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ARTICLE

Molecular characterization and promoter analysis of crustacean heat shock protein 10 in *Scylla paramemosain*

Jian Ding, Fang-Yi Chen, Shi-Ying Ren, Kun Qiao, Bei Chen, and Ke-Jian Wang

Abstract: Heat shock proteins (Hsps) are an evolutionarily conserved group of molecules present in all eukaryotic and prokaryotic organisms. Hsp10 and Hsp60 were originally described as the essential mitochondrial proteins involved in protein folding. Recent studies demonstrate that Hsp10 has additional roles including immune modulation. In our study, an homologous Hsp10 (*Sp*-Hsp10) was identified in the mud crab *Scylla paramemosain*, and its genomic DNA organization was determined. The cDNA sequence of *Sp*-Hsp10 contains an open reading frame of 309 bp, encoding a putative protein of 102 amino acid residues with approximately 10 kDa. The *Sp*-Hsp10 gene is located next to the *Sp*-Hsp60 gene and shares a 1916-bp intergenic region. The promoter activity of the *Sp*-Hsp10 flanking gene was analyzed using luciferase reporter assays in transfected endothelial progenitor cells. The upregulation of *Sp*-Hsp10 expression was detected after exposure of hemocytes to a heat shock of 1 h at 37 °C compared with unstressed hemocytes raised at 20 °C. To our knowledge, this is the first report characterizing the genomic organization of a new Hsp10 in a crustacean.

Key words: heat shock protein 10, *Scylla paramemosain*, promoter activity, heat shock protein 60, heat shock.

Résumé : Les protéines de choc thermique (Hsp) constituent un groupe de molécules conservées au cours de l'évolution puisqu'elles sont présentes chez tous les organismes tant eucaryotes que procaryotes. Hsp10 et Hsp60 ont initialement été décrites comme étant des protéines mitochondriales essentielles impliquées dans le repliement des protéines. Des études récentes ont montré que Hsp10 joue des rôles additionnels incluant la modulation immunitaire. Dans ce travail, un homologue de Hsp10 (*Sp*-Hsp10) a été identifié chez le crabe de vase *Scylla paramemosain*, et l'organisation de son ADN génomique a été étudiée. L'ADNc du *Sp*-Hsp10 contient un cadre de lecture de 309 pb et code pour une protéine putative de 102 acides aminés et d'environ 10 kDa. Le gène *Sp*-Hsp10 est voisin du gène *Sp*-Hsp60 et en est séparé par une région intergénique de 1916 pb. L'activité promotrice de la région bordant le gène *Sp*-Hsp10 a été analysée au moyen d'un gène rapporteur (luciférase) dans des cellules souches endothéliales transfectées. Une augmentation de l'expression de *Sp*-Hsp10 a été détectée après exposition des hémocytes à un choc thermique d'une heure à 37 °C tandis que les hémocytes témoins étaient maintenus à 20 °C. Au meilleur de la connaissance des auteurs, il s'agirait de la première description de l'organisation génomique d'un gène Hsp10 chez un crustacé. [Traduit par la Rédaction]

Mots-clés : protéine de choc thermique 10, *Scylla paramemosain*, activité promotrice, protéine de choc thermique 60, choc thermique.

Introduction

The heat shock response was discovered by Ritossa in 1962 in the *Drosophila* larva salivary gland chromosome puffs in response to sudden elevated temperature exposure and exposure to dinitrophenol or sodium salicylate (Ritossa 1962). The key proteins regulating this process were later characterized and referred to as heat shock proteins (Hsps) (Tissiéres et al. 1974). Hsps are highly conserved and essential to all living organisms (Lindquist and Craig 1988) and are classified into five major families based on their molecular mass, including the small Hsps (e.g., Hsp10), Hsp60, Hsp70, Hsp90, and Hsp100. In eukaryotic cells, folding and assembly of newly synthesized polypeptides in the cellular environment requires the assistance of molecular chaperone proteins such as Hsp70, Hsp60, and Hsp10 (Hendrick and Hartl 1995). The Hsp10 protein was discovered in the serum of pregnant women and thus is named the early pregnancy factor, which displayed immunosuppressive properties (Morton et al. 1974; Morton et al. 1977; Athanasas-Platsis et al. 2003). Mammalian Hsp10 is also shown to have anti-inflammatory activity by inhibiting lipopolysaccharide-induced toll-like receptor signaling possibly via interactions with extracellular Hsp60 (Johnson et al. 2005). Recently, Hsp10 was reported as a stress gene in response to heat (van der Giezen et al. 2005) and other stressors. The expression level of Hsp10 is also induced by cadmium exposure in COS-7 cells (Lee et al. 2002). Another report indicates that increased Hsp10 expression protects myocytes against SI/RO, metabolic ischemia (NaCN), and H_2O_2 injury (Lin et al. 2004).

