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Developmental expression of the *High Mobility Group B* gene in the amphioxus, *Branchiostoma belcheri tsingtauense*

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ABSTRACT High-Mobility Group (HMG) B proteins are abundant and highly conserved non-histone proteins, which play an important architectural role in the assembly of nucleoprotein complexes and in the regulation of transcription. These proteins have also been shown to play key roles during embryonic development and cell differentiation. Here we report a full-length cDNA sequence of the HMG protein, *AmphiHMGB* from Amphioxus. Sequence analysis indicates that this putative AmphiHMGB protein contains four domains: HMG-box A, HMG-box B, basic region, acidic carboxyl-terminal tail and a linker. Phylogenetic analysis suggests that AmphiHMGB falls outside the vertebrate clade. *HMGB* gene duplication occurred near the base of the vertebrate gene clade. The dynamic expression of *AmphiHMGB* during embryonic development reveals for the first time that it may involve differentiation of neural ectoderm, mesoderm and endoderm in this animal.

KEY WORDS: *AmphiHMGB, development expression, amphioxus, evolution*

High mobility group (HMG) proteins are a group diverse, ubiquitous nuclear proteins (Bustin, 1999; Muller *et al.,* 2001). They were first identified by their abundance and small molecular weight (Bustin and Reeves *et al.,* 1996). There are three subfamilies of HMG: HMGB (HMG-1/-2), HMG-I/Y and HMG-14/-17. HMGB subfamily are abundant and highly conserved non-histone proteins that may exist in all eukaryotic cells nuclei. HMGB contains two homologous basic DNA-binding domains (HMG boxes A and B) as well as a basic region linking an acidic carboxyl-terminal tail (Bustin and Reeves, 1996; Lee and Thomas, 2000). HMGB plays an important architectural role in the assembly of nucleoprotein complexes and regulation of gene transcription (Scaffidi *et al.,* 2002; Fages *et al.,* 2000; Muller *et al.,* 2001; Ge *et al.,* 1994; Boonyaratanakornkit *et al.,* 1998; Guazzi *et al.,* 2003). In addition, HMGB appears to be able to function as a cytokine (Muller *et al.,* 2001; O'Connor *et al.,* 2003). Members of the HMGB class probably play key roles in embryonic development and cell differentiation (Muller *et al.,* 2004; Agresti and Bianchi, 2003; Spada *et al.,* 1998; Vaccari *et al.,* 1998; Sparatore *et al.,* 1996). It has been shown that HMGB is involved V(D)J recombination of immunoglobulin genes as well as in invasion of cancer cells (Van Gent *et al.,* 1997). We isolated a cDNA clone encoding HMGB in amphioxus, by random screening of amphioxus neurula cDNA library. The cDNA clone is 833 bp long and contains a 666 bp open reading frame, which encoded a putative protein of 222 aa (GenBank accession number: AY578709). It has very high identities with the *AmphiHMGB* reported by Liu *et al.,* (2004) except the published sequence is 149 bases shorter. Its shorter part includes the basic region and linking acidic carboxyl-terminal tail that are required for HMGB family. They are different by only 3 sites over about 600. Given the high level of polymorphism in amphioxus populations (Canestro *et al.,* 2002) we think that they are same gene. This is almost certainly a mistake in the published work of Liu *et al.,* The further analysis shows that a full-length *AmphiHMGB* cDNA has been identified here. NCBI database BLAST search indicated that the nucleotide sequence shared high sequence similarity with HMGB homologues from other species. At the protein level, amphioxus HMGB shows identities of 41, 49, 50, 50, 50, 50 and 50% with the HMG protein of sea urchin, lamprey, rainbow trout, frog, chicken, mouse and human respectively. AmphiHMGB shared higher sequence identities with the homologue in vertebrates. The putative protein sequence contains four domains: HMG-box A, HMG-box B, basic region, acidic carboxyl terminal tail and a linker, all of which are conserved in HMG superfamily members (Fig. 1). Thus, our data from AmphiHMGB further confirm that HMG proteins are highly conserved in evolution. Phylogenetic analysis was performed on 16 homologues genes from vertebrate and invertebrate species by the neighbor-joining method. The number of bootstrap replicates was 1000. Residues 1-163 were used for the alignment (Fig. 2) because other sequences were highly variable and no homologous residues can be identified (Sharman *et al.,* 1997). Results of phylogenetic analysis indicated that amphioxus HMGB falls out-

Abbreviations used in this paper: AmphiHMGB, Amphioxus HMGB gene; HMG, high mobility group.

