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The Single Amphioxus Mox Gene: Insights into the Functional Evolution of Mox Genes, Somites, and the Asymmetry of Amphioxus Somitogenesis

Fn1 **Carolina Minguillón and Jordi Garcia-Fernàndez**[1]

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Mox genes are members of the "extended" Hox-cluster group of Antennapedia-like homeobox genes. Homologues have been cloned from both invertebrate and vertebrate species, and are expressed in mesodermal tissues. In vertebrates, Mox1 and Mox2 are distinctly expressed during the formation of somites and differentiation of their derivatives. Somites are a distinguishing feature uniquely shared by cephalochordates and vertebrates. Here, we report the cloning and expression of the single amphioxus Mox gene. *AmphiMox* is expressed in the presomitic mesoderm (PSM) during early amphioxus somitogenesis and in nascent somites from the tail bud during the late phase. Once a somite is completely formed, *AmphiMox* is rapidly downregulated. We discuss the presence and extent of the PSM in both phases of amphioxus somitogenesis. We also propose a scenario for the functional evolution of Mox genes within chordates, in which Mox was co-opted for somite formation before the cephalochordate–vertebrate split. Novel expression sites found in vertebrates after somite formation postdated Mox duplication in the vertebrate stem lineage, and may be linked to the increase in complexity of vertebrate somites and their derivatives, e.g., the vertebrae. Furthermore, *AmphiMox* expression adds new data into a long-standing debate on the extent of the asymmetry of amphioxus somitogenesis.

Key Words: **amphioxus; chordates; Mox; Extended-Hox; presomitic mesoderm (PSM); tail bud; somitogenesis.**

INTRODUCTION

Homeobox genes play crucial roles in morphogenetic processes. Changes in the regulation and number of homeobox genes have been instrumental in body plan evolution. Recent hypotheses based on phylogeny and sequenced genomes suggest that the Antennapedia superclass of homeobox genes derives from four ancient arrays of genes, originated by gene and cluster duplications (Pollard and Holland, 2000). One of these arrays has been named "extended Hox" and includes the Hox cluster and the Hox-like genes Evx and Mox. Hox and Evx genes have been deeply analyzed in several phyla, and their role in morphological processes as well as their evolutionary story have been described elsewhere (Ferrier *et al.,* 2000; Ferrier and Holland, 2001; Schilling and Knight, 2001). The study of the cephalochordate amphioxus, the closest living relative to vertebrates, has been particularly valuable to ascertain the ancestral state of these genes in chordates. Briefly, the ancestral role of Hox genes in chordates may be the patterning of the neural tube, whereas Evx functions in the posterior part of the embryo and the tail bud of chordates (Wada *et al.,* 1999; Ferrier *et al.,* 2001).

The Mox class, the other member of the "extended Hox" group, seems to play a key role in mesodermal derivatives, especially in vertebrates during the formation and differentiation of somites, transient segments of paraxial mesoderm uniquely present in cephalochordates and vertebrates.

In vertebrates, there are two Mox genes, Mox1 and Mox2. AQ: 1 They have been found in a wide range of vertebrates, namely mouse (Candia *et al.,* 1992), human (Futreal *et al.,* 1994; Grigoriou *et al.,* 1995), chicken (Rallis *et al.,* 2001), rat (Gorski *et al.,* 1993), *Xenopus* (Candia and Wright, 1995), and zebrafish (Neyt *et al.,* 2000). In the mouse, *Mox1* is first expressed at the onset of gastrulation, in a posterior domain of the embryonic mesoderm. Shortly after, when the somites begin to form, it is expressed in the presomitic

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mesoderm (PSM), in the epithelial and differentiating somites, and in the lateral plate mesoderm. In contrast, *Mox2* is not expressed before somite formation, and thereafter is expressed all over the epithelial somites. During somite differentiation, *Mox2* expression becomes restricted to the sclerotome and migrating myoblasts and their derivative muscles in the limb buds (Candia *et al.,* 1992; Candia and Wright, 1996). Moreover, knockout mice for the *Mox2* gene show limb muscle defects, which led the authors to regard this gene as a component of the genetic hierarchy controlling limb muscle development (Mankoo *et al.,* 1999). In *Xenopus*, a single Mox gene has been isolated (*XMox2*; Candia and Wright, 1995). As for mouse *Mox1*, the mesodermal-specific expression of this gene is found in undifferentiated dorsal, lateral, and ventral mesoderm, in the posterior part of neurula/tail bud embryos, and is more anteriorly detected in the dermatomes. In the tail bud tadpole, *XMox2* is expressed in tissues of the tail bud itself, a site of continuous gastrulation-like processes resulting in mesoderm formation. In chicken, two Mox genes have been isolated. *cMox2* is expressed in the somites of developing embryos, in presumptive migrating myoblasts from the dermomyotome to the limb buds, and in the ventral and dorsal parts of limb buds (Rallis *et al.,* 2001).

In invertebrate protostomes, Mox orthologues are also related to mesodermal derivatives. In *Drosophila*, the Mox gene *buttonless* (*btn*) is specifically expressed in 20 cells of a single type during embryonic development, the dorsal medial (DM) cells. These cells are located along the dorsal midline of the bridge that links the two halves of the mesoderm, which points to their mesodermal origin. The absence of *btn* gene function entails the initial commitment to the DM cell fate, but differentiation does not occur and DM cells are lost (Chiang *et al.,* 1994). In the gastropod mollusc *Haliotis rufescens*, the Mox gene orthologue (*Hrox1*) is not expressed in the early embryo, and transcripts are most prevalent during larval morphogenesis from trochophore to veliger. In many gastropods, the larval refractor muscle cells differentiate from the mesoderm of the early trochophore larva. Analysis of muscle-specific tropomyosin gene expression indicates that muscle differentiation and early *Hrox1* gene expression overlap in time (Degnan *et al.,* 1997), and may thus be linked. Mox homologues have been found in flatworms but there is no expression reported (Lukianov *et al.,* 1994) and no clear Mox gene orthologues are present in the *Caenorhabditis elegans* genome (The *C. elegans* Sequencing Consortium, 1998). A *bona fide* Mox gene orthologue has been reported in the cnidarian *Hydra magnipapillata* (*Hm-Cnox5*; Naito *et al.,* 1993), but no expression data are available.