To date, a large number of Hsp10 members have been identified, and the genomic DNA organization of Hsp10 is fully determined in several species. It is interesting to note that the genes encoding Hsp10 and Hsp60 are linked in a head to head manner and are shown to share a bidirectional promoter in *Rattus norvegicus* (Ryan et al. 1997), *Homo sapiens* (Hansen et al. 2003), pufferfish (*Fugu rubripes*), zebrafish (*Danio rerio*), and the nematodes *Caenorhabditis elegans* (Martin et al. 2002). Recently, this configuration was also found in the nematode *Strongyloides ratti* *Sr*-Hsp10 and *Sr*-Hsp60 genes. However, unlike Hsp10 genes from

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Table 1. List of primers used for *Sp*-Hsp10/*Sp*-Hsp60 cloning, expression, and promoter construction.

Primer*	Sequence (5′-3′)	Application
10RP	GGCACGAACCTTCTCAATACACCAGCC	5′ RACE
10F1	GAGTTTGGAGGCACGAAGGTGAC	Inverse PCR
10R1	CACTGCCACCACCTTGCCTATC	Inverse PCR
10F2	ATCCTGGCCAAGATGAAGAACGAG	Inverse PCR
10R2	CACGAACCTTCTCAATACACCAG	Inverse PCR
10F3	ATAGGCAAGGTGGTGGCAGTG	qPCR
10R3	AGTCCTTCTCCTCCAGCGTCA	qPCR
10R4	AAGCGTCCCGGTGATGCTCGTCG	Intergenic region amplification
60F1	GGCTACATCTCCCCCTACTTCATC	3′ RACE and genome amplification
60R1	CAATGGCAATGTCCTGGATGGTG	5′ RACE and genome amplification
60F2	ATGTACCGCGCTGCCTCCCTG	*Sp*-Hsp60 ORF amplification
60R2	CAGCGTGTCGTCCTTGGTGATTTG	5′ RACE and genome amplification
60F3	GTGCCAAGCTGGTGCAGGATGTGGCCAAC	Genome amplification
60R3	TCACATCATGCCTCCCATGCCAC	Genome amplification
60R4	GTTGGCCACATCCTGCACCAGCTTGGCAC	Intergenic region amplification
PF1	GAACGCGTCTGGAAGGAGAGATAATAC	Promoter construction
PF2	GAACGCGTCTCCAAAGATAACCCTG	Promoter construction
PF3	GCACGCGTAAGGAAACTAATTTGAG	Promoter construction
PF4	GAACGCGTCAGGTACTTTAAGCATG	Promoter construction
PF5	GAACGCGTCCTGTACCATGTGG	Promoter construction
PR1	GAAGATCTTGCGCCGTGCTGTGGTG	Promoter construction
18SF	CAGACAAATCGCTCCACCAAC	qPCR
18SR	GACTCAACACGGGGAACCTCA	qPCR

*The *Mlu*I and *Bgl*II sites are highlighted.

humans and *C. elegans*, which are interrupted by two introns each, *Sr*-Hsp10 contains only one intron. Furthermore, the *Sr*-Hsp60 gene has no introns, whereas its human and *C. elegans* homologs have eight or four introns, respectively (Tazir et al. 2009). Although Hsp10 sequences have been reported from many animal and bacterial species, large taxonomic gaps still remain. Furthermore, no previous studies concerning the genomic organization of Hsp10 or its induction following stress in crabs and other crustaceans has been recorded.

The mud crab *Scylla paramemosain* is an important commercial crustacean species in southeast China, distributed throughout the tropical and subtropical zones in the west Pacific and Indian oceans. To understand the role of Hsp10 in *S. paramemosain*, a new *Sp*-Hsp10 gene was cloned, and the genomic DNA organization of *Sp*-Hsp10 and *Sp*-Hsp60 was determined using a nested PCR strategy. We investigated the expression pattern of *Sp*-Hsp10 in the tissues tested and in hemocytes upon heat shock stimulation. Furthermore, the sequence of the putative promoter region of *Sp*-Hsp10 was determined and its activity characterized.