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side the vertebrate clade. Our data further suggest that HMG gene duplication occurs near the base of the vertebrate gene family clade (Fig. 2). Southern blotting results also showed that there might be only one copy of *AmphiHMGB* in Qingdao amphioxus (Fig. 3). The pattern of *AmphiHMGB* expression was determined by whole-mount *in situ* hybridization using *AmphiHMGB* antisense and sense DIG-labeled RNA probes, followed by histological section. *AmphiHMGB* expression was detectable from fertilization through the 72 h larva stages (Fig. 5). The mRNA transcripts were conspicuous in the cytoplasm of zygote and in blastomeres at the cleavage stage. At the blastula *AmphiHMGB* was expressed at a low lever. Its expression was detected throughout the endomesoderm at the gastrula stage. At the 9.5 h early neurula stage, *AmphiHMGB* was mainly expressed in the neural plate and presumptive notochord. As the embryo develops, transcripts of *AmphiHMGB* remain in the neural plate, notochord and mesoderm, differentiating paraxial mesoderm. At the 16 h neurula stage, *AmphiHMGB* transcripts were detected in the cerebral vesicle, neural tube, notochord, developing somites and endoderm. Expression continues in the cerebral vesicle, neural tube, epithelium of the gut and pharynx until at least the 72 h stage. No signal was detectable in the ectoderm and the resulting epidermis. In consistent with our theory that only a single *AmphiHMGB* appears in amphioxus, Northern hybridization only detected one band in all embryonic stages (Fig. 4).

