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SHORT COMMUNICATION

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The Mnx homeobox gene class defined by *HB9*, *MNR2* and amphioxus *AmphiMnx*

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Abstract The *HB9* homeobox gene has been cloned from several vertebrates and is implicated in motor neuron differentiation. In the chick, a related gene, *MNR2*, acts upstream of *HB9* in this process. Here we report an amphioxus homologue of these genes and show that it diverged before the gene duplication yielding *HB9* and *MNR2*. *AmphiMnx* RNA is detected in two irregular punctate stripes along the developing neural tube, comparable to the distribution of 'dorsal compartment' motor neurons, and also in dorsal endoderm and posterior mesoderm. We propose a new homeobox class, Mnx, to include *AmphiMnx*, *HB9*, *MNR2* and their *Drosophila* and echinoderm orthologues; we suggest that vertebrate *HB9* is renamed *Mnx1* and *MNR2* be renamed *Mnx2*.

Keywords Homeobox · Motor neuron · Amphioxus · Gene duplication

Introduction

The *HB9* homeobox gene (also known as *HLXB9*) was first isolated in humans (Deguchi and Kehrl 1991) and subsequently in other vertebrates (Saha et al. 1997; Tanabe et al. 1998; Harrison et al. 1999). *HB9* is marginally more closely related to Hox, ParaHox, En, Emx, and NK class homeobox genes than it is to the Prd, Prd-like or more divergent homeobox classes (Bürglin 1994). Its evolutionary origin is unclear, however. One early suggestion was that *HB9* is related to, or derived from, the *Drosophila* homeotic gene *proboscipedia* (Burglin 1994; Harrison et al. 1994). This now seems unlikely as *HB9* maps to 7q36 (near *En2* and *Gbx1*) and not within the human Hox clusters (Pollard and Holland 2000). An alternative possibility is that *HB9* is a member of a distinct class of homeobox genes, analogous to other defined classes such as Emx, Msx, Dlx, Mox, etc. Consistent with this suggestion, a second homeobox gene with high sequence identity to *HB9* over the homeobox was reported from chick, and named *MNR2* (Tanabe et al. 1998). Interestingly, *MNR2* and *HB9* are both expressed during motor neuron differentiation, and form part of a cascade of genes regulating this process (Tanabe et al. 1998; Arber et al. 1999). *HB9* is also expressed in the developing gut, brain, testis and lymphoid lineage (Harrison et al. 1994, 1999; Saha et al. 1997) and mutations in *HB9* are implicated in the human congenital condition sacral agenesis (Lynch et al. 1995; Ross et al. 1998). To investigate the relatedness between *HB9* and *MNR2*, and the evolution of their developmental roles, we have examined a homologous gene in a cephalochordate (amphioxus), the group of animals thought to be the sister group of the vertebrates.

Materials and methods

Homeobox probes from *AmphiGsx* and *AmphiXlox* (Brooke et al. 1998) were used to screen a *Branchiostoma floridae* embryonic cDNA library (Max-Planck-Institut, Berlin library identifier MPIMGp531). The *AmphiMnx* cDNA clone was one of several weak positives identified (clone coordinate 102P07). Molecular phylogenetic analysis was performed on conserved blocks, as follows. First, the deduced AmphiMnx protein sequence was aligned with HB9 (human, mouse, chick and *Xenopus*), MNR2 (chick), and a putative *Drosophila* orthologue using CLUSTALW implemented at www.ebi.ac.uk. Second, Gblocks 0.73b (Castresana 2000) was used to identify conserved blocks suitable for phylogeny construction. The partial *Xenopus* HB9 sequence was omitted for this step only. We relaxed the Gblocks homology detection criteria to 4/6 identities for conserved positions and for block flanking positions, because the *Drosophila* sequence is relatively divergent. Third, the resultant data set of 152 aligned amino acid positions was used for phylogenetic tree construction using Quartet Puzzling maximum likelihood, implemented using TREE-PUZZLE 4.0.2 with four γ-distributed variable rates and one invariant (Strimmer

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A

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AmphiMnx          TRRPRTAFTS QQLLELEKYF KENKYLSRPK RFEVATALML TETQVKIWFQ NRRMKWKRSK
Mouse HB9         C......... .......HQ. .L........ ......S... .......... ..........
Chick HB9         C......... .......HQ. .L........ ......S... .......... ........Q.
Chick MNR2        S......... .......NQ. .L........ ......S... .......... .........R
Drosophila Mnx    .......... ........Q. .Q........ .....SG... S......... ..........
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B

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Consensus         IYPWMK
Dfd               ------
Antp              L----R
Msx-1             RT---Q
HB9               -L-K-P
MNR2              LV-RLS
AmphiMnx          -M-R-D
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C