It is now clear that numerous gene families expanded by gene duplication on the vertebrate stem lineage. Its phylogenetic position as the sister group of vertebrates (Wada and Satoh, 1994; Cameron *et al.,* 2000), its simple and prototypical vertebrate-like body plan, and the preduplicative state of its genome situate amphioxus in a privileged position to trace the history of a given gene family in chordates. Mox genes are particularly relevant under a chordate evolutionary perspective, as they may be involved in somite formation and differentiation in vertebrates, and somites are an evolutionary innovation that originated just before the divergence of cephalochordates and vertebrates (Pourquié, 2001). Thus, the isolation and study of Mox in amphioxus may shed light on whether the ancestral role of Mox in chordates is linked to somite origin.

We isolated the single and prototypical amphioxus Mox gene. *AmphiMox* is the pro-orthologue of vertebrate Mox1 and Mox2, and is expressed during somite formation, in the PSM and in nascent somites from the tail bud. Once a somite is completely formed, *AmphiMox* is rapidly downregulated. Our results suggest that Mox was co-opted for somite formation before the cephalochordate–vertebrate split. Novel expression sites found in vertebrates after somite formation may be linked to the increase in complexity of vertebrate somites and their derivatives, e.g., the vertebrae.

MATERIALS AND METHODS

Gene Cloning

A 117-bp DNA fragment with similarity to the Mox homeobox was isolated from *Branchiostoma floridae* cDNA by PCR, using the degenerate primers SO1 and SO2, which recognize the first and third helix, respectively, of the Antennapedia-superclass homeobox sequence (Garcia-Fernàndez and Holland, 1994). Genomic and cDNA clones containing the *AmphiMox* gene were isolated by a combination of library screenings and PCR. Screenings of a single-animal genomic library (Ferrier *et al.,* 2000) and of a larval cDNA library (a gift of Linda Holland, Scripps Institute of Oceanography, San Diego) were performed in medium stringency conditions (60°C) in Church's buffer (Shifman and Stein, 1995). Introns length were determined by PCR.

Phylogenetic Analysis

The sequences used for the phylogenetic comparisons with the *AmphiMox* gene reported here (Accession No. AF490355) were obtained from public databases and aligned by using ClustalX. The homeodomain sequences were subjected to neighbor-joining by using the MEGA 2.0 package (www.megasoftware.net). The parameters used underwent pairwise deletion and Poisson correction. Topology robustness was assessed by 1000 bootstrap resampling of the data.

Obtaining Embryos

Ripe adults of the Florida lancelet were collected from Old Tampa Bay (Florida) during the summer breeding season. Males and females were spawned electrically in the laboratory and *in vitro* fertilization was performed on petri dishes. The selected developmental stages were raised following Holland and Holland (1993).

Whole-Mount in Situ Hybridization and Sectioning

In situ whole-mount hybridizations on amphioxus embryos and larvae were performed as described by Holland *et al.* (1996). A 1200-bp *Eco*RI fragment of the *AmphiMox* cDNA (lacking most of the trailer sequence) was used for *in vitro* transcription with digoxigenin-labeled dUTP. After hybridization and whole-mount photography, selected embryos were contrasted in 1% Poinceau S, 1% acetic acid, dehydrated through an ethanol series, and embedded in LR White medium (TAAB) resin. Serial 3-μm sections were obtained with a glass knife, mounted in DePeX, and photographed under Nomarski optics.

RESULTS

Isolation and Characterization of AmphiMox

A PCR survey of amphioxus embryo cDNA with "universal" Antennapedia-like homeobox primers yielded the isolation of Hox and ParaHox genes, plus a fragment similar to the Mox class of homeobox genes. The *AmphiMox* gene was further characterized by cDNA and genomic screenings (Fig. 1). F1

The genomic screening with the PCR fragment was performed in medium stringency conditions to isolate any gene belonging to the same or related subfamilies of homeoboxes. Three of six positive clones contained other homeobox genes, while the strongest positives encompassed the genomic region of *AmphiMox* (Fig. 1A). cDNA screening of a larval library gave two positive clones of 2.6 kb. The whole cDNA sequence and the predicted protein sequence of *AmphiMox* are shown in Fig. 1B. Further PCR strategies and selected genomic sequencing allowed the characterization of the genomic organization of the gene. *AmphiMox* putatively codes for a 240-amino-acid protein and is organized in three exons and two large introns of about 6 and 5.5 kb (Fig. 1C). For the species of known genomic organization but for *Drosophila*, this structure is conserved (Chiang *et al.,* 1994). The second intron is within the homeobox, between residues 44 and 45 of the homeodomain (Fig. 1B, triangles). This position bears an intron in many homeobox genes, which may reflect the ancestral condition of all homeobox genes or particular families (Bürglin, 1995). Alternatively, the specific nucleotide sequence of the homeobox here (residues QV, encoded by CARGTN) may be a hot spot for intron insertion.

Southern blots of genomic DNA obtained from single individuals were hybridized with a cDNA probe that detects exons 1 and 2 of the *AmphiMox* gene. One or two large bands were detected in each individual DNA, consistent with *AmphiMox* being a single copy gene (data not shown).

