SHORT COMMUNICATION

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Sequence and developmental expression of amphioxus *AmphiNk2–1*: insights into the evolutionary origin of the vertebrate thyroid gland and forebrain

Abstract We characterized an amphioxus NK-2 homeobox gene (*AmphiNk2–1*), a homologue of vertebrate *Nkx2–1*, which is involved in the development of the central nervous system and thyroid gland. At the early neurula stage of amphioxus, *AmphiNk2–1* expression is first detected medially in the neural plate. By the mid-neurula stage, expression is localized ventrally in the nerve cord and also begins in the endoderm. During the late neurula stage, the ventral neural expression becomes transiently segmented posteriorly and is then down-regulated except in the cerebral vesicle at the anterior end of the central nervous system. Within the cerebral vesicle *AmphiNk2–1* is expressed in a broad ventral domain, probably comprising both the floor plate and basal plate regions; this pattern is comparable to *Nkx2–1* expression in the mouse diencephalon. In the anterior part of the gut, expression becomes intense in the endostyle (the right wall of the pharynx), which is the presumed homologue of the vertebrate thyroid gland. More posteriorly, there is transitory expression in the midgut and hindgut. In sum, the present results help to support homologies (1) between the amphioxus endostyle and the vertebrate thyroid gland and (2) between the amphioxus cerebral vesicle and the vertebrate diencephalic forebrain.

Key words NK2 homeobox · Cephalochordata · Lancelet · Diencephalon · Endostyle

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Introduction

The proximate invertebrate ancestor of the vertebrates was probably a cephalochordate-like creature, and developmental genetic studies of the modern cephalochordate, amphioxus, can give insights into the question of the origin of the vertebrates (Holland and Holland 1998). One interesting group of genes for such studies comprises the NK2-class homeobox genes, which have a distinctive homeodomain with a tyrosine at position 54 and also often have a TN domain and an NK-2-specific domain. A previous study of an NK2-class homeobox gene from amphioxus (*AmphiNk2–2*) gave new insights into the evolutionary history of the vertebrate forebrain and gastric endocrine cells (Holland et al. 1998).

The present paper concerns a new NK-type gene from amphioxus, *AmphiNk2–1*, which is homologous to vertebrate *Nkx2–1* (also known as *TTF-1*, *Titf1*, and *T/ebp*; Lazzaro et al. 1991; Kimura et al. 1996; Pera et al. 1998; reviewed in Harvey 1996) and *Drosophila vnd* (Jimenez et al. 1995; Mellerick and Nirenberg 1995; Holland et al. 1998). During development of higher vertebrates, *Nkx2–1* is expressed in the thyroid gland, lung, and in ventral regions of the anterior nerve cord (Lazzaro et al. 1991; Pera and Kessel 1998); moreover, these structures selectively fail to develop in homozygous mutants with defective *Nkx2–1* (Kimura et al. 1996).

In the present study, we found that *AmphiNk2–1* is an early marker for the midline of the neural plate (unlike *AmphiNk2–2*) and subsequently becomes localized ventrally within the cerebral vesicle. This latter pattern is comparable to vertebrate *Nkx2–1* expression in ventral regions of the vertebrate forebrain (Shimamura et al. 1997; Pera and Kessel 1998). Together with previously published microanatomy (Lacalli et al. 1994), the neural expression domain of *AmphiNk2–1* supports the idea that at least most of the cerebral vesicle of amphioxus is homologous with the diencephalic part of the vertebrate forebrain. In addition, *AmphiNk2–1* is expressed in the developing endostyle, just as vertebrate *Nkx2–1* is expressed in the developing thyroid gland (Lazzaro et al.

1991), strongly supporting the homology between the amphioxus endostyle and the vertebrate thyroid gland.

Materials and methods

Males and females of the Florida amphioxus (*Branchiostoma floridae*) were collected in Tampa Bay, Florida. Eggs from electrically stimulated females were fertilized, and the embryos and larvae were raised in the laboratory at 23°C (Holland and Holland 1993). cDNA libraries were constructed from mRNA from 26-h embryos (Panopoulou et al. 1998) or from pooled 2- to 4-day larvae (Holland et al. 1996). To isolate the *AmphiNk2–1* gene, polymerase chain reaction (PCR) was performed with degenerate primers as described in Holland et al. (1998). The resulting 120-bp PCR product, which included part of the homeobox, was used to probe the cDNA library from 2- to 4-day larvae. One of the weakly hybridizing clones obtained was a partial cDNA of *AmphiNk2–1* missing about 300 bp at the 5′ end. To obtain the missing sequence, a 738-bp *Sal*I-*Bst*EII fragment of this partial clone was used to probe the 26-h library, which yielded one full-length cDNA clone. Developmental expression of *AmphiNk2–1* was studied by in situ hybridization. An antisense riboprobe about 1,600 bp long was synthesized from the partial cDNA. Riboprobe synthesis and in situ hybridization were according to Holland et al. (1996).