Materials and methods

Animal and sample preparation

Healthy 100 ± 10 g male crabs (*S. paramemosain*) were obtained from a crab farm in Xiamen, China. The crabs were carefully dissected on ice and their heart, gills, midgut gland, dermis, stomach, muscle, and eyestalks were collected for total RNA extraction. The haemolymph was resuspended in a cold anticoagulant solution (NaCl 140 mmol/L; glucose 100 mmol/L; citric acid 26 mmol/L; trisodium citrate 30 mmol/L; and pH 5.0) at a proportion of 1:1 (hemolymph:anticoagulant), followed by centrifugation at 500*g* for 5 min. The hemocyte cell pellets were used for total RNA extraction and in vitro cell culture. Total RNA was isolated using TRIzol reagent (Invitrogen). Genomic DNA was isolated from muscle using a Universal Genomic DNA extraction kit (TaKaRa) and removed from the RNA template with RNase-free DNase I (Roche).

Expression studies

Hemocytes (1×10^6/mL) were seeded in Leibovitz L-15 medium (Hyclone) containing 5% fetal bovine serum (FBS, Gibco), penicillin (100 IU/mL), and streptomycin (100 μg/mL) in 96-well cell culture plates (Nunc) and cultured at 20 °C. The osmolarity was adjusted with the addition of NaCl (0.12 g/L). After being cultured for 24 h, the hemocytes were subjected to heat shock at 37 °C for 1 h, and then the heat-shocked cells were allowed to recover at 20 °C for 2, 4, 6, and 8 h. Cell viability (over 98% during the whole time in the medium) was evaluated and further confirmed using the Trypan Blue exclusion method (Mascotti et al. 2000). First strand cDNA of the tissues tested and hemocytes samples were synthesized using the PrimeScript RT reagent kit (TaKaRa). Primers 10F3 and 10R3 (Table 1) were used for *Sp*-Hsp10 amplification. In addition, 18S-specific primers 18SF and 18SR were used as the control. All reactions were performed using an ABI PRISM 7500 SDS/relative quantification system. The reactions were run with the following PCR cycling conditions: 2 min, 50 °C; 10 min, 95 °C; 40 × (15 sec, 95 °C; and 1 min, 60 °C). PCR efficiency was determined through the slope of the standard curve using 10-fold dilutions of cDNA. All samples were tested in triplicate in the same plate and the resulting threshold cycle values recorded. The $2^{-\Delta\Delta Ct}$ method of relative quantification was adapted to estimate copy numbers of the *Sp*-Hsp10 gene. The SPSS V13.0 program was used for statistical analysis by using independent-samples *t* test. Differences were considered statistically significant at $p < 0.05$ (or $p < 0.01$).

Cloning of the cDNA sequences of *Sp*-Hsp10 and *Sp*-Hsp60

Hsp10 is a co-chaperone for Hsp60 in the protein folding process. We performed RACE–PCR to obtain the full-length cDNA sequences of both *Sp*-Hsp10 and *Sp*-Hsp60. Gene-specific primers for *Sp*-Hsp10 (10RP for 5′ RACE) were designed based on the partial cDNA sequence obtained from the SSH library (Chen et al. 2010). Two expressed sequence tags (accession Nos. CV161872.1 and CV224944.1) of Hsp60 from the crab *Callinectes sapidus* were obtained using sequence analysis. The primer 60F1 (60F1 for 3′ RACE) was designed based on the highly conserved sequence across species. Based on the result of 3′ RACE, two specific primers, 60R1 and 60R2, were designed for 5′ RACE. The full length cDNA sequences of *Sp*-Hsp60 were obtained by assembling their 5′ and 3′ RACE products and verified by the PCR amplification using gene-specific primers 60F2 and 60R3. The PCR products were separated in

agarose gel and purified using a gel extraction kit (QIAGEN), then cloned into vector pMD-18T by using a TA cloning kit (TaKaRa) for sequencing (Invitrogen).

Determination of the genomic DNA organization of *Sp*-Hsp10 and *Sp*-Hsp60

The 5′-flanking (promoter) regions of *Sp*-Hsp10 were cloned to gain further insight into its regulation at the transcriptional level. The extracted DNA (5 μg) was digested with restriction enzyme *Eco*RI, and then the digested DNA was ligated using T4 DNA ligase at a low concentration (2.5 ng/μL). Nested inverse PCR was used to amplify the 5′- and 3′-flanking sequences of *Sp*-Hsp10 using the specific nested primers 10F1 and 10R1, 10F2 and 10R2. Only the partial 5′-flanking structure of *Sp*-Hsp10 was cloned using inverse PCR. To characterize the full-length 5′-flanking intergenic region between *Sp*-Hsp10 and *Sp*-Hsp60, we cloned the DNA sequence of *Sp*-Hsp60 following a nested PCR strategy with the two primer pairs 60F3 and 60R2, 60F1 and 60R3. Then primers 60R4 and 10R4 were designed for the amplification of the bidirectional promoter region. Intron positions and transcription factors were identified by comparison with the corresponding cDNA sequence.

