Molecular cloning and expression analysis of female sterile homeotic gene (fsh) in the oriental river prawn *Macrobrachium nipponense*

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**ABSTRACT.** The gene *female sterile homeotic (fsh)* plays crucial roles in molecular function, including protein kinase activity and DNA binding, which are involved in biological processes such as terminal region determination and negative regulation of DNA-dependent transcription. Although *fsh* has been found in *Drosophila melanogaster*, little is known regarding its expression in crustaceans. In this study, a *fsh* gene homologue, designated as *Mnfsh*, was cloned and characterized from the testis of the oriental river prawn, *Macrobrachium nipponense*, by using EST analysis and the RACE approach for the first time. The full-length cDNA of *Mnfsh* was 2029 bp, consisting of a 5' UTR of 361 bp, a 3' UTR of 216 bp, and an ORF of 1452 bp encoding 484 amino acids. qRT-PCR analysis showed that the *Mnfsh* gene was expressed in the testis, ovary, muscle, heart, eyestalk, and abdominal ganglion, with
the highest level of expression in the ovary and the lowest in the heart. qRT-PCR analyses showed that the expression levels of MnfsH mRNA both significantly increased in the zoea stage, the VII larvae, and 1st day post-larvae after metamorphosis. In conclusion, the results of the present study indicate that MnfsH is an arthropod fsh homologue and probably also plays important roles in embryogenesis, organogenesis, and morphological differentiation of M. nipponense.

Key words: Female sterile homeotic (fsh); Macrobrachium nipponense; Gene cloning; Gene expression

INTRODUCTION

More than a dozen trithorax group (trxG) proteins are involved in activation of Drosophila HOX genes. The maternal-effect gene female sterile (1) homeotic [fs(1)h, also called fsh] is one of the trxG genes (Gans et al., 1975) and was identified as the most important trans-activator of Ultrabithorax (Ubx) activation by its strong genetic interactions with Ubx, trithorax (trx), and absent, small or homeotic discs 1 (ash1) mutations (Digan et al., 1986; Shearn, 1989). Additionally, it has been reported to be related to female reproduction and growth in Drosophila melanogaster (Gans et al., 1980; Haynes et al., 1989). Because Ubx expression impacts where the thoracic segments and abdominal segments connect in the Drosophila embryo, lack of functional fsh is known to cause complex developmental defects including homeotic transformation and early embryonic lethality (Forquignon, 1981; Huang and Dawid, 1990). Previous studies showed that zygotic mutations cause either lethality or female sterility, whereas maternal mutations cause segmental deletions and thoracic homeotic transformations (Rhee et al., 1998; Florence and Faller, 2008). Considering that crustaceans have a dramatically close evolutionary relationship with insects (Budd and Telford, 2009; Zhang et al., 2012), we hypothesize that MnfsH is involved in the regulation of embryonic development in crustaceans.

fsh recognition proteins have been isolated and characterized in other species such as Drosophila melanogaster (Haynes et al., 1989), Camponotus floridanus, and Harpgnathos saltator (Bonasio et al., 2010). However, little information is available to date on temporal expression of fsh mRNA during embryonic and larval development in crustaceans. The oriental river prawn, Macrobrachium nipponense, (Crustacea; Decapoda; Palaemonidae) is a commercial freshwater prawn species that is widely distributed in many Asian countries including China, Japan, Korea, Vietnam, and Myanmar (Hongtuo et al., 2012; Qiao et al., 2013). This species is considered an important fishery resource in China, with an annual production of 230,248 tons (Bureau of Fishery, Ministry of Agriculture, and P.R.C., 2011). In recent years, our laboratory has performed some molecular research on the M. nipponense development system (Zhang et al., 2012, 2013a,b, 2014). To further understand the molecular mechanism of maturation, other gene regulations involved in the developmental process also need to be examined in M. nipponense. In this study, we cloned a full-length fsh cDNA from oriental river prawn and analyzed its expression pattern throughout developmental stages, which could help improve our understanding of the regulatory mechanism of early embryonic and post-embryonic development in the oriental river prawn.
MATERIAL AND METHODS