Results and discussion

Structure of *AmphiNk2–1*

The cDNA sequence of *AmphiNk2–1* (GenBank accession number AF077840) is 2323 bp long and has a polyadenylation signal near the 3′ end. Beginning with the first ATG downstream from the in-frame stop codons, the longest open reading frame encodes 360 amino acids,

including a TN domain, a homeodomain, and an NK2-specific domain (Bodmer 1995; Harvey 1996). Figure 1 compares these domains between *AmphiNk2–1* and related proteins in other species. When all three conserved domains are taken into consideration, the closest match is between *AmphiNk2–1* and mammalian *NKX2–1*, which also share the following conserved stretches of amino acids between the TN domain and homeodomain: 80–83 (QLSH), 89–93 (YCNGN), and 111–120 (WYGANPDPRF) for the amphioxus protein. In addition, the amphioxus and mammalian proteins are similar in having glutamine-rich regions between the homeodomain and the NK 2-specific domain. In mammalian *Nkx2–1*, the glutamine-rich region apparently inhibits transcription of thyroid-specific target genes (De Felice et al. 1995), and the corresponding region of *AmphiNk2–1* may have a similar function.

Developmental expression of *AmphiNk2–1*

During amphioxus development, in situ hybridization first detects *AmphiNk2–1* expression at the early neurula stage (Fig. 2A, B). Expression is limited to an anteroposteriorly elongated group of cells in the middle of the neural plate. During subsequent development, these cells are precursors of cells that will comprise the ventral region of the nerve cord (floor plate and possibly basal plate). By the stage of the hatching neurula, additional expression becomes detectable in the anteroventral endoderm (Fig. 2C). As the neurula stage progresses, the lateral edges of the neural plate curl up and fuse middorsally to produce the nerve cord. By the mid neurula (Figs.

Fig. 1A–C Amino acid sequences of homeodomain (**A**), TN domain (**B**), and NK2-specific domain (**C**) of amphioxus *AmphiNk2–1* compared to corresponding domains encoded by related genes (with database accession numbers): human *NKX2–1* (X87141), mouse m*Nkx2–1* (S53724), flatworm Dth2 (X69202), *Caenorhabditis* ceh24 (AF026056), amphioxus *AmphiNk2–2* (AF032999), mouse m*Nkx2–2* (U31566), leech Lox10 (Z22635, only homeodomain determined), *Drosophila* vnd (X87141), and mouse Nkx2–9 (Y15741). For each conserved domain, identical amino acids are indicated by *dashes*; percentages of identical amino acids are given on the *right*

A

AmphiNk2-1	RRKRRVLFSQAQVYELERRFKQQKYLSAPEREHLAQLINLTPTQVKIWFQNHRYKCKRQD	100%
NKX2-1	--SM-H---------------M---A	92%
mNkx2-1	--SM-H---------------M---A	92%
Dth2	-----I------I----------------------------N--------------------SQ	92%
ceh24	---------------------A---T-----Q--NS-R-------------------E	88%
AmphiNk2-2	K--------K--T-------R--R----------R--R------------------AQ	85%
mNkx2-2	K--------K--T-------R--R----------S--R---------------M--AR	83%
Lox10	-----I------I-------R-------------TF-G----------------T-KSK	83%
vnd	K-------TK--T-------R--R----------S--R---------------T--AQ	82%
mNkx2-9	---------K--TL------R--R--------Q--R-LR-------------L--GR	80%

B

AmphiNk2-1	TPFSVTDILSPL	100%
NKX2-1	-----S------	92%
mNkx2-1	-----S------	92%
Dth2	absent	0%
ceh24	SK---NS-----	67%
AmphiNk2-2	-S-T-K---DMP	50%
mNkx2-2	-G---K---DLP	58%
Lox10	?	
vnd	SG-HIS---NLE	33%
mNkx2-9	absent	

