

A Mox homeobox gene in the gastropod mollusc *Haliotis rufescens* is differentially expressed during larval morphogenesis and metamorphosis

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Abstract We have isolated a homeobox-containing cDNA from the gastropod mollusc *Haliotis rufescens* that is most similar to members of the Mox homeobox gene class. The derived *Haliotis* homeodomain sequence is 85% identical to mouse and frog Mox-2 homeodomains and 88.9% identical to the partial cnidarian cnox5-Hm homeodomain. Quantitative reverse transcription-polymerase chain reaction analysis of mRNA accumulation reveals that this gene, called *HruMox*, is expressed in the larva, but not in the early embryo. Transcripts are most prevalent during larval morphogenesis from trochophore to veliger. There are also transient increases in transcript prevalence 1 and 3 days after the initiation of metamorphosis from veliger to juvenile. The identification of a molluscan Mox homeobox gene that is more closely related to vertebrate genes than other protostome (e.g. *Drosophila*) genes suggests the Mox class of homeobox genes may consist of several different families that have been conserved through evolution.

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1. Introduction

Proteins encoded by homeobox genes comprise a large family of conserved transcription factors that control the development of eukaryotes. Hox class homeobox genes are clustered within metazoan genomes and play a critical role in antero-posterior spatial patterning. Other classes of metazoan-specific homeobox genes are interspersed throughout the genome and have a diversity of functions in development [1]. One such class comprises the Mox homeobox genes [2], to date isolated only from vertebrates and represented only by mammalian and *Xenopus Mox-1* and *Mox-2* genes [3–5], which are expressed in the mesoderm during gastrulation [3,4].

Of the major protostome phyla, homeobox genes have been studied extensively in the arthropods and annelids, but less so in the molluscs (see [6] for a compilation of homeobox gene structure and expression). Most molluscs, along with many annelids, platyhelminthes, nemerteans, sipunculids, echiuroids and gnathostomulids, display a highly conserved, similar pattern of development that includes spiral cleavage and the formation of a trochophore larva [7,8]. Although this is one of the most common modes of metazoan development, there is

little understanding of the molecular mechanisms controlling spiralian cell specification and pattern formation (isolation and characterization of developmentally regulated genes in annelids is largely confined to the leech, which undergoes a derived form of development that does not include many of the ancestral spiralian features [9]). We previously identified eight homeoboxes that are expressed during larval development and metamorphosis of the archaeogastropod mollusc *Haliotis rufescens* (the red abalone) [10] as a first step towards understanding the role of this gene family in spiralian development. *Haliotis* is a particularly tractable model system with which to investigate events occurring at larval metamorphosis, since the initial morphogenetic chemical cues, chemosensory receptors and receptor-mediated signal transduction pathways controlling the induction of metamorphosis have been identified [11,12]. Here we report the first complete gastropod homeobox gene sequence, a Mox homologue (*HruMox*), from *Haliotis* and describe its temporal expression pattern during embryogenesis, larval development and metamorphosis.

2. Materials and methods

2.1. Abalone larvae and RNA isolation

Haliotis rufescens were induced to spawn by the addition of hydrogen peroxide to the sea water [13]. The resultant larvae were reared at 15°C in 5 µm-filtered, UV-irradiated flowing sea water. At 9 days post-fertilization (DPF), planktonic competent veliger larvae were induced to settle and initiate metamorphosis by the addition of 10⁻⁶ M γ-aminobutyric acid (GABA) to the cultures [14]. Total RNA was isolated from approximately 5000 of each stage of unfertilized eggs, embryos, larvae and postlarvae undergoing metamorphosis as described in [10]; a single cohort was used for quantitative RT-PCR and Northern blot hybridization analyses.