Animal and sample preparation

Sexually mature adult oriental river prawns, weighing about 2.0-5.6 g, were obtained from Tai Lake (120°13'44''E, 31°28'22''N) in Jiangsu Province, China from February to May 2012 and were reared in aerated freshwater for 72 h at 28°C before tissues were collected. For cDNA synthesis and tissue distribution pattern, the testis, ovary, muscle, heart, eyestalk, and abdominal ganglion were dissected from healthy mature individuals. Different developmental stages of embryos, larvae, and post-larvae were obtained from our laboratory. Each developmental stage of embryos was determined and collected following the criteria by Chen et al. (2012); the embryos were then observed and pictures were taken with a fluorescence microscope (OLYMPUS BX61, Japan). Larvae were collected every 4 days between 1 day post-hatching (L1) and LVIII (1 day before metamorphosis). Post-larvae were collected every 5 days between 1 and 20 days after metamorphosis (PL1-PL20) and every 10 days between PL20 and PL30. All samples were washed with 1X phosphate-buffered saline (0.01 M), frozen immediately in liquid nitrogen, and stored at -80°C until RNA extraction.

Isolation of total RNA and for real-time quantitative polymerase chain reaction

Total RNA was extracted from the testis of adult prawns using RNAiso Plus Reagent (TaKaRa, Japan) according to manufacturer protocols. The isolated RNA was treated with RNase-free DNase I (Sangon, China) to eliminate possible genomic DNA contamination. The concentration of each total RNA sample was then measured by BioPhotometer (Eppendorf, Germany), and 2 μL was analyzed on a 1% agarose gel to check the integrity. The cDNA was synthesized from 5 μg of total RNA by the PrimeScript™ RT-PCR kit (TaKaRa, Japan). Reaction conditions used were recommended by the manufacturer. The cDNA was kept at -20°C for real-time quantitative polymerase chain reaction (qRT-PCR).

Sample analysis and calculation

A gene-specific primer set was designed based on expressed sequence tags (EST) sequences (ALB_High_Quality_EST.seq.Contig868) of fsh homologue obtained from the M. nipponense testis cDNA library (Qiao et al., 2012). The cDNA ends of the fsh gene was isolated through rapid-amplification of cDNA ends (RACE) PCR with the 5'-full RACE kit (TaKaRa) and 3'-full RACE kit (TaKaRa) using a nested PCR strategy using a thermal cycler (Eppendorf) according to manufacturer protocols (Amparyup et al., 2008). The total RNA of the testis tissues was used as a template to amplify the cDNA ends. Gene-specific primers of Mnfsh were designed based on the known fragment. All primers used in this study are shown in Table 1.

The PCR products were gel-purified and ligated into the pMD18-T vector (TaKaRa) following manufacturer instructions. The recombined products were then transformed into Escherichia coli DH5α-competent cells (Qiagen, Germany), which were identified by blue/white screening and confirmed by PCR. Three of the positive clones were sequenced in both directions using an automatic DNA sequencer (ABI Applied Biosystems Model 3730), and these resulting sequences were verified and subjected to cluster analysis in National Center for Biotechnology Information (NCBI).
Table 1. Sequences of primers employed in this study for the fsh gene of Macrobrachium nipponense.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer sequence (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primers for the middle of the sequence</td>
<td></td>
</tr>
<tr>
<td>fsh middle 5' primer</td>
<td>ATCACACACCAACGCCCCGTCC</td>
</tr>
<tr>
<td>fsh middle 3' primer</td>
<td>GTGAGTGGCGTTAGCGGCCG</td>
</tr>
<tr>
<td>Primers for 3' RACE PCR</td>
<td></td>
</tr>
<tr>
<td>fsh 3' out primer</td>
<td>TGGCTCTACTCGCTTCACCA</td>
</tr>
<tr>
<td>3' RACE Outer primer</td>
<td>CTTCCTCGCCGCATAACA</td>
</tr>
<tr>
<td>3' RACE Inner primer</td>
<td>TACCGTCGTCCACTAGTGATT</td>
</tr>
<tr>
<td>Primers for 5' RACE PCR</td>
<td></td>
</tr>
<tr>
<td>fsh 5' out primer</td>
<td>AAATTCACACTGACCGCTAC</td>
</tr>
<tr>
<td>5' RACE Outer primer</td>
<td>GTGAGTGGAAAGGAATGTCG</td>
</tr>
<tr>
<td>5' RACE Inner primer</td>
<td>CATGCTACATCGACAGCTAC</td>
</tr>
<tr>
<td>Primers for qRT-PCR analysis</td>
<td></td>
</tr>
<tr>
<td>Mnfsh 5' primer</td>
<td>GTGACCAGGCTCCGCCAATC</td>
</tr>
<tr>
<td>Mnfsh 3' primer</td>
<td>TGACGGGCGTTGGTGCTGTGATG</td>
</tr>
<tr>
<td>β-actin 5' primer</td>
<td>TATGCACTTCCTCAGCAC</td>
</tr>
<tr>
<td>β-actin 3' primer</td>
<td>AGGAGCGCCAGTGTCAT</td>
</tr>
</tbody>
</table>