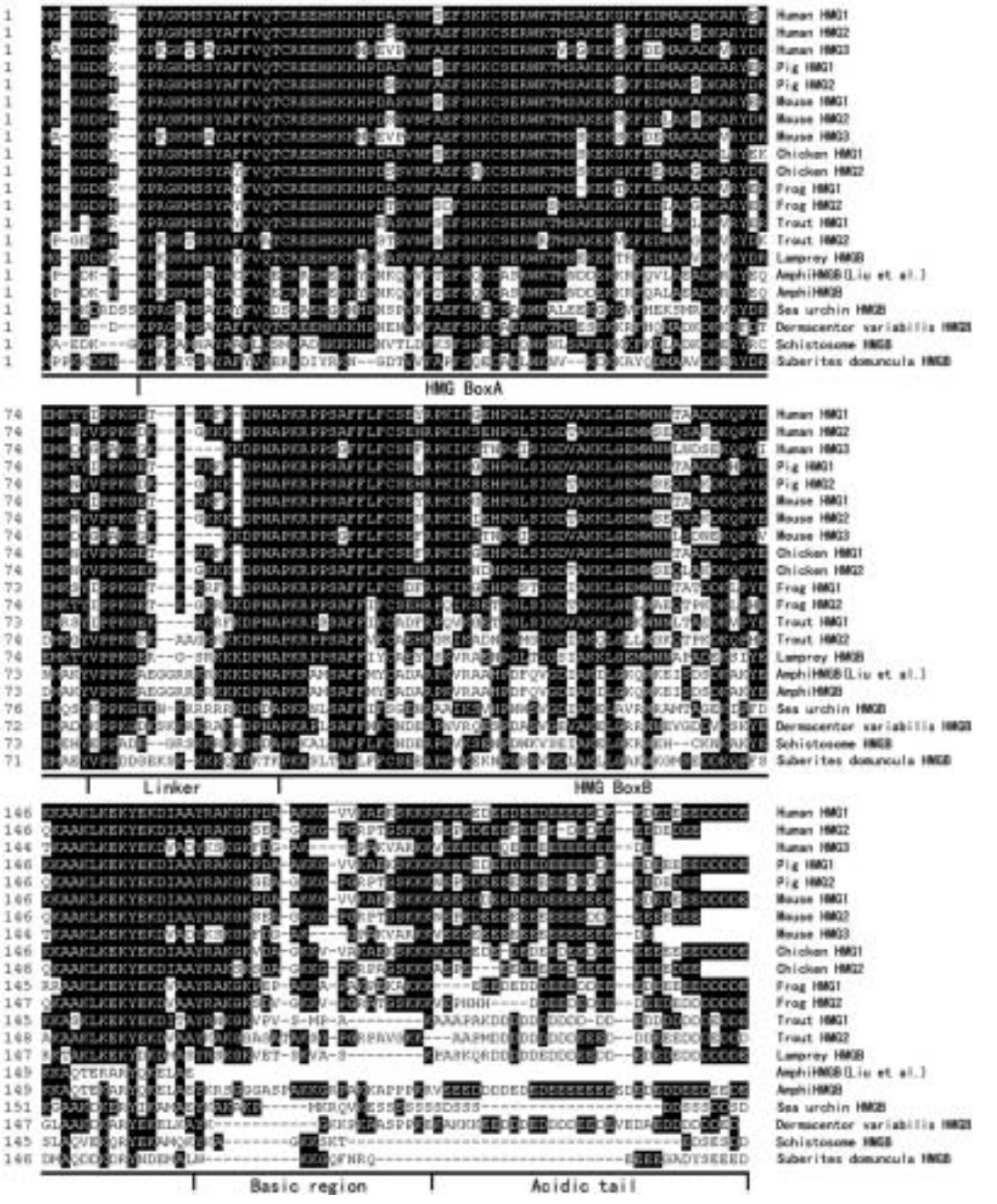

Fig. 1. Amino acid sequence alignment of the HMGB protein in *Branchiostoma belcheri* and similar organisms using Megalign (DNASTAR) by the clustal method. *Black background indicates the amino acids that match the consensus. Gaps introduced into sequences to optimize alignments are represented by (-). Sources of the sequences are from NCBI or EMBL: Human HMG1, AAQ91389; Human HMG2, NP_002120; Human HMG3, NP_005333; Pig HMG1, P12682; Pig HMG2, P1774; Mouse HMG1, NP_034569; Mouse HMG2, CAA47900; Mouse HMG3, NM_008253; Chicken HMG1, CAA76978; Chicken HMG2, AAA48819; Frog HMG1, S62355; Frog HMG2, D30765; Trout HMG1, S48708; Trout HMG2, I51067; Lamprey HMGB, CAA67363; AmphiHMGB(by Liu* et al.,*), AY172026; Sea urchin HMGB, AAA91277; Dermacentor variabilis HMGB, AAO92280; Schistosome HMGB, AAR85353; Suberites domuncula HMGB, AAR08136.*

In summary, we report here the isolation and characterization of HMGB from amphioxus. To our knowledge, this is the first report on the spatial and temporal expression characterization of *AmphiHMGB* gene in the amphioxus embryos revealed by *in situ* hybridization. Liu *et al.,* (2004) has reported the expression of *AmphiHMGB* is detectable throughout the embryonic development in amphioxus only by Northern analysis. However, our result