Blast searches against databases and comparison of the deduced amino acid sequence of AmphiMox with other Mox proteins confirmed that it belongs to the Mox family of homeobox-containing genes (Fig. 1D). Beyond the homeodomain, sequence similarities of AmphiMox to vertebrate and invertebrate sequences are restricted to scattered residues (data not shown).

Phylogenetic Analysis

Several Mox genes have been isolated from various species. The homeodomain of AmphiMox is more closely related to that of vertebrate Mox genes (85–90%) than to those of other invertebrate genes (77–84%) (Fig. 1D). To gain more insight into the relationships among Mox genes, we conducted a molecular phylogenetic analysis by the neighbor-joining method on the homeodomain of Mox proteins, using Hox-4 sequences as outgroups (Fig. 2). Vertebrate Mox proteins fell into two groups: Mox1 and Mox2. *AmphiMox* branches immediately outside these groups (as their sister group), between the vertebrate genes and the rest of invertebrate genes. The particular grouping of protostome genes does not agree with current ecdysozoa/lophotrocozoa clades, but the bootstrap values supporting these invertebrate protein groupings are very low. The positioning of AmphiMox, before the origin of the vertebrate groups 1 and 2, is supported by a high bootstrap value (74%; see Fig. 4), in agreement with the hypothesis that vertebrate genes have originated by duplication after the cephalochordate–vertebrate divergence. When we omitted the *Drosophila* and flatworm Mox homeodomains (as divergent and relatively long-branched sequences can disrupt the tree topology), the position of AmphiMox as the sister group of vertebrate Mox genes was further supported (bootstrap value raises to 88%; data not shown). Thus, *AmphiMox* may well represent a prototypical direct descendant of the preduplicative gene prior to vertebrate-specific duplications. F2

AmphiMox Gene Expression

AmphiMox expression is intimately linked to somite formation. Somitogenesis in amphioxus can be divided into an early phase, in which somites originate from the outpocketing of the archenteron (Fig. 3G), and a late phase, in which somites arise as solid blocks directly from the proliferating tail bud (Fig. 3J) (Schubert *et al.,* 2001; and references therein). F3

In whole-mount *in situ* hybridizations, no signal was detected prior to hatching. The signal was first visible in the early posthatching neurula (5-somite stage; Fig. 3A), in the newly formed somite (s5). At this stage, *AmphiMox* was also expressed in the most anterior part of the PSM, in a region comprising about one somite in length.

Somitogenesis in amphioxus is particularly asymmetrical. Left somites are formed earlier than right somites and therefore become located slightly rostral (Cerfontaine, 1906). This asymmetry was mirrored by *AmphiMox* expression. In a dorsal view of 5- to 6-somite embryos (Fig. 3B), somites number 5 on each side are morphologically visible (s5) and the posterior boundaries are clearly discernible

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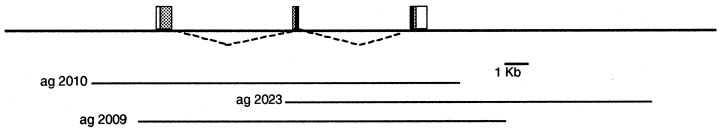

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TCACCGCAGAACATCACTGTCACCTGCGTCCTCACCTGTATGGAGGCAGCCATGTACATGAGCTCGGCAC     140
                                        M  E  A  A  M  Y  M  S  S  A  P
CCCCACCGTCACTCCACCACTACCCCTACGGGCACACGTCTCACTCAGGCCCGCAGGCGTCGCGCTACCC     210
  P  P  S  L  H  H  Y  P  Y  G  H  T  S  H  S  G  P  Q  A  S  R  Y  P
TCCGGTGCCCACCACGGCCGGCGGGCTCCACGCTCTCGCCGAGGCCAGCCATGTATCTATGTACTCTTCC     280
 P  V  P  T  T  A  G  G  L  H  A  L  A  E  A  S  H  V  S  M  Y  S  S
CAGGCGATGAGCTGGTCCCAACGCCCCGAGCAGGTCTCCCTCCACAACTCGGGCATGCACGAAAACCCGT     350
Q  A  M  S  W  S  Q  R  P  E  Q  V  S  L  H  N  S  G  M  H  E  N  P  Y
ATTACGGCCAAGAACAGGACTTGTCCTGCAACAAGACCTTGGAGGAGCGAACCAACATGGCAGTGCCCAC     420
  Y  G  Q  E  Q  D  L  S  C  N  K  T  L  E  E  R  T  N  M  A  V  P  T
GTCTTGTGTTCTGAACTCGAACGTTGTGAACGACGACGACAGGCTGTCCTGCTCTAGTGTGTCCCCCACA     490
 S  C  V  L  N  S  N  V  V  N  D  D  D  R  L  S  C  S  S  V  S  P  T

AACAATAGAGAAAACTCAGACTCGAGTTTTAAGCTGGACCTGTCGGCCAAGCCGAGGAAAGAGCGCACAG     560
N  N  R  E  N  S  D  S  S  F  K  L  D  L  S  A  K  P  R  K  E  R  T  A
CCTTCACCAAACAGCAGATCATGGAGCTGGAGAACGAGTTCCGCCATCACAACTACCTGACGAGGCTCCG     630
  F  T  K  Q  Q  I  M  E  L  E  N  E  F  R  H  H  N  Y  L  T  R  L  R

