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EXPRESSION NOTE

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An amphioxus Msx gene expressed predominantly in the dorsal neural tube

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Abstract Genomic and cDNA clones of an Msx class homeobox gene were isolated from amphioxus (*Branchiostoma floridae*). The gene, *AmphiMsx*, is expressed in the neural plate from late gastrulation; in later embryos it is expressed in dorsal cells of the neural tube, excluding anterior and posterior regions, in an irregular reiterated pattern. There is transient expression in dorsal cells within somites, reminiscent of migrating neural crest cells of vertebrates. In larvae, mRNA is detected in two patches of anterior ectoderm proposed to be placodes. Evolutionary analyses show there is little phylogenetic information in Msx protein sequences; however, it is likely that duplication of Msx genes occurred in the vertebrate lineage.

Key words Amphioxus · Msx gene · Neural crest · Placodes · Vertebrate evolution

The Msx class of homeobox genes encode highly conserved homeodomain proteins implicated in the development of a wide range of embryonic tissues (reviewed by Davidson 1995). Multiple Msx genes have been isolated from vertebrates, including mouse (three genes), chick (two genes), *Xenopus* (two genes) and zebrafish (five genes); single Msx genes have been reported from invertebrates, including *Drosophila*, ascidian, sea urchin and leech (Ma et al. 1996; Master et al. 1996; Dobias et al. 1997; Ekker et al. 1997; Isshiki et al. 1997). The three mouse Msx genes are all expressed in the dorsal neural tube, while *Msx-1* and *Msx-2* are also expressed in other tissues during embryogenesis, including cranial neural crest cells, placodes, limb buds, tooth buds, genital tubercle, pituitary and hair follicles. Expression in non-neural tissues is clearly functional, as demonstrated by gene targeting (Satokata and Maas 1994) and antisense experiments in mice (Foerst-Potts and Sadler 1997), identification of a human mutation (Jabs et al. 1993) and ectopic expression in chick embryos (Ferrari et al. 1998). Determining how vertebrate Msx genes evolved their multiple roles may be addressed by comparison to their closest invertebrate relatives: cephalochordates or amphioxus. For many vertebrate gene families involved in the control of development, amphioxus has just single homologues (Holland 1996); amphioxus can therefore provide information about possible ancestral roles and about timing of gene duplication events.

We first amplified a 220-bp fragment from amphioxus genomic DNA using Msx-specific polymerase chain reaction (PCR) primers described by Holland (1991). This yielded eight identical recombinant clones. Screening of a *Branchiostoma floridae* genomic library (Garcia-Fernàndez and Holland 1994) with a PCR clone lead to isolation of the 3' exon of the gene, including the full homeobox sequence. Using this to screen a cDNA library made from 5- to 24-h embryos (kindly provided by Dr J. Langeland, University of Kalamazoo, Mich) yielded three overlapping cDNA clones from the same gene, which we denote *AmphiMsx*. A genomic Southern blot revealed that *AmphiMsx* is present in a single copy in the amphioxus genome (data not shown). The longest cDNA contained the full open reading frame, shown in Fig. 1A. The deduced start codon matches the Kozak consensus and is the first methionine downstream of an in-frame stop codon. The gene contains an intron 5' to the homeobox, as expected for Msx genes; the existence of other introns more 5' would not have been detected since this region was not cloned from genomic DNA. The homeodomain encoded by *AmphiMsx* has much higher sequence identity to the Msx class (between 92 and 95%) than to related homeodomain classes such as Dlx, NK1, NK2, NK3,

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The sequence reported in this paper has been deposited in the GenBank data base under the accession number AJ130766

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A

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MAQSTLPTSSNSAFSKPTTSAPSSASSPTSTEKLPFSVASLMADK  75
PKETEQNQSDSGPPPLQSPGGPQSPASPPATVPTAPAQPPSRPSD 150
FSVEGILSKPCSSETAAAEKGHDPTGFAAARFPWLQSSRYSPPPR 225
DRLPTPNKCTLRKHKTNRKPRTPFTTQQLLALERKFRQKQYLSIA 300
ERAEFSASLNLTETQVKIWFQNRRAKAKRLQEAELEKLKMAAKPM 375
LPPALGMTFPSPFYAAASPFHRPGLPVQACQIGPYTYYPSHTYAG 450
FIHSS                                          455
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B