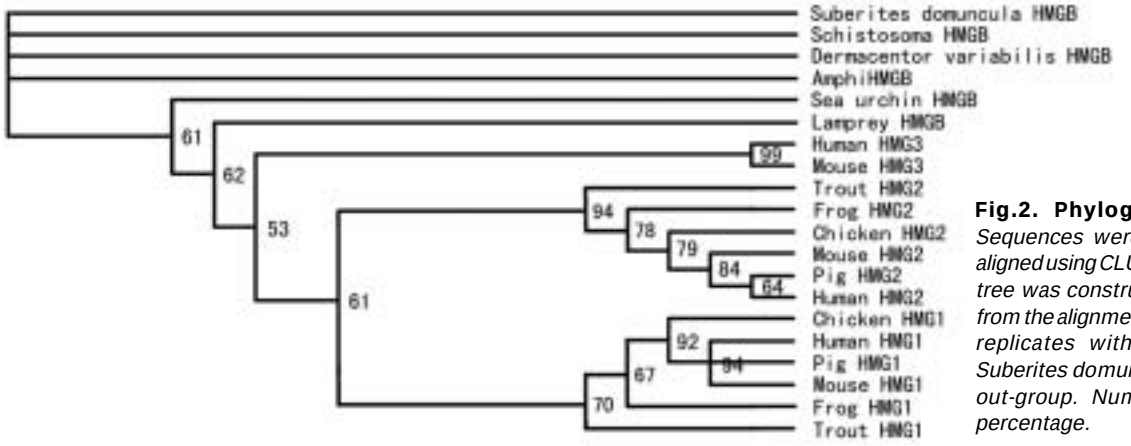

Fig.2. Phylogenetic tree of HMGB. *Sequences were extracted from NCBI and aligned using CLUSTALX. The neighbor-joining tree was constructed using sequence 1-163 from the alignment in Fig. 1 and 1000 bootstrap replicates with the treepuzzle program. Suberites domuncula HMGB was used as an out-group. Numbers represent bootstrap percentage.*

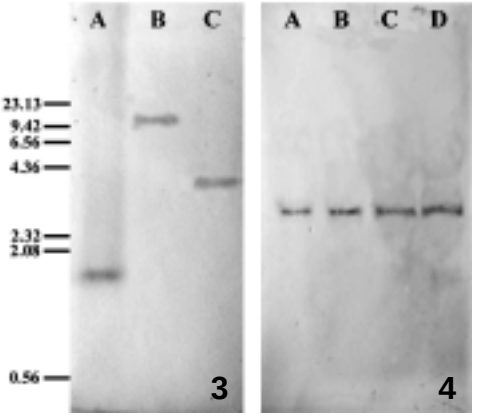

Fig. 3 (Left). Amphioxus *HMGB* Southern blot analysis. *Lanes A-C were digested with restriction enzymes Bam HI, EcoRV, HindIII respectively.*

Fig. 4 (Right). The expression of amphioxus HMGB in different developmental stages was characterized by Northern blot. *Lanes A-D show 3 h post-fertilization, 6 h gastrula, 12 h and 24 h neurulae respectively.*

is different with their data. In our study, we detected the expression correlates with the development of the cerebral vesicle, neural tube, myogenic somites, gut epithelium and pharynx. The developmental expression pattern of *AmphiHMGB* indicates that it might be involved in differentiation of neural ectoderm, mesoderm and endoderm in this animal. Our data are similar to those of other HMGB vertebrate homologues. Pauken *et al.,* (1994) reported the HMG1 mRNA during early mouse embryogenesis. HMG proteins were transcribed in developing rat brain and *Xenopus* nerve system, respectively (Angelova *et al.,* 1993). Guazzi *et al.,* (2003)