CAGGTACGAGATCGCCGTCAAACTGGACCTCACCGAGCGTCAGGTCAAGGTGTGGTTCCAGAACCGGCGG     700
 R  Y  E  I  A  V  K  L  D  L  T  E  R  Q  V  K  V  W  F  Q  N  R  R
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M  K  W  K  R  T  K  G  G  A  A  M  R  D  K  E  A  L  K  E  L  L  P  R
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ATTGTGAGCAGGTACTACAAGGCTGGTAGGATGGAAGTTTTGCTACTCAGATCTTTTGGTTCTTGTTAGC    2170
GGTTTTCAAGAGGGTCAACACTACTTGGAAAGAAAACAGCTGCAACGTTGTAAATGGACCAGTGAAACTG    2240
TATCCAGATAATGAGAATCGACCGTGTTGTTGTAACGTGGAAACCCTGTCAACAGCGGACTAAGAAAAAT    2310
GCCGGTTTGACCCTATAACAGAGATAACCCGCTTTCCACTGATCTTTTCAGTTTACGTTTCGCCTCTGCA    2380
CCTGTCAGCATTTGTTTTCACCCGTCTGTGTTCTACTGATATCAACTTGTTTAGTTACACATGGTGTATG    2450
GAGGCATGTTTATTGTTGTACCTTTGTACTTTCTTATGTTGCATAGTTCGGACTATGGACAGTAATGCAC    2520
TATTGTAAATACATGTTTGTGTACCGGAAAATAAAAAAAAAAACCG                             2566
```

C

exon	position in cDNA	acceptor splice site	donor splice site	intron length
1	...509		...CTCAG/gtgagaaaaa	6 Kb
2	510-673	ttctaaccag/ACTCG...	...GTCAG/gtgagacatg	5,5 Kb
3	674-829	ctcgttcaag/GTCAA...		

D