C

AmphiNk2-1	GNSPRRVAVPVLVKDGKPC	100%
NKX2-1	QQ-----------------	89%
mNkx2-1	QQ-----------------	89%
Dth2	absent	0%
ceh24	DEEEEESEK---KPS-VFG	21%
AmphiNk2-2	LP-----------R-----	84%
mNkx2-2	LP-----------R-----	84%
Lox10	?	
vnd	LP-----------RN----	79%
mNkx2-9	PGLL---M-----H-RP-S	53%

2D, 3 C), the ventral expression in the nerve cord becomes segmented in the anteroposterior axis and is strongest anteriorly (in the region of the nerve cord called the cerebral vesicle). Cross sections show expression in many ventral cells of the nerve cord (Fig. 2E, arrow). The rostrocaudal segmentation of neural expression, although transitory, may indicate that *AmphiNk2–1* is playing a role in the development of metameric neural structures. By the mid neurula stage, pharyngeal endoderm cells strongly express *AmphiNk2–1* (Fig. 2E) ventrally and on the right side in the rudiment of the endostyle. In more posterior gut regions, endodermal expression is midventral (Fig. 2F).

At the late neurula stage (Fig. 2G), detectable neural expression is limited to the anterior end of the nerve cord (the cerebral vesicle). In the gut epithelium, expression remains intense ventrally and on the right side of the pharynx in the developing endostyle. More posteriorly, there is expression throughout the gut wall, except middorsally (Fig. 2H).

In the 24-h embryo (Fig. 2I), detectable neural expression continues in the anterior part of the cerebral vesicle; in some embryos, there is also strong expression more posteriorly in the cerebral vesicle (data not shown). A cross-section through the cerebral vesicle (Fig. 2J) reveals that transcripts of *AmphiNk2–1* are limited to the ventral half of the nerve cord (including the floorplate and flanking regions). Anteriorly in the gut, there is strong expression throughout the endostylar thickening that comprises the right wall of the pharynx. However, there is no detectable expression in the club-shaped gland (Fig. 2K, arrow), which is another derivative of the pharyngeal wall. More posteriorly in the gut, there is relatively weak expression in the wall of the midgut and hindgut (Fig. 2I).

In the 48-h larva (Fig. 2L), expression in the cerebral vesicle and hindgut is weaker, and no further expression is detectable in the midgut, while expression remains intense throughout the endostyle. During the next few days, detectable endodermal expression becomes restricted to the endostyle. In the embryos and early larvae of amphioxus, *AmphiNk2–1* is expressed throughout the endostyle tissue and is not limited to the posterior region of the endostyle, where Fredriksson et al. (1984) demonstrated iodine-binding cells. After the first week of larval development, the endostylar expression can no longer be detected by in situ hybridization.

The in situ results for amphioxus *AmphiNk2–1* can be compared and contrasted with the developmental expression of related genes in other animals. The mesodermal expression of *Dth-2* in the peripheral parenchyme of flatworms (Garcia-Fernàndez et al. 1993) and of *ceh24* in the muscles of the gut and vulva of *Caenorhabditis* (Harfe and Fire 1998) has no clear counterpart in other animals studied to date. Moreover, the midgut and hindgut expression of *AmphiNk2–1* in amphioxus does not clearly correspond to endodermal expression of *NKX2–1* in vertebrates, but might possibly be related to the expression of a related gene, Lox10, in the midgut of the