2.2. Isolation of *HruMox* cDNA

Partial *HruMox* (previously termed *Hrox1*; see [10]) homeobox cDNA sequence was obtained previously by PCR amplification of larval cDNA using degenerate homeobox primers [10]. For the present study, an *HruMox*-specific primer (hm1-5'-CTGACACGGC-TACGACGATAT-3') was used to amplify the 3'-end of cDNA synthesised from RNA isolated from 7 day old larvae. 3'-RACE (30 cycles of 94°C for 45 s, 61°C for 60 s, and 72°C for 2.5 min), and cloning and sequencing of PCR products, were performed as described in [15]. The 5'-end of the gene was obtained by direct PCR amplification from a λgt10 7-day-old larval cDNA library using a second *HruMox*-specific primer (hm2-5'-CACTGCAAGTTCA-TATCGTCC-3') and a λgt10-specific primer (5'-CTTTTGG-CAAGTTCAGCCTGGTTAAG-3'). Prior to PCR, 2 × 10⁶ recombinant λgt10 plaques in 1.0 µl of 20 mM Tris-HCl (pH 7.4), 100 mM NaCl, 10 mM MgSO₄ were diluted 1:10 in H₂O and boiled for 10 min. One microlitre of diluted, recombinant phage DNA was amplified with hm2 and the λgt10-specific primer (30 cycles of 94°C for 45 s, 59°C for 60 s, and 72°C for 2.5 min), and the predominant product was purified, cloned and sequenced as described in [10].

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2.3. Quantitative RT-PCR analysis

Quantitative RT-PCR analysis of *HruMox* mRNA prevalence was modified from the protocol of [16]. A 1239 nt in vitro transcript was synthesized from 1 µg of linearized pHMX1-1A (a recombinant pSKII plasmid containing the 3'-RACE product) with T7 polymerase. Plasmid DNA was digested by the addition of 5 U RNase-free DNase I (Promega), and newly synthesized RNA extracted with an equal volume of phenol/chloroform and ethanol precipitated. RNA integrity was assessed by formaldehyde denaturing gel electrophoresis, and concentration was determined spectrophotometrically [17]. The transcript was diluted to a final working concentration of 4 × 10⁷ molecules/µl.

cDNA was synthesized from 250 ng total RNA from different developmental stages. Synthesis was performed at 37°C for 1 h in a 20 µl reaction consisting of 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl₂, 5 µM random hexamers, 1 mM dNTPs, 20 U RNasin and 200 U reverse transcriptase [10]. For the standard curve, 2-fold dilutions of the synthesized RNA (7.8 × 10⁴ to 4 × 10⁷ molecules/µl) were reverse transcribed. Two microlitres of the cDNA reaction, equivalent to 25 ng total RNA and 7800 to 4 × 10⁶ molecules of in vitro transcripts, were amplified in a 50 µl reaction consisting of 0.25 µM ³²P-end-labelled [17] primers hm1 and hm4 (nucleotides 1508–1484, Fig. 1; 5'-GGCAACCTATGAGTATTAAGACAG-3'), 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2 mM MgCl₂ and 2.5 U *Taq* polymerase. *HruMox* cDNA was PCR amplified for 23 cycles of 94°C for 45 s, 59°C for 60 s, and 72°C for 1.5 min. Denatured PCR products were electrophoresed on a 5% acrylamide, 6 M urea gel [17]. The gel was dried, an autoradiogram obtained, and radioactive bands were excised from the gel and quantitated by liquid scintillation counting. This experiment was repeated 3 times from the same RNA preparations; control reactions, containing no reverse transcriptase, were run in parallel for the developmental and standard series.

2.4. Northern blot hybridization

A digoxigenin random-prime-labelled *HruMox* cDNA probe was synthesized from a 752 bp PCR product (nucleotides 756–1508, Fig. 1) according to the procedure provided by the supplier (Boehringer-Mannheim). This probe was used in Northern hybridization analysis as described previously [18].

3. Results and discussion

3.1. Isolation and sequence analysis of *HruMox*

Degenerate oligonucleotide primers identified *HruMox* (previously termed *Hrox1*, see [10]) and other homeoboxes expressed during larval morphogenesis and metamorphosis [10]. Further *HruMox* cDNA sequence was determined by isolating and cloning a 1166 bp 3'-RACE product amplified from 7-day-old larval RNA and a 836 bp PCR product amplified from a λgt10 7-day-old larval cDNA library. A composite 1885 bp *HruMox* cDNA sequence was obtained from the three sequences as follows: λgt10 library-derived sequence corresponded to nucleotides 1–786; the previously isolated homeobox sequence [10] to nucleotides 744–812; and the 3'-RACE sequence to nucleotides 756–1885 (Fig. 1). Nucleotide sequence overlap confirmed that the three cDNAs correspond to the same *HruMox* transcript.