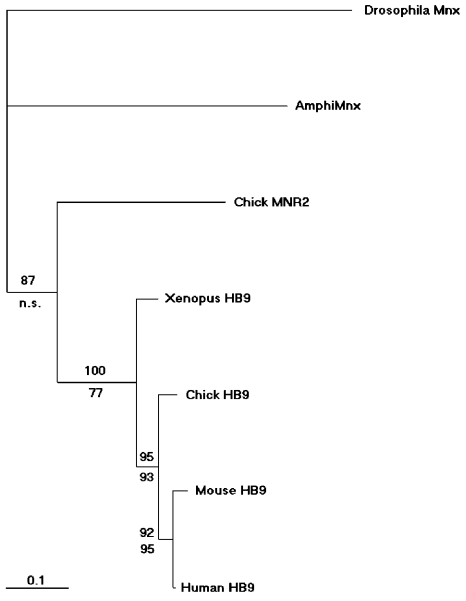

Fig. 1 **A** Alignment of the AmphiMnx homeodomain to those encoded by representative *HB9* and *MNR2* genes, plus an orthologue detected in the *Drosophila* genome sequence. *Dots* denote identical residues. **B** The consensus hexapeptide (Bürglin 1994) aligned with the clear hexapeptides of *Drosophila* Dfd and Antp, the weak hexapeptides of mammalian Msx-1 and mammalian and chicken HB9, the equivalent region of chicken MNR2, and the weak hexapeptide of AmphiMnx. **C** Phylogenetic tree constructed from conserved blocks identified in complete protein sequences. *Numbers* denote Quartet Puzzling support values (*below nodes*) or Neighbour Joining (NJ) bootstrap percentage support (*above nodes*). *AmphiMnx* is descendent from a precursor gene that duplicated to yield *HB9* and *MNR2*. Sequences used in phylogenetic analysis: human HB9 (AF107452, AF107453), mouse HB9 (AF153046), chick HB9 (AF066861), *Xenopus* HB9 (AF072382), chick MNR2 (AF066860), *Drosophila* Mnx (AAF50503). There are several versions of human HB9 in GenBank; that reported by Heus et al. (1999) was selected as likely to be accurate, due to close similarity to mouse HB9. The complete *AmphiMnx* cDNA sequence is deposited with GenBank (accession number AF308821)

and von Haeseler 1996), and also by Neighbour Joining applied to a Dayhoff PAM distance matrix, implemented using PHYLIP 3.573c (Felsenstein 1993). Since the alignment is considerably larger than just the homeodomain, it was not possible to use other homeobox classes to root the tree. Whole-mount in situ hybridisation to amphioxus embryos was performed as described by Holland (1999).

Results and discussion

We report a cDNA clone from the cephalochordate amphioxus that encodes a protein with high sequence similarity to vertebrate HB9 and MNR2. The 1.8 kb clone includes an open reading frame with the potential to encode a protein of 296 amino acids, including a homeodomain with high sequence identity to those encoded by *HB9* and *MNR2* (Fig. 1 A). A sequence upstream from the homeodomain has weak similarity to the hexapeptide motif characteristic of most 'ANTP superclass' homeobox genes, such as the Hox, ParaHox and NK genes (Fig. 1B). A second peptide motif close to the N-terminus is conserved specifically with the HB9 and MNR2 proteins. There is extensive sequence similarity between *HB9*, *MNR2*, the amphioxus gene reported here, a sea urchin gene *PIHbox9* (Bellomonte et al. 1998) and a homeobox gene we identified by a tblastn search of the complete *Drosophila* genome sequence (Adams et al. 2000). We suggest that these genes form a homeobox class related to, but distinct from, other ANTP classes. We denote this the Mnx class, and the amphioxus gene *AmphiMnx*.

Molecular phylogenetic analysis was used to investigate the relation between *HB9*, *MNR2* and *AmphiMnx*. The maximum likelihood and neighbour joining methods yielded the same tree topology, shown in Fig. 1C. This clearly indicates that *HB9* and *MNR2* are related by gene duplication, and that this duplication predates the divergence of amphibians and amniotes. We conclude that *MNR2* is unlikely to be chick-specific; we predict it is in the genomes of other vertebrates. The amphioxus gene described here, *AmphiMnx*, diverged prior to this gene