leech (Nardelli-Haefliger and Shankland 1993) and in the mid- and hindgut primordia of *Drosophila* (Mellerick and Nirenberg 1995; Nirenberg et al. 1995).
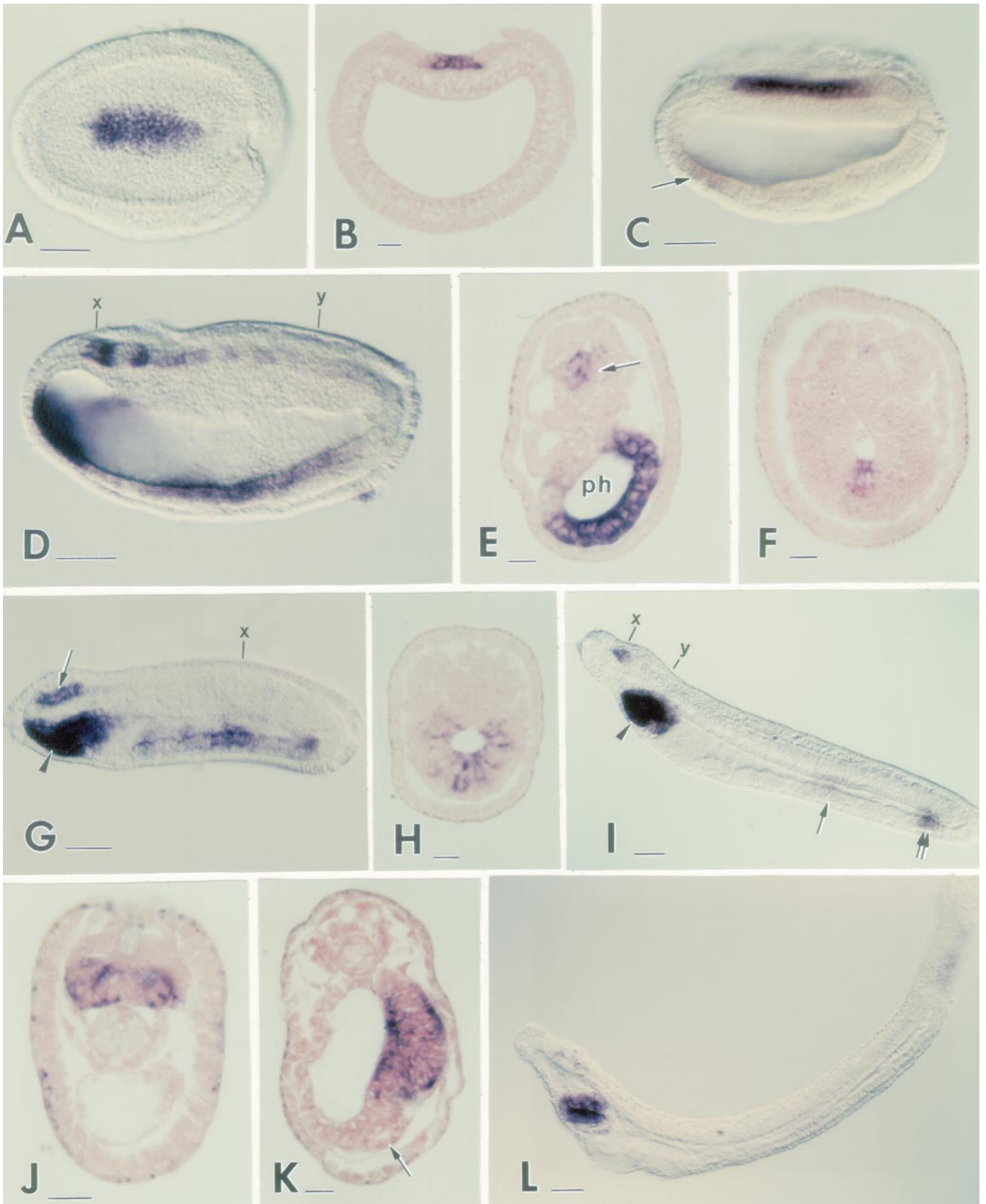
The role of amphioxus *AmphiNk2–1* in foregut development seems to lack counterparts in other invertebrates, but has striking parallels with vertebrate *Nkx2–1* in the developing thyroid gland. During amphioxus development, foregut expression of *AmphiNk2–1* occurs in the endostyle, which is the thickened, glandular right side of the pharynx supplying secretions to trap small food particles in the gut. At larval metamorphosis (after about a month of development), the endostyle shifts to a ventral position, grows in a posterior direction, and assumes the shape of a glandular groove running midventrally along the floor of the pharynx. As in the larva, the endostyle of the adult produces secretions for trapping and transporting ingested particulate food.

The homology between the adult amphioxus endostyle and the vertebrate thyroid gland was first proposed by Müller (1873) from insights he gained by watching an amphioxus-like endostyle in larval lampreys become converted to a thyroid gland in adult lampreys. Since then, the proposed homology has gained additional support from studies suggesting that the amphioxus endostyle includes thyroglobulin and thyroperoxidase and can metabolize iodine to form iodothyronines (Ericson and Fredriksson 1990). Even so, the homology between the amphioxus endostyle and the vertebrate thyroid gland has sometimes been questioned (Burrow 1989) because no endocrine function has ever been demonstrated for the endostyle.