The longest open reading frame (ORF) is in frame with the deduced homeodomain (Fig. 1). This 265 residue ORF spans nucleotides 195–989 and has a predicted relative molecular mass of about 30 × 10³. The homeodomain is located towards the carboxyl end of the ORF (residues 163–222). Derived protein sequence outside the homeodomain is Ser (14.6%) and Pro (8.3%) rich. Comparison of the *HruMox*-deduced homeodomain with other homeodomains in the GenBank/EMBL database reveals greatest similarity to vertebrate Mox 2 sequences (85%) [4,6,19,20] and a partial cnidarian Cnox5-Hm sequence (88.9%) [21] (Fig. 2). Among bilaterian homeodomains, the planarian Dutarh6 [22] and *Drosophila*

buttonhead (btd) [23] homeodomains are 81.7 and 75% identical to *HruMox*, respectively. Among Antennapedia (Antp) class homeodomains, the most similar (65%) is the deformed (Dfd) homeodomain [24] (Fig. 2). *HruMox* is similar in size to the mouse Mox 1 and Mox 2 proteins, which are 252 and 298 amino acids, respectively. Analysis of mouse Mox 1 and Mox 2 non-homeodomain sequences reveal that they are also relatively rich in Ser (9.5 and 13.5%, respectively) and Pro (8.7 and 5.8% respectively) residues. While *HruMox* has a number of short amino acids motifs (3–4 residues) in common with either mouse Mox protein, these are not usually located in the same relative position in the proteins nor present in both Mox 1 and 2.

3.2. Temporal accumulation of *HruMox* mRNA during larval development and metamorphosis

A dioxigenin-labelled cDNA probe, corresponding to nucleotides 756–1508 (Fig. 1), hybridized to a single, 1.9 knt transcript that is expressed in 3-day-old larvae and 2-day post-GABA-induced postlarvae (Fig. 3A). This transcript is similar in size to the composite *HruMox* cDNA sequence (Fig. 1).