```
AmphiMox          PRKERTAFTKQQIMELENEFRHHNYLTRLRRYEIAVKLDLTERQVKVWFQNRRMKWKRTK    100

h/mMox1           A---------E-LR---A--A---------------N---S-----------------V-     85
cMox1             S---------E-LR---A--A---------------N---------------------V-     87
h/m/r/c/XMox2     ----------E--R---A--A---------------N---------------------V-     90
Dmbtn             N-------S-T-LKQ--A--CYS-------------A-E-----------------C--I-     77
Gt-Dutarh2        Q-------S-G--L---K--AV-----------L--A-N-N---I-----------C--S-     77
Hrox1             ----------H--Q---K--NV-----------L--S-E-S-----------------V-     83
Hmcnox5                     -----VRN-------------S-S-S-----------------V-     84
```

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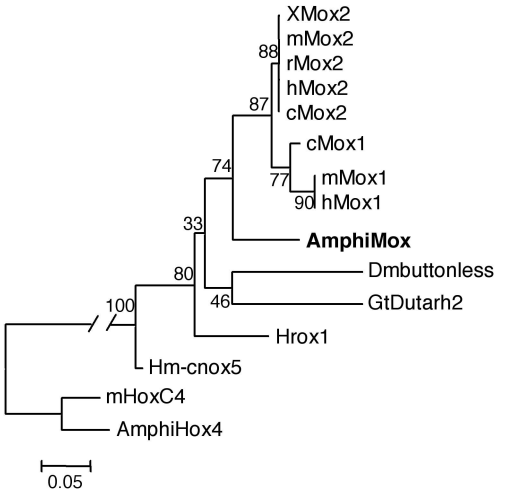

FIG. 2. Neighbor-joining phylogenetic tree relating the homeodomain of AmphiMox protein with that of other available Mox proteins. Sequence and species abbreviations are the same as in Fig. 1. The tree is rooted by using the AmphiHox4 and the mouse HoxC4 homeodomain sequences. The numbers refer to bootstrap values over 1000 replicates.

(arrowheads). However, the *AmphiMox* signal is asymmetrical: *Mox* RNA is detected in the right somite 5, but no longer in the left one, which has been formed earlier. Left and right somites 5 are morphologically similar, but the left one is older and has already shut-down Mox expression, whereas the right one has not silenced Mox yet. In a magnification (Fig. 3C), the posterior border of left somite 6 can be observed (arrowhead). Nevertheless, somite 6 is not yet morphologically visible on the right side. *AmphiMox* was expressed both in the newly formed left somite 6 but also in the anterior right PSM, which gives rise to right somite 6. The picture emerging is that *Mox* is expressed in the anterior-most part of the PSM, maintained in the forming somite, and downregulated shortly after the overt differentiation of the somite (schematized in Fig. 4A). F4

To further localize the expression of *AmphiMox*, we performed transverse sections of prestained embryos of an age similar to that of the specimen shown in Fig. 3B. Section through level i in Fig. 3C shows *AmphiMox* expression in the formed left somite 6 and in the forming right somite 6 (Fig. 3D; schematized in Fig. 3E). Section through a more posterior level (ii in Fig. 3C) reveals the outpocketing of the right side of the archenteron, to give rise a new somite. Note that Mox is already expressed there (Fig. 3F; schematized in Fig. 3G). In contrast, in the left side, the archenteron has not yet initiated the outpocketing to form the next left somite. Accordingly, Mox is still not expressed (Fig. 3G; schematized in Fig. 4B).

In 18-h embryos (11- to 12-somite stage), somites are formed directly from the tail bud. *Mox* is strongly expressed in the newly formed somite 11 on the right side of the embryo (Fig. 3H). The posterior border of the somite is discernible (arrowhead). In addition, residual levels of expression were detected in somite 10. An optical section through level x in Fig. 3H distinguishes between right and left sides of the tail bud area (Fig. 3I; schematized in Fig. 3J). Left somite 12 has just formed (s12l) and strongly expresses *Mox*. Right somite 11 (s11r, which is slightly older) still expresses *Mox*. The future right somite 12 begins to detach from the right side of the tail bud (asterisk), and also expresses *Mox*, but to a lesser extent than its left counterpart (s12l), that was built in advance.

From our data, *AmphiMox* was not expressed in the tail bud itself, but in nascent somites deriving from its most anterior part. Late phase somite formation in amphioxus is slowed down with respect to the early phase. If *AmphiMox* is not expressed in the tail bud itself, but in nascent somites, in a certain moment neither a right nor a left somite will be forming. In that particular instant, *Mox* signal would then be confined to already formed somites on both sides, but it would not be detectable in-between. This holds true, as shown by the dorsal view of an embryo slightly older than that in Fig. 3I, in which two somites are labeled for *Mox* expression in the right side (the anterior one weakly), and only one somite in the left side (Fig. 3K). Remarkably, no signal was detected in any midline structure.

In larval stages, *AmphiMox* behaves according to the dynamics described above. The signal is detected only in

FIG. 1. (A) Genomic organization of the *B. floridae AmphiMox* gene. The coding region is shown in gray and the homeobox is shown in black. The introns are represented by dotted lines. Horizontal lines represent the phage clones encompassing the genomic region. (B) Nucleotide and deduced amino acid sequence of *AmphiMox* cDNA (GenBank Accession No. AF490355). The first in-frame methionine and the noncanonical polyadenylation signal are underlined. The 60 residues of the homeodomain are shown in bold, and the intron positions are indicated by black triangles. (C) Conservation of the acceptor/donor splice sites. Exon positions are shown according to the cDNA nucleotide numeration. Capital and small letters correspond to exon and intron sequences, respectively. (D) Alignment of the AmphiMox homeodomain with other Mox homeodomains. Dashes indicate identity to the AmphiMox sequence. The numbers on the right represent the percentage of identity to the AmphiMox sequence. Identity to the partial cnidarian Mox homeodomain refers to the available residues. The abbreviations used for species and genes are: Dutarh2, *Girardia tigrina*; Hm-cnox5, *Hydra magnipapillata*; Hrox1, *Haliotis rufescens*; m, mouse; r, rat; h, human; X, *Xenopus laevis*; c, chicken; Dm, *Drosophila melanogaster*. Sequences were obtained from public databases.