In the vertebrates, *Nkx2–1* is expressed in the developing thyroid gland (Lazzaro et al. 1991) where it encodes a protein that can bind to the regulatory region of at least three thyroid-specific target genes (thyrotropin receptor, thyroglobulin, and thyroperoxidase; Damante et al. 1994; Mascia et al. 1997). Moreover, mice with a null nutation in *Nkx2–1* have defects that include the failure to develop thyroid glands (Kimura et al. 1996). In sum, *Nkx2–1* apparently has an early developmental function during commitment of endodermal thyroid cell precursors and a later function as a transcriptional activator of thyroid-specific genes. The present demonstration that *AmphiNk2–1* is conspicuously expressed in the developing endostyle of amphioxus is additional support for the proposed homology between the amphioxus endostyle and vertebrate thyroid gland (Fig. 3B-D).

AmphiNk2–1 is expressed in the developing central nervous system of amphioxus, and comparable expression patterns have been reported for *Caenorhabditis ceh-24* (Harfe and Fire 1998), leech *Lox10* (Nardelli-Haefliger and Shankland 1993), *Drosophila vnd* (Nirenberg et al. 1995) and vertebrate *Nkx2–1* genes. These genes tend to be expressed ventrally (i.e. medially) and anteriorly within the central nervous system (sometimes following a relatively wide-spread expression along the anteroposterior neuraxis).

In the developing central nervous system of chordates, there are interesting parallels between the expres-

sion of amphioxus *AmphiNk2–1* and vertebrate *Nkx2–1*. In the neural plate of amphioxus, *AmphiNk2–1* expression begins along the anteroposterior axis, which presumably contributes to the floor plate of the nerve cord later in development. *Drosophila vnd* (Mellerick and Nirenberg 1995) and amphioxus *AmphiNk2–1* (present results) are the only genes expressed in a segmented pattern in the nerve cord (Fig. 3A,C), although it is not clear whether this periodicity arose independently in the two organisms or is an ancestral feature that has tended to be lost in many animals. During chick development, the initial neural expression of *Nkx2–1* is in the floor plate along much of the nerve cord (Pera and Kessel 1998; Qiu et al. 1998). Later in the development of both amphioxus (present study) and the chick (Pera and Kessel 1998; Qiu et al. 1998), neural expression of these genes is downregulated except in the forebrain, where transcripts are distributed throughout the ventral part of the nerve cord.

In mouse embryos (Fig. 3B), *Nkx2–1* is expressed in the telencephalon, which has no obvious homologue in amphioxus, and in the diencephalon, which appears to be homologous to most of the amphioxus cerebral vesicle (Lacalli et al. 1994). In the mouse diencephalon, the expression domain of *Nkx2–1* marks both floor plate and basal plate regions and is bounded dorsally by a complementary, non-overlapping expression domain of *Nkx2–2* (Shimamura et al. 1995). As diagrammed in Fig. 3E, the respective amphioxus homologues of these two genes have a similar complementary expression in the developing cerebral vesicle (Holland et al. 1998; present results), and *AmphiNk2–1* thus appears to be a marker for the floor plate and basal plate regions of the diencephalic forebrain.

In the vertebrates, early neural expression of *Nkx2–1* is induced by sonic hedgehog from the dorsal foregut and forming notochord (Ericson et al. 1995; Shimamura and Rubenstein 1997). Recently, Shimeld (1999) demon-

Fig. 3A–E Diagrams showing expression domains (*stippled*) for NK2-class genes related to amphioxus *AmphiNk2–1*; in all side views, anterior is toward *left*. **A** Side view of stage 10 *Drosophila* embryo with *vnd* expression in procephalic rudiment, ventral nerve cord (*vn*) and midgut primordium (*mg*). **B** Side view of 9-day mouse embryo with *mNkx2–1* expression in thyroid (*th*) and in parts of telencephalon (*te*) and diencephalon (*di*). **C** Side view of 15-h amphioxus embryo with *AmphiNk2–1* expression in dorsal nerve cord, both in cerebral vesicle (*cv*) and more posteriorly, and in gut, including endostyle (*en*). **D** Side view of 20-h amphioxus embryo with *AmphiNk2–1* expression in cerebral vesicle (*cv*) of dorsal nerve cord and in gut, including endostyle (*en*). **E** Cross-section of preceding embryo at level indicated by *arrowhead* in **D**; labeled features are Hatschek's right diverticulum (*rd*), notochord (*no*), and cerebral vesicle (*cv*) with expression domain of *AmphiNk2–2* (*fine stippling*) (from Holland et al. 1998) and *AmphiNk2–1* (*coarse stippling*)