HruMox transcript prevalence through development was

TGACGACTCAGTCCACAGCTGGAGTAGTGTTTTTCTTCTCTAACCGGAGACTCCGTCCTCC	59
ACCGAGTACTTCACTTGTGGAATATAGCAGGGTATTGCTACTAGCCCACTGACAAAC	119
CATTGCAAAAGGATCAATCGCATTTTGGAAATGTGCGTTGAGGCAGGGTTAATTTTCCTC	179
AACCGAGGACCCCGATGGAAGGTCGCGAGCGGATAGGAGCAAGGACAAGTGTTCCTCCGGG	239
M E G P E R I G A R T S V P G	15
ATGGCCACCGCTCTGCAACTATAGCATGGGAGCYLTACTACAGAGCCAAAGTCCGCTT	299
M A T A S C N Y S M G R G L Q R P S P F	35
GACTGCATGCACAGTGGTGTAGGACTGACCTCTGGTAATCTTGGGTATGGTTCATATTC	359
D C M H S G V G L T S G N L G Y G S Y S	55
AATGACTGGGATGGTACACGGTACCTATTCAACCCCAATAGTGTCAACACAGACCGGCAG	419
N D W G L V H G T Y S P H S V N T D R Q	75
CTGAACAGATCACAAGGGTCTTACCTTTCTATGTCCTCTTCAAAGACTATTAATCAAT	479
L N R S Q G S Y L S M S S S K D Y S I N	95
ACCTTCAATTATCACCTCTCACCTTCTGCCCCAATCGGGGAGGGGGCGGCTTCGAC	539
T S T L S P S H P S A P Q A S G R G A F D	115
ACGACAGGGGAAACCGACTCATATAATCTATGTGTAACCCCTCGACGCAAGTCAACA	599
T T G G T D S Y N S M C N P S T Q V T T	135
TGTATAACCGGAACTAGACAAGAAAGAAACAAACAAAGTSCCCCGATCCTTAT	659
C I T P E L D K K E N K P K D S P D P Y	155
AAATTGGACCTTTCGGTAAACCAAGGAAGGAGGAGGACAGCTTTACCAACATCAGATC	719
K L D L S V K P R K E R T A F T K H Q I	175
CAGGAATCGAAGGAGTTCACGTACACAACCTATCTGACACGGCTACGACGATGAA	779
Q E L E K E F N V H L L R R Y E	195
CTTGCAGTGTCCCTGGAACTTTCTGAAAGACAGGTGAAGGCTGGTTCAAAACAGAAGA	839
L A V S L E L S E R V K V W F Q N R R	215
ATGAATGGAACCGCTGAAGGGGACTAAGCTCGTAAAGGACAAAGTAGATGGGCAACTC	899
M K W K R V K G T K L V A A G L V D G Q L	235
AAGCCAGTAATGGCCCTAATGTGACTTCTACAGGTGTCACTGACGCAGATATGGACGAA	959
K P V M A P N V T S T S V T D A D M D E	255
AGCTTGACTGAACAAGGCCCGCTGCTCACTGATACAGGATTTGATTAAGTTTCTGTGA	1019
S L T E Q E P P A H *	265
TAACCATGAGGAATATGATCTCTGAATAGGTCCTGTCTCTTTTCCCATGAGCCAAAT	1079
GGAAGCAGAGCTTGACTCAAGTCAAGCTGTTACGACAAATCAGGAAGTCAAGACGTT	1139
GCAGGCCCTCAATGAACTTCGGTTTCAATAGGCTTGACATTAAGTCAAGTCAAGTCAAGT	1199
CTTCATGAATATGCTGGTGAAGAATGTGACAGACGGGATGTGCCATTAACATTAACATTA	1259
TGTTGACAAATGGCTGACATGTACCATGTGTAATGTTACAGAAATTAATATCAAGTCT	1319
CTATGATGGCAAAATGTTGATACATATAAAGCCCTCCCTGGTCCGCGACGACGACG	1379
AGGAGAAATCTCATGTTTACCTTTCCTAAAATTTCTCAATGATGTTGACGACGACGAC	1439
AGACGATGAAATCCCCACGCAATAAGACAGAAATTAATATACCTCTTAATTAATCA	1499
TAGGTTGCCATTTGTTGACCGTAACTGACAGCAAGTCAAGTGCATGATTAATTAAGCA	1559
GCAGCTTAAACTACACCGGTCATGTAGCAACAAAATGTGTTACCGAATTCCTCGAAA	1619
GATTTGGTTATTTCACTGTGAGGGAAGAAAGTAACAACTTACCTCCGATTTGGATGTT	1679
CATTTGAAAAAGTAATGTTTTCACGCTCCATAGAACATGCGCCATCTGGTGGCG	1739
AAAAAATCGGACGAGTACACTGCTCTCTTCTGATTTGCTCAAGTGAACGTTCAAGG	1799
TTTGGTACAGGAAACTTATCAGGATAAGTATCCAAGTTTATTTTACAAACATGCT	1859
CTGTAATAAACAATAATTTAGAAGA	1885

Fig. 1. *Haliotis HruMox* cDNA and predicted amino acid sequences. The homeodomain sequence is in bold and the polyadenylation signal is underlined. Nucleotide and amino acid numbering is to the right. The *HruMox* DNA sequence can be found in the GenBank/EMBL database under accession number X75217.

	10	20	30	40	50	60	
HruMox	PRKERTAF	TKHQIQE	LEKEFNV	HNHNYL	TRLRRYEL	AVSLSE	RQVKVWFQ
cnox5-Hm	--N--VRN	-----S	-----S	-----S	-----S	-----S	88.9/88.9
m Mox-2	-----E	--R--A--AH	-----I	--N--D--T	-----I	-----I	85.0/90.0
X Mox-2	-----E	--R--A--AH	-----I	--N--D--T	-----I	-----I	85.0/90.0
m Mox-1	A-----E	--LR--A--AH	-----I	--N--D--T	-----I	-----I	83.3/88.3
Dutarh6	Q-----S	--G--L--A	-----A	--N--N--I	-----C	--S--	81.7/85.0
btd	N-----S	--T--LQ--A	--CYS	-----I	--A--T--	-----C	75.0/83.3
Dfd	-KRQ---	Y-R--L--	--HYNR---	R--I--I	--HT--V---	I--I-----	KDN 65.0/80.0
Antp	RKRG-QTY	-RY-TL---	--HFNR---	R--I--I	--HA--C--T---	I--I-----	KEN 55.0/70.0