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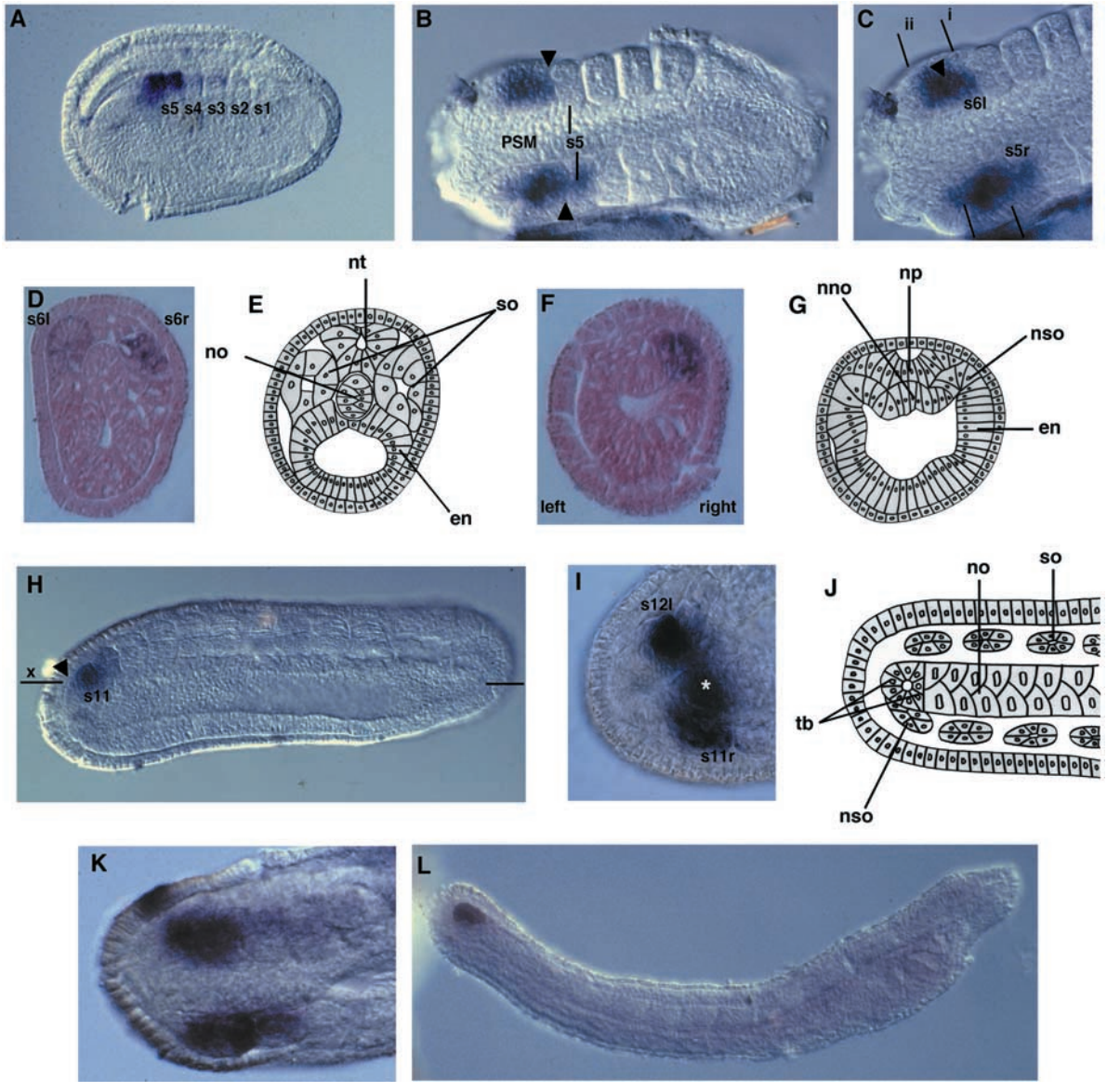

COLOR

FIG. 3. Embryonic and larval expression of *AmphiMox*. Lateral views (A, H, L) have anterior to the right and dorsal up. In dorsal views (B, C, I, J, K), anterior is to the right. In transverse sections (D–G), dorsal is up from posterior. (A) Five-somite early posthatching neurula. The signal is detected in the forming somite and in the PSM in a region comprising approximately a somite in length, where the next somite will be formed. (B) Dorsal view of a 5- to 6-somite embryo. The posterior border of left and right somites 5 (s5) are indicated by arrowheads. The signal is asymmetrically detected in the right somite 5 and posteriorly on both sides of the PSM. (C) In a magnification of (B), the posterior border of left somite 6 is discernible (arrowhead) and *AmphiMox* signal is clearly asymmetrical (right-side signal is more rostral than the left-side one), paralleling the asymmetry characteristic of amphioxus somite formation. (D) Cross-section through level i in (C) shows *AmphiMox* signal in the left somite 6 (s6l) and the right somite 6 (s6r). (E) Schematic representation of a cross-section through level i in (C). (F) Cross-section through level ii in (C) exhibits *AmphiMox* signal in the right side of the dorsolateral wall of the archenteron that begins to bud to form a somite. (G) Schematic representation of a cross-section through level ii in (C). (H) Side view of an 18-h embryo (11–12 somites). The signal is still confined to the newly formed somite 11 on the right side (s11), whose posterior border is already visible (arrowhead). (I) Frontal optical dissection through level x in (H), showing expression in left somite 12 (s12l) and right somite 11 (s11r). Future right somite 12, which is beginning to detach from the tail bud, also shows *AmphiMox* expression (asterisk). (J) Schematic representation of a frontal section through level x in (H). (K) Dorsal view of an embryo slightly older than that in (I). Mox expression is restricted to formed somites on both sides, and is absent from midline structures. (L) In larval stages (36 h), the signal continues restricted to the posterior-most somite formed from the tail bud region. Abbreviations: en, endoderm; nno, nascent notochord; no, notochord; np, neural plate; nso, nascent somite; nt, neural tube; so, somite; tb, tail bud.

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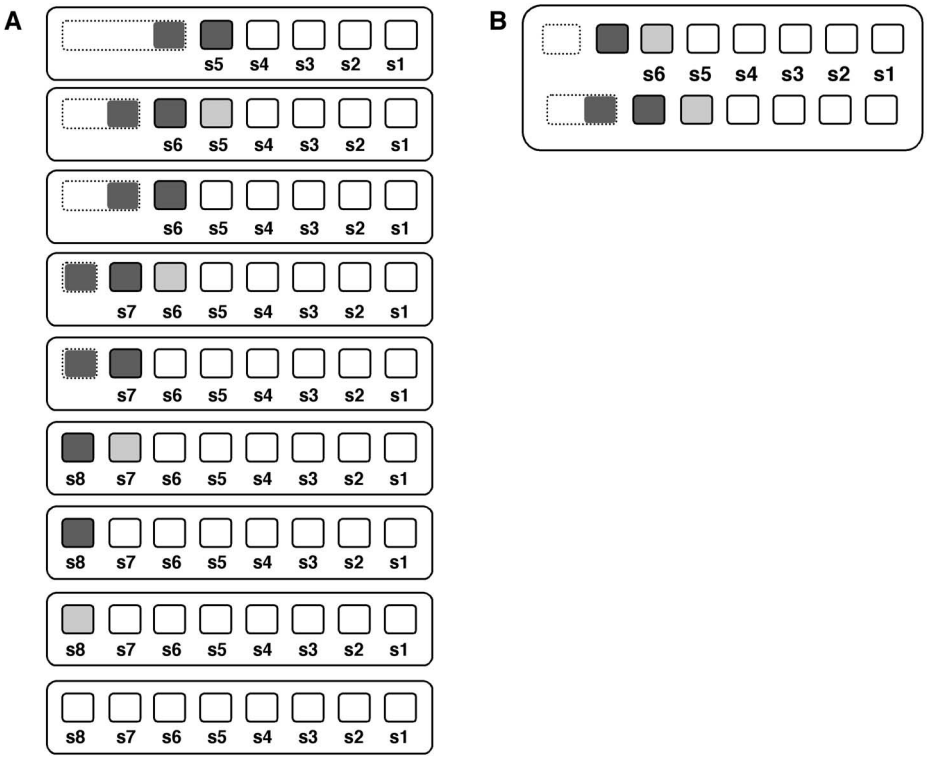

FIG. 4. Schematic representation of the dynamics of *AmphiMox* expression during the early phase of amphioxus somitogenesis. Anterior is to the right and left side is up in (B). Levels of *AmphiMox* expression are represented with graded gray. Rounded squares represent somites, and discontinuous rectangles represent the PSM. Expression within the PSM represents budding off somites from the dorsolateral wall of the archenteron. (A) Single row of somites. Somitogenesis proceeds from up toward down. (B) Both rows of somites at a given instant of somitogenesis. *AmphiMox* is switched on in the anterior most part of the PSM, just before somite formation, maintained in the newly formed somite and downregulated soon after. Note that left row somites are proceeding earlier and are thus located slightly rostral.

the posterior-most somite formed from the tail bud, and it is undetectable in more anterior (older) somites (Fig. 3L).

DISCUSSION

The Single Amphioxus Mox Gene Is a Pro-orthologue of Vertebrate Mox1 and Mox2

One cannot safely assert that a gene is in single copy in an organism without sequencing the complete genome. There is not a single technique to rule out without doubt the presence of a given gene or additional members of a given gene family. Nevertheless, we argue that a single Mox gene is present in the *B. floridae* genome, supported by four approaches. First, PCR with degenerate oligonucleotides that theoretically amplify any Mox-like gene yielded only *AmphiMox* after the sequencing of scores of independent clones. Second, the genomic screening with the PCR fragment was performed in such a way that if other Mox genes had been present in the genome they would have been isolated; still, a single Mox gene was obtained. The isolation of homeobox genes from other Mox-related families validates this strategy: other Mox-like genes would have been isolated as they would be more similar to the probe used (Mox homeobox fragment). Third, the two independent cDNA clones isolated resulted in the same *AmphiMox* gene, although the screening was performed with a probe that included the well-conserved helix III–IV of the homeobox to facilitate the isolation of other Mox-like genes. Fourth, DNA was isolated from single animals for genomic Southern, to minimize the high level of polymorphism of natural populations of *B. floridae* (Cañestro, 2001). One or two bands in each individual agree with the heterozygosity or homozygosity of a single *AmphiMox* locus.

A single Mox gene in amphioxus is consistent with the presumed preduplicative state of the amphioxus genome, which preceded genome duplications at the base of vertebrate origin (Holland *et al.*, 1994; for a discussion see Wolfe, 2001). Thus, *AmphiMox* resembles the ancestral Mox gene, before vertebrate-specific gene and genome duplications. Its vertebrate prototypical nature is reinforced by our phylogenetic analyses: the homeodomain of AmphiMox is halfway