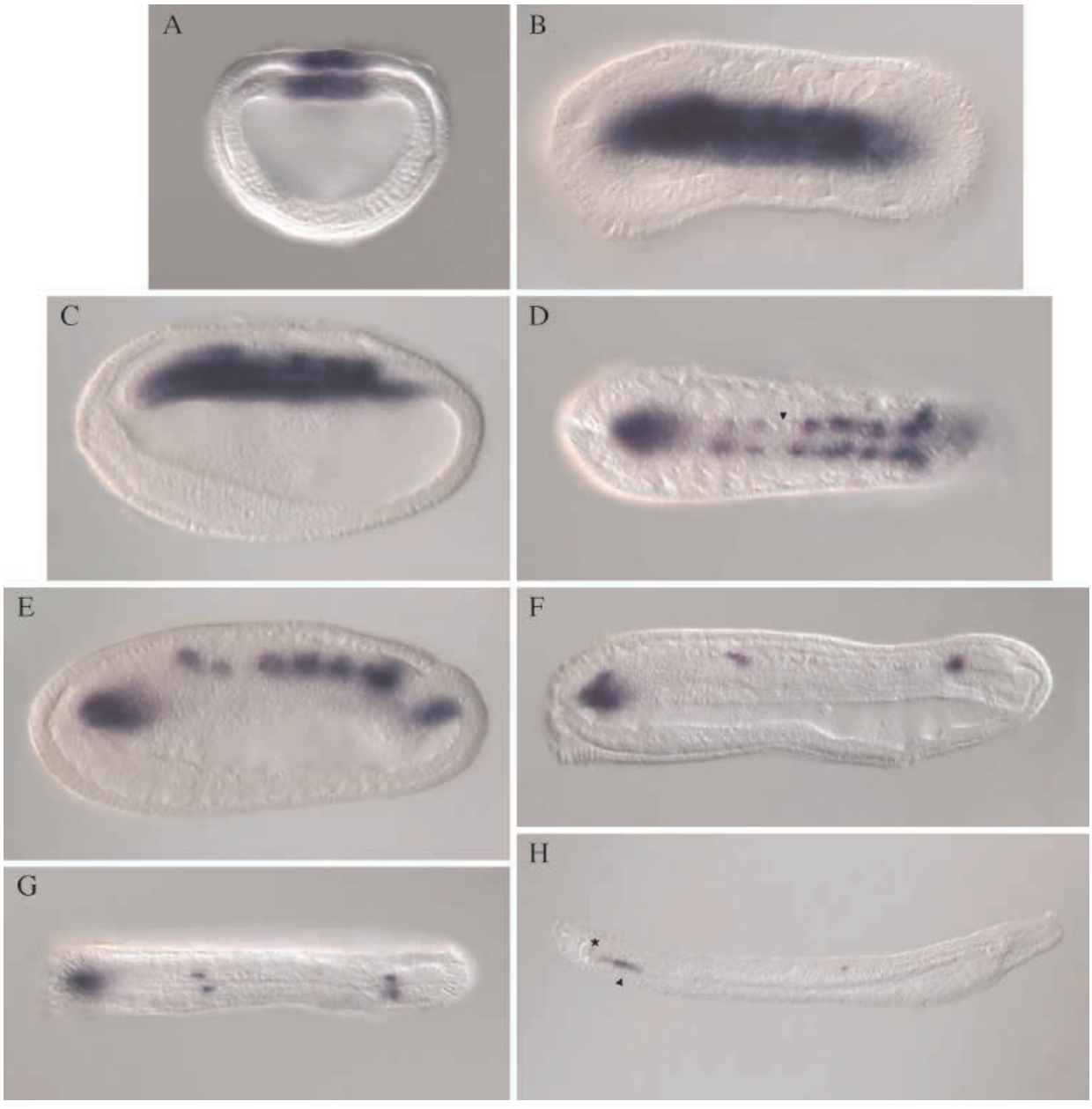

Fig. 2A–H Expression of *AmphiMnx* detected by whole-mount in situ hybridisation. Anterior to the *right*, except **A**. **A** Embryo 10 h post fertilization, posterior view; **B** 15-h embryo, dorsal view; **C** 15-h embryo, lateral view; **D** 19-h embryo, dorsal view; **E** 19-h embryo, lateral view; **F** 24-h embryo, lateral view; **G** 24-h embryo, dorsal view; **H** 35-h larva, lateral view. *Arrowhead* in **D** marks gap in repetitive series of neuronal spots. In **H**, *arrowhead* marks dorsal wall of gut, *star* marks posterior mesoderm

duplication event. Therefore, *HB9* and *MNR2* are vertebrate-specific duplicates, in an analogous way to *En1* and *En2*, or *Otx1* and *Otx2*, or *Emx1* and *Emx2*, etc. To reflect this evolutionary relationship, we propose that *HB9* be renamed *Mnx1*, and *MNR2* be renamed *Mnx2*.