Fig. 2. Alignment of *Haliotis HruMox* deduced homeodomain sequence with other closely related metazoan homeodomains: Mouse (m) and *Xenopus* (X) Mox homeodomains [3,19], the most similar cnidarian (cnox5-Hm) [21], planarian (Dutarh6) [22] and *Drosophila* (btd) [23] homeodomain, and homeodomains of two members of the *Drosophila* HOM-C complex, Dfd [24] and Antp [30]. Dashes indicate identity with *HruMox*. The first value to the right corresponds to percent peptide similarity to *HruMox* and the second value is the percent identity with conservative amino acid changes included (I-V-L; T-S; R-K; D-E; N-Q; F-Y-W).

determined from three replicate quantitative RT-PCR analyses. Positive control RT-PCR amplification of a *Haliotis* larval tropomyosin mRNA from the same RNA samples indicated that the reverse transcription of cDNA was occurring at approximately identical efficiency in all samples (not shown). Negative control experiments on both developmental and in vitro RNAs, containing no reverse transcriptase, yielded no amplification products (not shown).

HruMox mRNA was not detected in the egg or embryo (Fig. 3B,C); that is, no *HruMox* products were detected following saturation RT-PCR amplification (35 PCR cycles) of 1 µg maternal, 16-cell (4 h post-fertilization) and gastrula (16 h post-fertilization) total RNAs (not shown). The transcript was first detected in the newly hatched trochophore larva, indicating a significant increase in *HruMox* mRNA prevalence between 16 and 24 h (Fig. 3B,C). During larval morphogenesis from trochophore to veliger, mRNA levels remained relatively constant at about 150 *HruMox* mRNAs/ng total RNA. Development of the veliger larva was completed about 3 days after fertilization at 15°C. After this stage, *HruMox* transcript prevalence began decreasing, such that by 7 days there were about 65 transcripts/ng total RNA (Fig. 3C). Larvae not induced to initiate metamorphosis exhibited further decreases in the amount of *HruMox* mRNA. In the metamorphosing post-larvae, *HruMox* mRNA prevalence increased at 1 day (≈115 *HruMox* mRNA/ng total RNA) and 3 days (≈85 *HruMox* mRNA/ng total RNA) post-GABA-induction (Fig. 3C). By 6 days post-induction of metamorphosis, transcript accumu-

lation had decreased to approximately 40 molecules/ng total RNA.

In vertebrates, *Mox* genes appear to be involved in initial mesoderm differentiation, being expressed prior to factors involved in terminal differentiation [3,4]. In *Xenopus*, *X. Mox-2* gene expression is activated when mesoderm induction occurs at gastrulation; transcripts are first detected in ventral, lateral