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AmphiMsx NRKPRTPFTTQQLLALERKFRQKQYLSIAERAEFSASLNLTETQVKIWFQNRRAKAKRLQ
Msx-1    ----------A-----------------------S--S--------------------
Msx-2    ----------S-----------------------S-----------------------
Msx-3    ----------A---------H-------------S--S--------------------
msh      --------------S--K---E-------------S--R--------------------
SpMsx    ----------S-----------------------------------------------

AmphiDll M-----IY-SF--QQ-N-R-QRT---ALP----LA-Q-G--Q-----------S-Y-K-M
NK1/S59  P-RA--A--YE--VS--N--KTTR---VC--LNLAL--S-------------T-W-KQN
NK2/vnd  K--R-VL--KA-TYE---R---QR---AP--EHLASLIR--P---------H-Y-T--A-
NK3/bap  KKRS-AA-SHA-VFE---R-A-QR---GP--S-MAK--R-------------Y-T--K-
NK4/tin  K----VL-SQA-V-E--CR--L-K--TG---EIIAQK---SA----------Y-S--GD
Hlx-1    RSWS-AV-SNL-RKG--KR-EIQK-VTKPD-KQLA-M-G--DA---V------M-WRHSK
Tlx-1    KK----S--RL-ICE--KR-HRQK--AS----ALAKA-KM-DA---T------T-WR-QT
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Fig. 1 A Complete deduced protein sequence for AmphiMsx. The *arrowhead* denotes the position of an intron in the gene; sequence motifs (eh1, hexapeptide and homeodomain, in that order from amino to carboxy termini) are *underlined in bold*. The full nucleotide sequence is available from the EMBL database, accession number AJ130766. **B** Alignment showing high sequence similarity of the AmphiMsx homeodomain to Msx class homeodomains (Mouse Msx-1, Msx-2, Msx-3, *Drosophila* msh). For comparison, representative homeodomains from related classes are also shown: Dlx (AmphiDll), NK1 - NK4 (*Drosophila* S59, vnd, bap, tin), Hlx-1 and Tlx-1. *Dashes* indicate amino acids identical to AmphiMsx

NK4, Tlx or Hlx (between 37 and 60%; Fig. 1B). Phylogenetic analysis also supports assignment to the Msx class (not shown). The deduced protein sequence has two other conserved protein motifs characteristic of Msx and several other homeodomain proteins: the eh1 motif (Fig. 1A, bold and underlined) and a sequence related to the hexapeptide of Hox proteins (Fig. 1A, bold and underlined). The eh1 motif acts as a transcriptional repressor in En class proteins and is present in Msx, NK-1, NK-2 and gsc homeodomain proteins (Smith and Jaynes 1996) plus, surprisingly, fork-head proteins (Shimeld 1997). Both the eh1 and the hexapeptide motif are thought to mediate interaction with protein cofactors to modulate transcriptional activity or specificity.

We constructed molecular phylogenetic trees of Msx proteins using several alternate alignments and phylogenetic methods. ClustalW (Thompson et al. 1994) and MUST (courtesy of Hervé Philippe, CNRS, Paris) were used for constructing alignments and the Neighbor-joining and Parsimony methods of MUST, PHYLIP (Felsenstein 1993) and MEGA (Kumar et al. 1993) for constructing phylogenies. *AmphiMsx* was consistently found to be closely related to vertebrate Msx genes, usually as an outgroup although this was not well-supported by bootstrapping (not shown). Taken together with the Southern analysis and the fact that we cloned the same Msx gene by PCR, cDNA and genomic library screening, this leads us to suggest that the gene duplications leading to multiple vertebrate Msx genes took place on the vertebrate lineage, after the cephalochordates had diverged. We also conclude that the amount of phylogenetic information retained by Msx protein or DNA sequences is insufficient to resolve relationships between chordate members of this gene family, due to invariance of some motifs combined with very low conservation in other regions of the protein.