Nucleotide sequence and bioinformatic analyses

Searches for protein sequence similarities were conducted with NCBI’s basic local alignment search tool (BLAST) algorithm (http://www.ncbi.nlm.nih.gov/BLAST/). The protein prediction was performed using the ORF Finder (http://www.ncbi.nlm.nih.gov/orffinder/). The ProtParam program (http://www.expasy.ch/tools/protparam.html) was used to compute physical and chemical parameters of the amino acid sequence. The motif was performed with the Motif Scan program (http://hits.isb-sib.ch/cgi-bin/motif_scan/). Multiple alignments of fsh were generated using the DNAMAN program (http://www.lynnon.com/). A phylogenetic tree was constructed using the neighbor-joining (NJ) algorithm with 1000 bootstraps (http://www.megasoftware.net/; Tamura et al., 2011).

Temporal and spatial expression patterns analyzed by qRT-PCR

The Mnfsh mRNA expressions at different stages from embryo to post-larva and various adult tissues were measured by a SYBR Green qRT-PCR in a CFX96TM Real-Time System (Bio-Rad, USA). Gene-specific primers (Table 1) were used to amplify the fsh gene, and the PCR products were sequenced to verify the specificity of the PCR primers. The β-actin primers (Table 1) were used to amplify the β-actin fragments that were used as an internal control (Zhang et al., 2010). Amplifications were performed on a 96-well plate with a 20 μL reaction volume containing 10 μL of 2X SYBR Green Premix Ex Taq (TaKaRa), 0.2 μL of each primers (10 μM), 2 μL of template, and 7.6 μL of PCR-grade water. The PCR temperature profile was 95°C for 30 s followed by 40 cycles of 94°C for 15 s, 60°C for 20 s, and 72°C for 20 s, with a 0.5°C/5 s incremental increase from 60°C to 95°C. Each sample was run in triplicate along with the internal control gene. To ensure that only one PCR product was amplified and detected, the dissociation curve analysis of amplification products was performed at the end of each PCR. The relative copy number of Mnfsh mRNA was calculated according to the 2^-ΔΔCT comparative cycle threshold (CT) method (Livak and Schmittgen, 2001).

Statistical analysis

All data are reported as means ± SD (standard deviation) (N = 3). Statistical analysis
was performed using the SPSS software 19.0. Statistical significance was determined using one-way ANOVA and Tukey’s multiple range tests. Significance was set at P < 0.05.

RESULTS
cDNA cloning and sequence characterization of \textit{fsh}

The full-length cDNA sequence of \textit{Mnfsh} was determined by merging the sequences of the 3’ and 5’ RACE products. The coding nucleotide sequence and corresponding predicted amino acid sequences were analyzed, and the \textit{Mnfsh} nucleotide and deduced amino acid sequences are shown in Figure 1. The \textit{Mnfsh} cDNA was comprised of 2029 bp, containing 361 bp in the 5’-untranslated region, 1452 bp in the open reading frame (ORF), and 216 bp in the 3’-untranslated region with two potential polyadenylation sites (AATAAA) located upstream of the poly(A) tail. The deduced \textit{Mnfsh} contains 483 amino acid residues and shows 51% identity with the female sterile homeotic protein of \textit{Harpegnathos saltator} (GenBank Accession: EFN88794.1) and about 50% identity with that of \textit{Camponotus floridanus} (GenBank Accession: EFN69688.1). Analysis of the deduced protein sequence of the \textit{Mnfsh} showed that it was comprised of 483 amino acids with a predicted molecular mass of 52.641 kD and an isoelectric point of 8.78. Conserved sequence and characteristic motifs of two bromodomains were identified in the deduced amino acid sequences of \textit{Mnfsh}. The sequence data have been deposited in the GenBank DNA database under the accession number KC517379.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Mnfsh_sequence.png}
\caption{\textit{Mnfsh} nucleotide (above) and deduced amino acid (below) sequences. The 3’ UTR and 5’ UTR are shown by lowercase letters. ORFs are shown by capital letters; the start (ATG) and stop (TAG) codons are double underlined, polyadenylation signal (AATAAA) is in the boxes, polyadenylation is represented by a single underline, and the two putative bromodomains are shaded.}
\end{figure}

Similarity comparison and phylogenetic tree analysis

The sequence alignment was used to determine the percent identity and similarity of amino acid residues with other fsh genes. Sequence comparisons of the Mnfsh-deduced amino acids showed identity of 51, 50, and 74% to the female sterile homeotic protein of Harpegnathos saltator (Accession No. EFN88794.1), Camponotus floridanus (Accession No. EFN69688.1), and D. melanogaster (Accession No. NP_727228.1), respectively. Multiple alignments revealed that Mnfsh displayed a high degree of identity with fsh of other arthropods (Figure 2). Therefore, we deduce that the obtained Mnfsh sequence from the foregoing analysis was the fsh of M. nipponense.