between those of vertebrates and other invertebrates, and clearly emerges as the sister group of vertebrate Mox1 and Mox2. Hence, *AmphiMox* may well be a direct descendant of the ancestral Mox gene before vertebrate gene duplication and shed light on the ancestral function of Mox genes in the lineage leading to vertebrates.

Amphioxus Somitogenesis and the Extent of Amphioxus PSM

The muscular segments of amphioxus are the most evident segmented structures of the adult. They comprise a single row of muscular somites along either side of the notochord. The coelomic cavities of the most anterior somites of amphioxus are formed by enterocoely by outpocketing of the dorsolateral wall of the archenteron (Fig. 3G). This early paraxial mesoderm is formed during gastrulation and is used until the end of early somitogenesis. The number of somite pairs formed in this phase ranges from 8 in *B. floridae* (Holland *et al.,* 1997) to 14 in *B. lanceolatum* (Conklin, 1932). More caudal somites are formed by schizocoely (more similarly to vertebrates), by a splitting process of solid blocks from the proliferating tail bud. Early amphioxus somitogenesis is reminiscent of the formation of the trunk anterior somites of fish and amphibians, from the sequential segmentation of a preexisting territory that involutes during gastrulation, and not from dividing stem cells in the node/primitive streak of amniotes (for further discussion and views, see Pourquié, 2001). The second-phase somites of amphioxus originate from proliferating stem cells in the tail bud, as in vertebrates.

AmphiMox is expressed before the formation of the somite, from somite 5 onwards, maintained in the forming somite and downregulated shortly after the overt segmentation of the somite, regardless of their origin. Vertebrate Mox genes are conspicuously expressed in the PSM. Vertebrate PSM comprises several somites in length. In chicken, between the entry of a cell from the stem cell population of the rostral primitive streak or the tail bud to the PSM and its incorporation into a somite, it experiences 12 oscillations of cycling gene expression (Palmeirim *et al.,* 1997; reviewed in Maroto and Pourquié, 2001). During the early phase of amphioxus somitogenesis, the dorsolateral wall of the archenteron is the PSM itself, where *AmphiMox* is expressed in an anteroposterior sequence (schematized in Fig. 4A). In contrast, no such territory is found in amphioxus tail bud-derived somites, since *Mox*-positive cells arise directly from the tail bud. This is consistent with *AmphiWnt5* expression in the tail bud and nascent somites (Schubert *et al.,* 2001) and suggests that the patterning phenomena that occur in vertebrate PSM should take place in the amphioxus tail bud itself, or in small compartments of it.

AmphiMox is expressed similarly in both anterior-most and posterior-most somites, regardless of their origin, with the exception of the first four somites, in which Mox expression is not detected. These somites are unique in that they bud off at once from the archenteron, and not in an anteroposterior sequence. Their appearance has also been discussed by classical morphologists. Hatschek (1878) observed embryos with a single pair of somites, whereas Conklin (1932) never detected them and claimed that always more than one somite was present in all the embryos he analyzed. Mox may not be involved in these particular somites. Alternatively, we may have missed the short period between *Mox* activation and silencing in these somites, although we carefully examined large numbers of younger embryos. In summary, our data indicate that *AmphiMox* is expressed before the formation of the somite at least from somite 5 onwards, suggesting that the mechanisms involved in the early specification of the somites are shared by both types regardless of their origin, be it gut wall or tail bud.

Asymmetry of Amphioxus Somitogenesis from Somite 5 Onwards, as Revealed by AmphiMox Gene Expression

Another subject of discussion among classical amphioxus morphologists was the asymmetry of amphioxus somites. In this regard, Conklin and Hatschek agreed that somites are roughly symmetrical in position until the 7- or 8-somite stage. Notwithstanding, Cerfontaine (1906) postulated that the asymmetry is present from the first pair, with a slight delay in the development of the right side with respect to that of the left side. Our results do not resolve the issue of asymmetry in the very early somites (1–4), but they do for somite 5 onwards, in agreement with Cerfontaine observations, as the asymmetry is already detected in a 5- to 6-somite embryo (Fig. 3B). In this embryo, left-side somites are slightly further forward with respect to the right-side ones and the *AmphiMox* expression signal is asymmetrical: left somite 5 has switched off *Mox*, whereas transcripts are still detectable on the right somite 5, indicating that the former has been formed slightly earlier, and is thus located more anteriorly.

A Proposed Scenario for Mox Functional Evolution within the Chordates

The use of molecular markers for tracing homologies and gaining evolutionary insights is controversial, especially when comparisons concern distantly related taxa. Moreover, the data on protostome *Mox* gene expression are scarce and do not allow sensible conjectures concerning a conserved role of this gene class across metazoans. The only common feature is a loose relation of Mox genes to mesodermal derivatives (Chiang *et al.,* 1994; Degnan *et al.,* 1997).

In contrast, when the overall body plans of two animals are relatively similar, body part homologies can be confirmed by developmental gene expression domains, which