The expression pattern of *AmphiMsx* was examined in amphioxus embryos and larvae by whole-mount in situ hybridisation. Expression was first observed in late gastrulae in two lateral stripes in the ectoderm (Fig. 2 A). In neurulae, prior to neural tube closure, expression becomes confined to the lateral neural plate, with expressing cells excluded from the midline. This expression is most intense posteriorly, adjacent to somite 5 (Fig. 2B). As neurulation continues, expression is activated along much of the neural tube but remains excluded from the anterior and posterior extremes (Fig. 2C, D). There is slight asymmetry between left and right expression, matching asymmetry of the adjacent somites, and from a dorsal aspect the intensity of expression can be seen to vary along the anterior/posterior axis in approximate registry with the somites (Fig. 2D). Over the next few hours, the amphioxus embryo elongates before beginning the morphogenetic movements that produce the asymmetric larval head. During this time, *AmphiMsx* expression resolves into a semi-regular series of reiterated patches of expression (Fig. 2F), before decreasing and becoming undetectable in the neural tubes of early larvae (Fig. 2I and data not shown). Reiterated expression is reminiscent of the transient expression of chick *Msx-2* and mouse *Msx-3* in alternate rhombomeres (Graham et al. 1993; Shimeld et al. 1996), though we think it unlikely that this indicates some rhombomeric characteristics are found in a larger portion of the amphioxus neural tube. More likely, this represents a confinement of Msx expression to a specific subpopulation of neural cells. Sec-