Whole-mount in situ hybridisation to amphioxus embryos revealed that *AmphiMnx* has a dynamic expression pattern in neurectoderm and mesendoderm. Expression is first detected at 10 h post-fertilization (newly hatched gastrula) along the length of the neural plate and in the underlying mesendoderm (Fig. 2 A). The extensive mesendodermal expression persists until 15 h (Fig. 2B,C), but is mostly lost by 19 h, except at the anterior and posterior extremities (Fig. 2E). The anterior patch fades by 24 h (Fig. 2F); posterior expression persists in endoderm cells dorsal to the gut lining (arrowhead) and weakly in extreme posterior mesoderm (star) in swimming larvae, between 35 h (Fig. 2H) and 60 h (data not shown).

The neurectodermal expression is extensive and uniform when first detected (10 h), but from 15 h to 19 h it rapidly resolves into a series of bilateral pairs of spots forming two punctate irregular stripes along the neural tube (Fig. 2D). The most rostral patch of expression, opposite somite 1, is the largest and most intensely stained. This probably represents two adjacent expressing cells on

each side: one close to the midline and just behind the cerebral vesicle, the other more lateral. The next three pairs of spots are also close to the midline of the neural tube, and are positioned opposite the centre of somites 2, 3 and 4. There is then a noticeable gap in the series, opposite somite 5 (arrowhead, Fig. 2D,E). The two subsequent pairs of spots are located opposite somite boundaries 5/6 and 6/7, and comprise single cells lateral to the midline on each side. It is noteworthy that throughout the series, spots are slightly out of register between left and right sides of the body (left spots being more rostral), reflecting the asymmetrical nature of somites and their innervation in amphioxus (Fig. 2D).

Definitive identification of the neurectodermal cells expressing *AmphiMnx* is not possible, but a strong contender emerges from comparison with neuroanatomical descriptions and previous gene expression studies. Using serial transmission electron microscopy on amphioxus larvae, Lacalli and Kelly (1999) identified two distinct classes of motor neuron in the nerve cord. Ventral compartment (VC) motor neurons innervate the bulk of the myotome and have irregular spacing in the nerve cord, with no clear segmental pattern. In contrast, the dorsal compartment (DC) motor neurons that innervate mitochondria-rich superficial fibres of the myotome follow an irregular reiterated pattern, with two pairs of neurons opposite somite 2, just posterior to the cerebral vesicle, and subsequent pairs opposite somite boundaries 2/3, 3/4 and 4/5. There is then an alteration in the series, with no DC motor neurons opposite the somite 5/6 boundary. The study of Lacalli and Kelly (1999) did not extend more posterior to somite 6, so it is unclear if the segmental series of DC motor neurons recommences. The distribution of DC motor neurons in 8- to 12-day amphioxus larvae is strikingly similar to the pattern of *AmphiMnx* expression in 19-h embryos, despite the large temporal difference between the two studies. The principal difference is that the *AmphiMnx* positive cells are located approximately half a somite length more rostral than the DC motor neurons, as described for 8- to 12-day larvae. This could reflect a slight shift in the relative position of the nerve cord and the somites during amphioxus development.

The *AmphiMnx* expression pattern shows intriguing similarities to, and differences from, the expression of another putative motor neuron marker, the amphioxus LIM-domain gene *islet* (Jackman et al. 2000). At comparable stages of development, both *AmphiMnx* and *islet* are expressed in several neuronal cells at the level of somite 1, and then in three subsequent smaller patches. For both genes, there is then an alteration in the repeating series around the level of somite 5, before two further paired patches of expression opposite somite boundaries 5/6 and 6/7. For *AmphiMnx*, the alteration at the somite 5 level is manifested as a lack of expression, whereas for *islet* it consists of a change in the shape and dorsoventral position of the expressing cells. These latter *islet*-positive cells do not match the description of DC motor neurons as given by Lacalli and Kelly (1999). We suggest, therefore, that *AmphiMnx* is expressed in the developing DC motor neurons and that these, in turn, comprise a subset of the *islet*-expressing cells in the amphioxus nerve cord.

In vertebrates, the *MNR2* (*Mnx2*) gene acts upstream of *islet1, islet2* and *HB9* (*Mnx1*) in the *shh*-induced pathway of motor neuron differentiation. Based on gene expression patterns, we propose that a functional relationship between Mnx class and islet class homeobox genes also exists in the cephalochordate amphioxus, and by inference existed in the common ancestor of cephalochordates and vertebrates. This is despite the fact that *AmphiMnx* diverged before the gene duplication that yielded *HB9* (*Mnx1*) and *MNR2* (*Mnx2*), and that amphioxus *islet* diverged before the gene duplication that yielded *islet1* and *islet2* (Jackman et al. 2000). We conclude that the single *Mnx* gene of early chordates played a role in motor neuron development, and that this role was been maintained and elaborated by *HB9 (Mnx1*) and *MNR2* (*Mnx2)* after gene duplication in the vertebrate lineage.

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