Figure 2. Alignment of Mnfsh amino acid sequences with other species using DNAMAN. Species names are abbreviated at the left and represent the following sequences obtained from GenBank: M. rotundata (XP_003705173.1), B. impatiens (XP_003490841.1), C. floridanus (EFN69688.1), D. melanogaster isoform A (NP_727228.1), H. saltator (EFN88794.1), and M. nipponense (in this study, KC517379).

Continued on next page
Figure 2. Continued.

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M. rotundata

M. impatiens

C. floridanus

D. melanogaster

H. saltator

M. nipponense

M. rotundata

M. impatiens

C. floridanus

D. melanogaster

H. saltator

M. nipponense

Continued on next page
A condensed phylogenetic tree was constructed with MEGA 5.0 based on the NJ method using the complete fsh sequence deposited in NCBI in order to study the relationship between Mnfsh and other well-defined fsh of arthropods. The NJ tree showed that the Mnfsh was within the crustacean clade (Figure 3), which is consistent with the evolution of species.

**Figure 3.** The neighbor-joining phylogenetic tree based on the sequences of fsh from different species with MEGA 5.0. Species names and types of fsh are listed on the right of the tree. Accession numbers in GenBank as follows: M. nipponense (in this study, KC517379), B. impatiens (XP_003490841.1), M. rotundata (XP_003705173.1), C. floridanus (EFN69688.1), H. saltator (EFN88794.1), D. melanogaster isoform A (NP_727228.1), and D. melanogaster isoform B (AAF46312.3).
Gene expression of fsh in different tissues and stages

Knowing that β-actin acts as an internal control in all tissues, qRT-PCR was employed to investigate the distribution of Mnfish in different tissues. Mnfish was expressed in all examined tissues of adult prawns, but at varying levels. Prominent expression was observed in the ovary (6.87-fold), followed by the eyestalk (4.91-fold), muscle (3.94-fold), and testis (2.83-fold) at moderate levels; the lowest expression was in the abdominal ganglion (1.11-fold) and heart (the calibrator) (Figure 4).

Figure 4. Relative expression levels of Mnfish transcripts quantified in various adult tissues. Bars represent the triplicate mean ± SD (N = 3). Bars with different letters differed significantly (P < 0.05). O: ovary, E: eyestalk, M: muscle, T: testis, AG: abdominal ganglion, H: heart.

qRT-PCR results showed that the Mnfish expression level significantly changed during the embryonic, larval, and post-larval stages (Figure 5). The temporal profile ranged from a peak of high expression to a minimum of moderate expression. Peak expression was observed in the mature ovum (O) and higher than that at the first embryonic stage (cleavage stage, CS). Then, it sharply dropped by 98.24% at the blastula stage (BS). Subsequently, it remained stable until the nauplius stage (NS). As the embryo developed, the expression level of Mnfish remarkably increased from the NS to zoea stage (ZS). In the larvae, the expression level increased roughly and reached the highest at the LVII. However, it abruptly decreased in late larvae (LVIII, just before metamorphosis). Larvae underwent metamorphosis, at which time the larvae transition into post-larvae resemble miniature adults. During the post-larvae stage, the highest expression of Mnfish was found at the PL1, and it quickly decreased with progression of the post-larvae stage, but maintained low level after PL10.
Figure 5. Mnfsh expression, normalized to β-actin, quantified in Macrobrachium nipponense in the ovum, embryos, and larvae before metamorphosis and post-larvae after metamorphosis. Bars represent the triplicate mean ± SD (N = 3). Bars with different letters significantly differed (P < 0.05). O: ovum, CS: cleavage stage, BS: blastula stage, GS: gastrula stage, NS: nauplius stage, PS: protozoea stage, ZS: zoea stage. LI: first larva, PL1: first day post-larva after metamorphosis, PL5: fifth day post-larva after metamorphosis, PL10: tenth day post-larva after metamorphosis, PL15: fifteenth day post-larva after metamorphosis, PL20: twentieth day post-larva after metamorphosis.