Fig. 2A–L *AmphiNk2.1* expression in embryos and larvae of *Branchiostoma floridae*. Whole mounts with anterior toward *left* and 40-μm *scale lines*; cross-sections are viewed from tail of embryo and have 10-μm scale lines. **A** Early neurula (9.5 h) in dorsal view with expression medial in neural plate. **B** Cross-section of preceding neurula with expression medial in neural plate. **C** Side view of hatching neurula (11 h) with expression in neural plate and anteroventral endoderm (*arrow*). **D** Side view of mid neurula (15 h) with neural expression breaking up into segments and with strong endodermal expression. **E** Section through *x* in **2D**, showing expression in ventral part of neural tube (*arrow*) and in ventral and right wall of pharynx (*ph*). **F** Section through *y* in **D** showing expression in ventral wall of hindgut. **G** Side view of late neurula (17 h) with expression anteriorly in neural tube (*arrow*), in pharynx (*arrowhead*), and in more posterior gut regions. **H** Cross section through *x* in **G** showing expression in gut wall, except dorsally. **I** 24-h embryo with anterior labeling in neural tube, in pharynx (*arrowhead*), in midgut (*arrow*), and in hindgut (*twin arrow*). **J** Cross-section through *x* in **I**, with expression in ventral part of neural tube (dark secretory granules in epidermis do not indicate expression). **K** Cross-section through *y* in **I**, with expression in right wall (endostyle) of pharynx, but not in club-shaped gland (*arrow*). **L** 48-h larva with strong expression in endostyle and weak expression in cerebral vesicle and hindgut
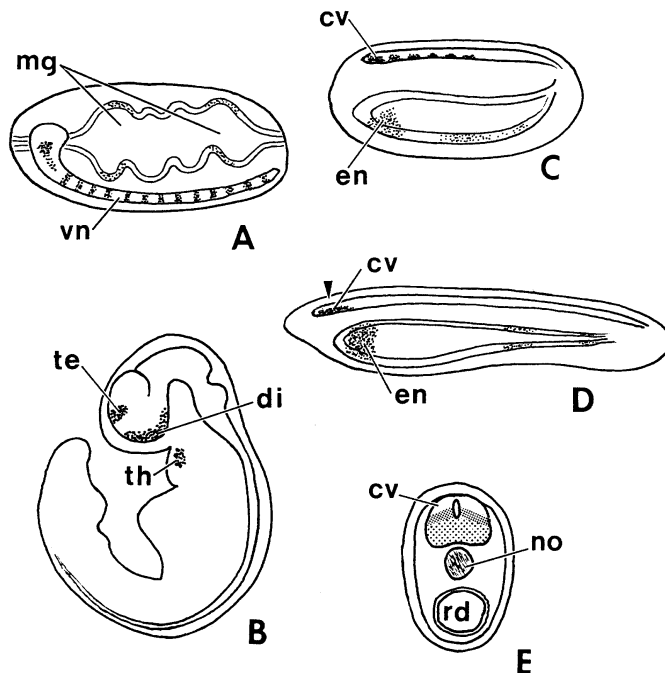
strated that *AmphiHh,* an amphioxus homologue of vertebrate *sonic hedgehog,* is expressed in the dorsal endoderm and prospective notochord at the early neurula stage. It is thus likely that, in parallel with the vertebrates (Qiu et al. 1998), amphioxus uses AmphiHh to induce *AmphiNk2–1* expression in overlying regions of the neural plate.

The forebrain expression of vertebrate *Nkx2–1* (Price 1993; Shimamura et al. 1995) and amphioxus *AmphiNk2–1* may also have parallels with expression of *Nkx2–1* homologues at the anterior end of the nervous system in some developing protostomes: *Drosophila vnd* (Nirenberg et al. 1995) and leech *Lox10* (Nardelli-Haefliger and Shankland 1993) are expressed, respectively in the procephalic rudiment and in the supraesophageal ganglion. Thus the developmental expression of *Nkx2–1* homologues near the anterior end of the central nervous system in a diversity of animals is an additional indication that the basic genetic mechanisms for patterning the anterior nervous system may already have been present

in the common ancestor of protostomes and deuterostomes (Bruce and Shankland 1998). This would be consistent with an ancient origin of NK2-class genes related to *Drosophila vnd* and to mammalian *Nkx2–1* and *Nkx2–2* from a single ancestral gene involved in development of the central nervous system and gut (Nardelli-Haefliger and Shankland 1993).

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