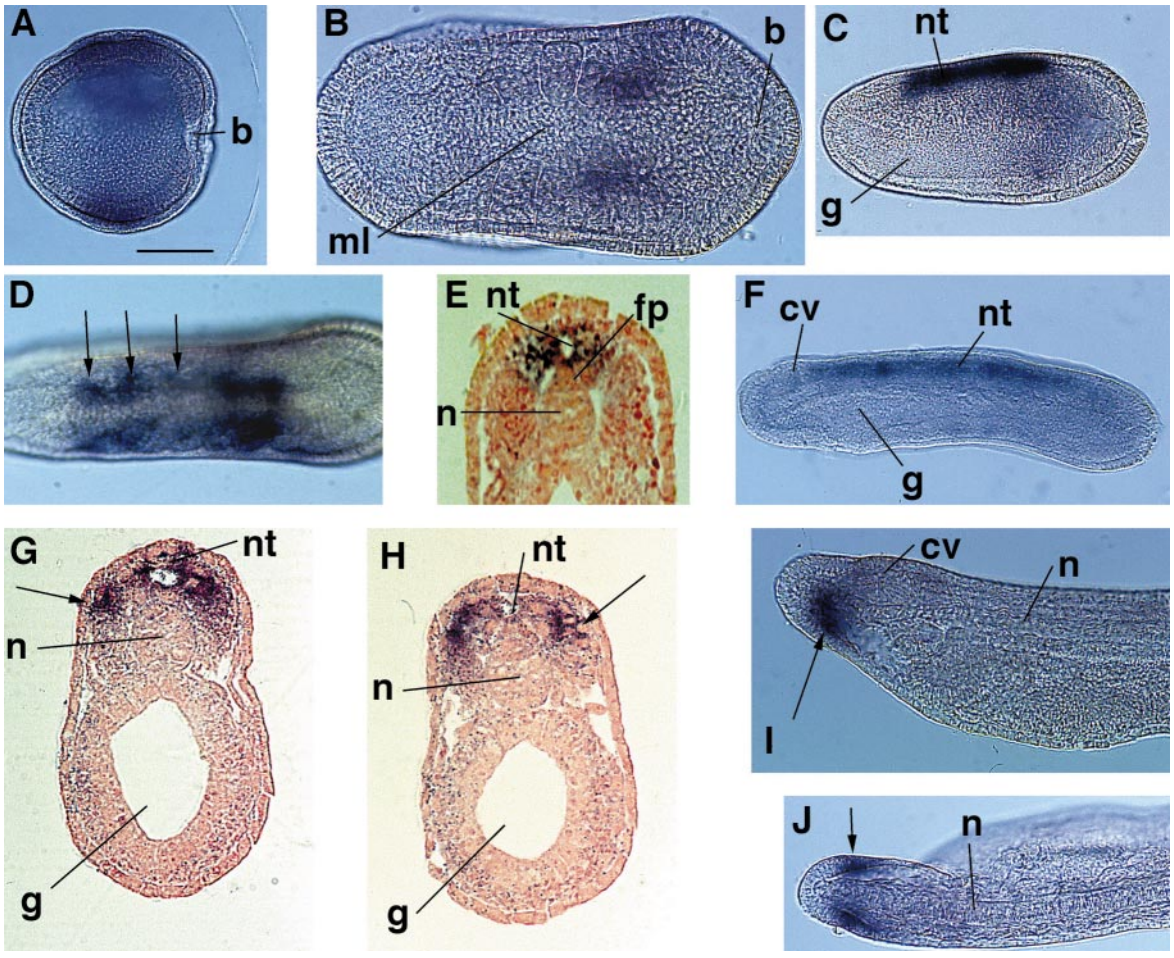

Fig. 2 A–J Expression of *AmphiMsx* during amphioxus embryonic and larval development detected by whole-mount in situ hybridisation. All embryos are oriented with anterior *to the left*, all sections with dorsal *to the top*. **A** Late gastrula in which the blastopore has almost closed. *AmphiMsx* expression is present in two broad domains in the lateral ectoderm. **B** Dorsal view of 5-somite neurula. *AmphiMsx*-expressing cells are present in the lateral neural plate and excluded from the midline. Expression is strongest posteriorly, adjacent to somites 4 and 5. **C** Lateral and **D** dorsal views of 7-somite embryos. Transcripts are confined to the neural tube from the level of somite 1 to somite 6. The dorsal view reveals that at this stage expression can be resolved into a semi-regular alternation of cells with high (*arrows*) and low levels of staining. **E** Transverse section through an embryo at approximately the 7-somite stage, note exclusion of staining from the floor plate cells overlying the notochord. **F** Larva beginning to undergo the transformations that create asymmetric anterior morphology. Patchy expression is visible in the neural tube. **G, H** Transverse sections through an embryo with about 9 somites. *AmphiMsx*-expressing cells are visible in the lateral neural tube and in small numbers in the dorsal somite (*arrows*). **I** Lateral and **J** dorsal views of a larva in which the mouth has just opened. *AmphiMsx* expression is confined to a small group of anterior ectodermal cells, rostral to the cerebral vesicle (*arrow*; *b* blastopore, *cv* cerebral vesicle, *fp* floor plate, *g* gut, *ml* midline, *n* notochord, *nt* neural tube). The *scale bar* on **A** is equivalent to 40 µm for **A, C, D, F, I** and **J**, 25 µm for **B** and 17 µm for **E, G** and **H**

tions of hybridised neurulae revealed that expression is confined to dorsal cells in the neural tube and is excluded from the floor plate (Fig. 2E). Sections of older embryos revealed that the reiterated expression is due to groups of cells in the lateral neural tube. Reconstruction of sectioned embryos showed that left and right patches of stained cells did not precisely align (Fig. 2G, H and data not shown).

Sectioning 18-h embryos also revealed a small number of expressing cells in the dorsal somite, outside the neural tube (arrow on Fig. 2H). These expressing cells are not detected either before or after the 18-h stage. It is tempting to speculate that these cells are of neural origin, having separated from the neural tube during neurulation; if so, these cells may be homologous to the neural crest cells of vertebrates. However, since we could not detect these cells at earlier or later developmental stages we were not able to determine if they originated in the neural tube or if they subsequently migrated away from the dorsal somite.

Expression of *AmphiMsx* was also investigated during early larval development. In animals raised at 23°C the mouth opens at about 36 h and feeding commences. At this stage, expression is confined to a small, bilaterally symmetrical patch of cells in the anterior ectoderm (Fig. 2I, J). This patch of cells is anterior to the cerebral vesicle and adjacent to the notochord, in a position where

an anterior sense organ (the corpuscles of de Quatrefages) will develop (de Quatrefages 1845). We suggest that this expression domain may be homologous to mouse *Msx-1* or axolotl *Msx-2* gene expression in ectodermal placodes (Grindley et al. 1995; Metscher et al. 1997).

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