have properties of special quality and relative position (Holland and Holland, 1999). This reasoning harmonizes well with the case of cephalochordates vs vertebrates. The prototypical vertebrate-like body plan of amphioxus and its preduplicative vertebrate-like genome have fashioned the use of amphioxus as a reference for establishing the ancestral role of a given gene within chordates. This has been a fruitful venture (Holland, 1999). In addition, segmentation within the paraxial mesoderm to form somites is a distinguishing feature uniquely shared by cephalochordates and vertebrates (Saga and Takeda, 2001). In the latter, mesodermal expression is also characteristic of Mox genes, which are mainly involved in somitogenesis and the formation of somite derivatives. Thus, Mox genes may participate in the early specification of somites, before overt and terminal differentiation of their derivatives. However, neither *Mox1* nor *Mox2* knockout mice confirm this hypothesis. *Mox2* knockout mice show alterations only in limb musculature (Mankoo *et al.,* 1999) and no alterations in the axial skeleton. *Mox1* knockout mice show vertebral abnormalities, like hemi-vertebrae, tail kinks and craniovertebral fusions (cited in Stamataki *et al.,* 2001). Thus, these genes do not seem to be critical for early somite determination but rather for late somite differentiation. Interestingly, *AmphiMox* is turned on in the PSM, just before the formation of a new somite, but is switched off soon after the somite border is formed (Fig. 4A). This points to similarities but also differences with respect to vertebrate Mox genes. The amphioxus data help unravel the ancestral function and co-option of Mox genes within vertebrates. First, our expression data suggest that the original function in the common ancestor of cephalochordates and vertebrates was at the onset of somite specification and formation. It is tempting to speculate that activation of Mox in a suitable territory of the embryonic mesoderm was coincident with or causal to the evolutionary design of somites. Second, the expression of Mox genes in vertebrates during somite differentiation is not detected in amphioxus, which may be due to the simpler structure of cephalochordate somites compared with the increased level of complexity of vertebrate somites and their derivatives.

Vertebrate somites undergo complex transformations, like epithelial to mesenchyme transition, delamination of cells from the dermamyotome and migration of those to form the limb musculature. In contrast, amphioxus is a predominantly epithelial animal (Whittaker, 1997). Early somites arise as evaginations from the gut walls that pinch off, resulting in a single-layered epithelium surrounding a cavity, the myocoel. The somites of amphioxus are subdivided into distinct regions, although there is no histological evidence for sclerotome and no cells migrate away from it (Shimeld and Holland, 2000). On the contrary, the large medial compartment of each somite is the myotome, which retains its epithelial organization throughout development. All myotomal cells differentiate in place and become the striated muscle cells that constitute the segmental muscle blocks along the body length (Holland *et al.,* 1995a). Mox genes were probably co-opted after duplication at the origin of vertebrates, leading to or facilitating the acquisition of vertebrate-specific roles after somite formation. This co-option and diversification was refined independently on each Mox gene (Mox1 and Mox2), giving rise to their specific and unique roles, highlighted in their respective knockout phenotypes. Genes other than Mox are also expressed in the already formed somites in vertebrates but not in amphioxus [e.g., *Amphisnail* (Langeland *et al.,* 1998) and *AmphiPax1/9* (Holland *et al.,* 1995b)]. Notably, the Mox1 protein binds to the Pax1 protein *in vitro* (Stamataki *et al.,* 2001). The expression pattern of these genes in vertebrates and the phenotype of the mutants are also consistent with the hypothesis of functional association of the gene products *in vivo*. However, AmphiPax1/9 and AmphiMox cannot cooperate *in vivo*, as they are not coexpressed at any developmental stage. Therefore, the assembly of both gene products occurred after gene duplications in the vertebrate stem lineage. This is compatible with the view that complete gene networks are co-opted, and with new networks being built up after gene or genome duplications, thus increasing the complexity of vertebrate somites.

The above proposed scenario for Mox functional evolution can be tested experimentally. First, it predicts that *Mox1*/*Mox2* double knockout mice will overcome vertebrate gene redundancy and reveal the ancestral function of Mox genes. Thus, somitogenesis will be severely disrupted in these mutants. Second, the sister group of cephalochordates and vertebrates, urochordates, would not express Mox in the progenitors of muscle cells. Ascidians do not form proper somites but muscle cells determined by maternal factors (Satoh, 1994). The absence of overt segmentation in ascidians may be a secondary derived condition, since in another group of urochordates, larvaceans, each muscle cell is innervated by reiterated neuronal cells and so may represent one segment. We failed to find Mox-like genes in ascidian genome project public databases. From tunicates to cephalochordates to vertebrates, there is an increasing level of mesodermal complexity, from muscle iteration, to proper but simple somites, to highly differentiated somites, and formation of vertebral tissue and other somite derivatives. Mox may well have been involved in this gradation: a single Mox gene co-opted in suitable mesodermal territories, and duplicated genes individually co-opted after gene duplication at vertebrate origins.

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