DISCUSSION

In the present study, the complete cDNA sequence of fsh was cloned from the M. nipponense and verified by the homology PCR approach and RACE techniques. Conserved sequence and characteristic motifs of bromodomains were identified in the deduced amino acid sequences of Mnfsh. Compared with other invertebrate nucleotide sequences (Haynes et al., 1992; Tamkun et al., 1992; Thorpe et al., 1996), the highest degree of sequence similarity was found near the two internal bromodomain repeats. The bromodomain, a motif initially identified in the Drosophila gene brahma (Tamkun, 1995) that is an extensive family of evolutionarily conserved protein modules originally found in proteins associated with chromatin and in nearly all nuclear histone acetyltransferases, has been discovered to function as an acetyl-lysine binding domain (Zeng and Zhou, 2002). The bromodomain consists of a 60-110 amino acid residue motif that has the potential to form two helices (Haynes et al., 1992; Marmorstein and Berger, 2001); it is conserved in eukaryotes and is commonly found in transcriptional regulatory proteins with diverse transcription-related functions (Jeanmougin et al., 1997). An additional homology among the fsh sub-class has been observed at the extreme C-termini of the proteins and designated as the extra terminal (ET) domain (Lygerou et al., 1994). The functions are not yet known for the ET domain. Our study cloned the full-length cDNA of the Mnfsh gene, which encodes a protein with two bromodomains, but the ET domain was not observed. A potential explanation is that the evolution between species led to the lack of ET domain in Mnfsh.

In the present study, the transcripts of fsh in M. nipponense were detected in all examined tissues; previous studies also found similar results (Chintapalli et al., 2007; Florence and Faller, 2008). The expression level of Mnfsh mRNA in the ovary was significantly higher than that in the testis, and it was explained that fsh is a maternal-effect gene. Interestingly, peak
expression observed also in the ovary, followed by eyestalk, indicating that the effective functioning of the eyestalk, which is a secretory organ, in oriental river prawn could be affected by $fsh$ when necessary.

In the present study, $Mnfsh$ mRNAs were expressed at every stage including the cleavage stage during the embryonic development of $M. nipponense$; this result is similar to those of previous studies that revealed that the gene plays an important role in development and reproduction. In particular, the mutations result in sterility and homeotic transformations (Gans et al., 1975, 1980; Digan et al., 1986), and its direct role in homeotic gene activation has been ascertained by complex phenotypes in mutant embryos in $Drosophila$ (Huang and Dawid, 1990). The embryonic developmental process of $M. nipponense$ can generally be divided into six stages: CS, BS, GS, NS, PS, and ZS. Our results found that the O had the greatest expression of $Mnfsh$, which indicates that $Mnfsh$ mRNA may have been maternally provided in the ovum, which provides strong evidence that it is a maternal-effect gene. After fertilization, $Mnfsh$ gene expression began to decline from the transition of cells from gametes to zygotes. CS expression was the highest in the embryonic developmental process, and then sharply decreased in the BS, retaining a low level in the NS. Furthermore, the relative expression of mRNA during the NS to ZS rose to 30% of the maximum, when organogenesis was most active, which was consistent with a previous finding that the organogenesis in the embryos started at the NS (Zhang et al., 2010). Therefore, our results demonstrated that $Mnfsh$ is closely related to histo-differentiation and organogenesis during the embryonic developmental process. After ZS, $M. nipponense$ ruptured out from embryonic membranes in 2 days and molted. In the larvae, the expression increased roughly and reached the highest level at the LVII stage; however, it abruptly decreased in the late larvae (LVIII, just before metamorphosis). After metamorphosis, the expression of $Mnfsh$ also began to increase in PL1. It is commonly known that, in the molting process, crustaceans experience morphological and appendage characteristic changes (Magalhães, 1989; Zhang et al., 2012). Therefore, our results indicate that the $Mnfsh$ gene has a close relationship with metamorphosis of oriental river prawn.

In conclusion, our results indicate that $Mnfsh$ may play important roles in embryogenesis, organogenesis, and morphological differentiation of $M. nipponense$. This study advances our understanding of the multiple biological functions of the $Mnfsh$ gene. Additionally, our results enhance the understanding of the multiple biological functions of the $Mnfsh$ genes and indicate that $Mnfsh$ may play important roles in embryogenesis, organogenesis, and morphological differentiation of $M. nipponense$. However, the detailed molecular mechanism of the $Mnfsh$ gene is still unknown in oriental river prawn; thus, its specific function needs to be further researched.

ACKNOWLEDGMENTS

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