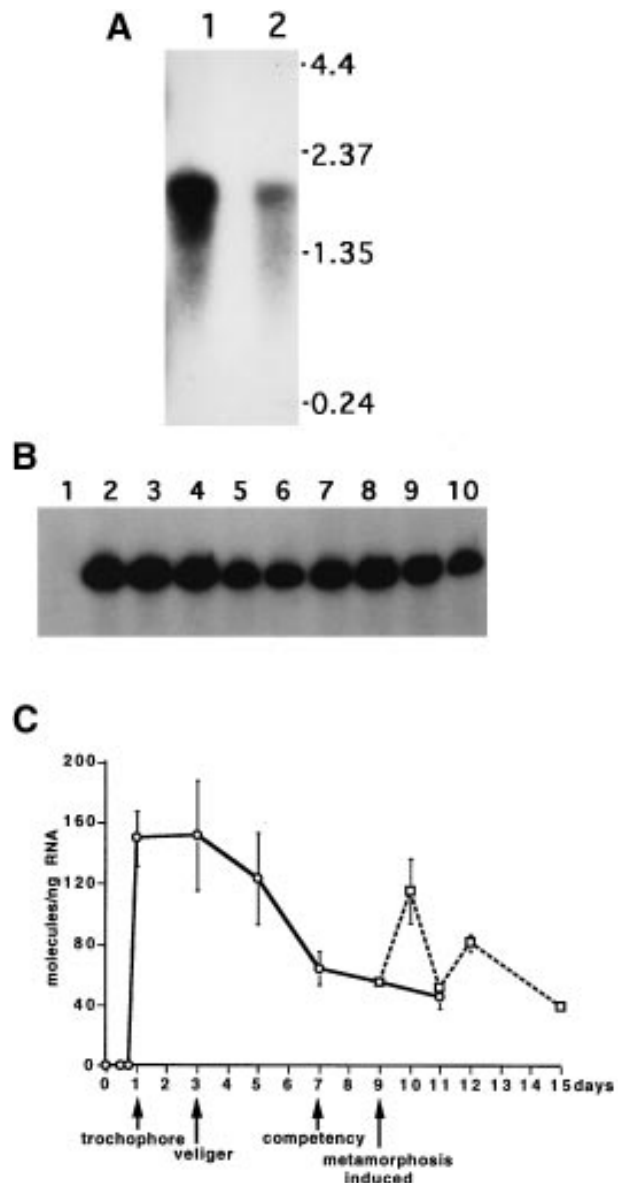


Fig. 3. Northern and quantitative RT-PCR analysis of *HruMox* transcript accumulation through embryonic and larval development, and metamorphosis. Competent veliger larvae were induced to begin metamorphosis by the addition of 10⁻⁶ M GABA 9 days post-hatching. A: Northern blot analysis. Lane 1, 3-day veliger larva; 2, 2-day post-GABA treatment. 10 µg poly(A) RNA was loaded in each lane. B: Autoradiograph of a gel showing the 752 bp RT-PCR products generated from a developmental time course. Lane 1, 16 h late gastrula; 2, 1-day trochophore larva; 3, 3-day veliger larva; 4, 5-day veliger; 5, 7-day veliger competent to settle; 6, 11-day veliger; 7, 1-day post-GABA postlarva; 8, 2-day post-GABA postlarva; 9, 3-day post-GABA postlarva; and 10, 6-day post-GABA postlarva. C: RT-PCR quantitation of *HruMox* transcript prevalence. Radiolabelled PCR products from the developmental series were compared to the standard curve generated from known amounts of in vitro sense transcripts, and the number of *HruMox* RNA molecules/ng total RNA determined and plotted. Triplicate experiments were performed. —○—, embryonic and larval expression; ---□---, postlarval expression at metamorphosis. One standard deviation unit is shown.

and paraxial mesoderm [4]. Likewise in mouse, *Mox-1* and -2 expression is in the mesoderm, beginning at gastrulation or shortly after the formation of the mesodermal germ layer [3]. In many gastropods, the larval retractor muscle cells differentiate from the mesoderm of the early trochophore [25,26]. Analysis of muscle-specific tropomyosin gene expression [15], indicates that muscle differentiation is occurring at about the same time that *HruMox* is first expressed. Tropomyosin transcripts begin accumulating about 24 h post-fertilization [15] and myofibrillogenesis begins in trochophore mesodermal cells about 42 h post-fertilization (Degnan and Morse, unpublished data).

Induction of *Haliotis* metamorphosis [27–29] results in a dramatic restructuring of the veliger larva, including the destruction of redundant larval tissues, the biogenesis of new juvenile (postlarval) structures, and the recruitment and restructuring of larval tissues into the juvenile body plan [12,15,18]. Transient increases of *HruMox* mRNA abundance at metamorphosis suggests that this transcription factor-encoding gene may be playing a role in one or more of the above processes. For example, within 48 h of induction of metamorphosis (24 h after the first increase in *HruMox* mRNA level) a set of median mesodermal cells begins expressing the tropomyosin gene [15]; these cells will eventually give rise to the juvenile columellar muscle.

3.3. Conclusions

The detection of a *Mox* homeobox-containing gene in the gastropod mollusc *Haliotis rufescens* that has greater sequence identity with *Mox* homeodomains in evolutionarily distant taxa (i.e. vertebrates, cnidarians and planarians) than *Drosophila* homeodomains suggests that there are multiple families within this class of homeobox genes. Either the *Drosophila btd* gene has diverged from other *Mox* class members or a genuine *Drosophila* orthologue of *HruMox* and vertebrate *Mox1/Mox2* has not been isolated.

Despite substantial morphological differences among disparate metazoan taxa, many classes of structurally conserved homeodomain proteins are functionally conserved [1,6]. The expression of *HruMox* during those larval and postlarval stages of *Haliotis* development when mesodermal differentiation occurs suggests that this homeobox gene may be functioning in a manner similar to that observed in vertebrates [3,4]. Comparison of *HruMox* spatial expression patterns with muscle-specific structural gene expression and myofibrillogenesis [15] should provide insight into the role of this gene in gastropod development.

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