, like its vertebrate homologs, might function in neuronal cell specification and axon guidance.

For amphioxus, nothing definite is yet known about the upstream regulators and downstream targets of *AmphiNk2-2*. However, for vertebrates, it is known that neural expression of *Nkx2-2* is under the control of *Sonic hedgehog* and *Pax-6* (Ericson et al. 1997). In the vertebrate diencephalon, *Sonic hedgehog* transcription is most intense just ventral to the expression domains of *Nkx2-2* (Shimamura et al. 1995), and experimental manipulation of the former influences the position of the latter (Barth and Wilson 1995). Thus, in the amphioxus forebrain, it seems likely that an amphioxus homolog of *Sonic hedgehog* acts upstream from *AmphiNk2-2*. Amphioxus may prove to be a useful model for elucidating such gene interactions, because both its anatomy and its genome are vertebrate-like, yet relatively simple.

**Acknowledgements** We are deeply indebted to John M. Lawrence for laboratory facilities at the University of South Florida and to Dr. David Mindale and his staff for help with the automated sequencing at the University of Michigan. This work was supported in part by NSF research grant IBN 96-309938 to NDH and LZH and in part by NIH research grants NS 29119-04 (NINDS) and HL 54732-01 (NHBL) to RB.

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