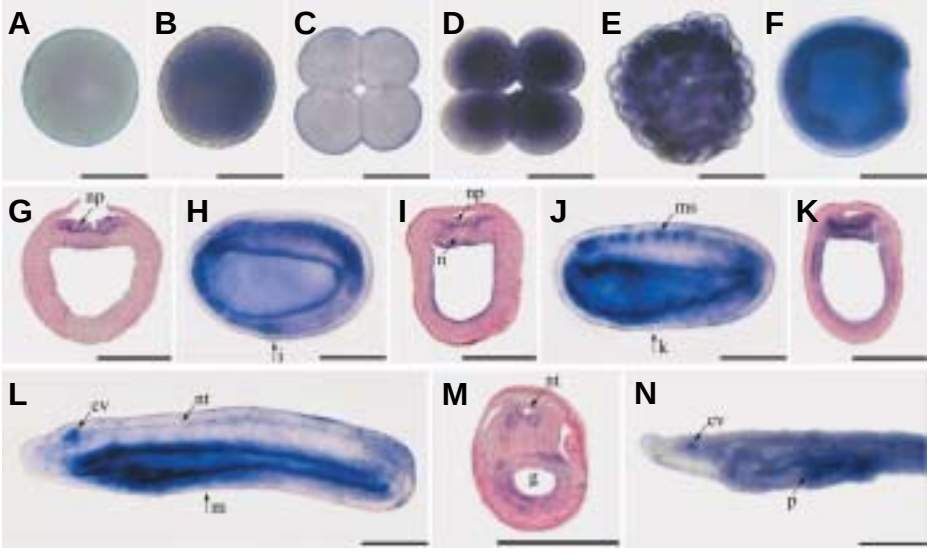

Fig.5. Developmental expression of *AmphiHMGB* detected by whole-mount†*in situ* hybridization. (B,D,E,F) *show zygote, 4-cell, blastula and gastrula stages respectively.* **(A,C)** *Zygote and 4-cell stage with a sense probe. From (G) to (N), embryos are oriented with anterior to the left and dorsal to the top.* **(G)** *Cross section of a 9.5 h early neurula. AmphiHMGB expression is visible in the neural plate and pre-notochord.* **(H)** *A 12 h neurula in which the transcripts are detected in the neural tube, notochord, endoderm and mesoderm.* **(I)** *Transverse section through region indicated by arrowhead in* **(H)**. *Distinct expression is apparent in the neural plate and differentiating myogenic somite.* **(J)** *Neurula with 9-10 somites; expression is detected in the cerebral vesicles, neural tube, notochord and epithelial cells lining the alimentary canal. Expression appears in myogenic somite at this stage.* **(K)** *Transverse section through region indicated by arrowhead in* **(J)**. **(L)** *Knife-shaped larva; expression of AmphiHMGB continues in the cerebral vesicles, neural tube, notochord and epithelium of the gut, but is down regulated in the myogenic somite and up regulated in the forming pharynx.* **(M)** *Transverse section through region indicated by arrowhead in* **(L)**. **(N)** *Anterior part of 72 h larva. It shows expression in the neural tube, cerebral vesicles and pharynx. Abbreviations: cv, cerebral vesicle; g, gut; ms, myogenic somite; n, notochord; np, neural plate; nt, neural tube; p, pharynx. i, k, m, the levels in Figs. 3H, 3J and 3L at which the cross-sections in Figs. 3I, 3K and 3M are made. Scale bar, 50 μm.*

reported that HMGB1 expressed in the mouse embryonic cortical plate and areas of the continuing neurogenesis, with temporally and spatially subcellular expression patterns. Thus, it will be interesting to study the functional roles of HMG1/2 fro-embryonic development and tissue differentiation in amphioxus and other animals.

Experimental Procedures

Adult amphioxus (*Branchiostoma belcheri tsingtaunese*) was collected from Shazikou near Qingdao City. Ripe adults were spawned in the laboratory as described (Tung *et al.,* 1958). Synchronously developing embryos and larvae were cultured and collected in different developmental stages. Embryos and larvae were fixed in 4% paraformaldehyde at room temperature for 30 minutes or at 4ºC overnight, dehydrated in graded ethanol and stored in 70% ethanol at –20°C. Some adults, embryos and larvae in different developmental stages were frozen in liquid nitrogen for DNA and RNA extraction. The cDNA library was constructed in vector λ ExCell (Pharmacia Biotech) using the mRNA from neurulae in our laboratory. The fragments were obtained from a large scale sequencing of the cDNA library. Among them a HMGB homologous fragment in amphioxus was found. Phylogenetic analysis was performed by the neighbor-joining method and phylogenetic tree was constructed with the treepuzzle program. DIG-labeled sense and antisense probes were synthesized from the obtained *AmphiHMGBa* fragments containing clone. Whole-mount *in situ* hybridization was carried out according to Holland *et al.,* (1999). Then some hybridized embryos and larvae were cut into 6 mm systematic histological sections after double embedded in agar-paraffin. Total mRNA was extracted from 3 h, 6 h, 12 h and 24 h amphioxus embryos respectively using Catrimox-14^{TM} RNA Isolation Kit Ver.2.11 (Takara). Genomic DNA was isolated from five amphioxus adults and then digested with four restriction enzymes (*Bam HI, EcoR*V, HindIII). Southern blot and Northern blot were done with the Dig High Prime DNA Labeling and Detection Starter Kit I (Roche).

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