Luciferase reporter assay

To investigate the regulatory domain of *Sp*-Hsp10, a series of DNA fragments containing full-length or partial sequences of the 1916-bp intergenic region were cloned into the promoterless pGL3-basic vector (Promega) containing the luciferase reporter gene. The fragments were amplified using high-fidelity Pfu DNA polymerase with the specific primer pairs. A *Mlu*I site was added to the 5′ end of the forward primers, and a *Bgl*II site was added to the reverse primer PR1. The PCR products were digested with *Mlu*I and *Bgl*II and inserted into a pGL3-basic plasmid cut with the same enzymes. The resultant constructs were verified with sequencing using the vector primers RVprimer 3 and GLprimer 2 (Invitrogen). For the transfection experiment, plasmids were purified from *Escherichia coli* DH5a cells with an endo-free plasmid purification kit (QIAGEN).

Endothelial progenitor cells (*Pseudosciaena crocea*, CCTCC) were cultured in MEM medium (Hyclone) supplemented with 0.1 mmol/L nonessential amino acids, 1.0 mmol/L sodium pyruvate, penicillin (100 IU/mL), streptomycin (100 μg/mL), and 5% FBS (Gibco) in a humidified atmosphere of 5% CO_2 at 25 °C. The cells were seeded in 24-well plates (Nunc) and transfected with plasmids using Lipofectamin 2000 (Invitrogen) following the manufacturer's instructions. In total, 2000 ng of the promoter construct and 50 ng of the pSV-β-galactosidase plasmid (Promega) were used for each transfection experiment. Six hours after transfection, the medium was replaced with new MEM supplemented with 5% FBS and cultured for 18 h at 25 °C. Duplicate plates were subsequently placed in an incubator at 37 °C for 1 h, whereas control plates remained at 25 °C. All plates were further incubated for 23 h at 25 °C prior to harvesting. The cells were washed twice with ice-cold PBS (without calcium) and lysed in 150 μL of 1× lysis buffer (Promega). Luciferase activity in the cell lysates was determined using a Wallac Victor 2 1420 multilabel counter (Perkin Elmer) and normalized to β-galactosidase activities. β-galactosidase activity was detected using a FlexStation 3 microplate reader (Molecular Devices). Transfections and the measurements were performed in duplicate and the experiment repeated three times. The data were statistically analyzed by the SPSS V13.0 program. Differences between independent samples were tested with the independent-samples *t* test.

Sequence analyses

The *Sp*-Hsp10 and *Sp*-Hsp60 genes and amino acid sequences were analysed and compared by using the BLAST search program available on the NCBI website (http://www.ncbi.nlm.gov/BLAST/) and ENA sequence search program (http://www.ebi.ac.uk/ena/). Multiple amino acid sequence alignments were created by using the Clustal V1.83 program. Phylogenetic and molecular evolutionary analyses according to amino acid sequences of *Sp*-Hsp10 were conducted with MEGA version 5, and a tree was conducted based on the Poisson correction model of neighbor-joining algorithm.

Results

Molecular cloning of a cDNA sequence encoding *Sp*-Hsp10 and *Sp*-Hsp60

The full-length cDNA sequence of *Sp*-Hsp10 comprised 734 bp and its coding region started from 79 bp downstream at the 5′ end to 309 bp long followed by a 344-bp 3′ untranslated region (UTR). The cDNA contained a Kozak consensus-like sequence (GCAATGG) upstream of the initiation codon ATG. The *Sp*-Hsp10 had a 309-bp ORF, encoding a predicted protein of 102 amino acid residues. No signal peptide was predicted in the N terminus (Fig. 1A). Based upon the reported sequence information, the full-length *Sp*-Hsp60 cDNA was also obtained using PCR and it consisted of 2431 bp with an ORF of 1731 bp, flanked by 67 bp of 5′ UTR and 643 bp of 3′ UTR (Fig. 1B).

The phylogenetic tree based on the putative Hsp10 protein sequences among different species is shown in Fig. 2, with *E. coli* set as outgroups. The Hsp10s from different crab species were clustered in a subgroup of invertebrates with high bootstrap values (100). Amino acid alignment revealed a high sequence identity (92.2%) of *Sp*-Hsp10 to Hsp10 from the crab *C. sapidus*, and 87.4% to Hsp10 from the fiddler crab (*Uca pugilator*). The amino acid sequences of the Hsp10 genes are highly conserved in both prokaryotes and eukaryotes (Gupta 1995). The amino acid sequence in *Sp*-Hsp10 was 58.0% identical to *Drosophila* Hsp10, 53.9% to *Branchiostoma* Hsp10, and 57.8% to human Hsp10.

Tissue distribution of *Sp*-Hsp10 and its expression after stress in the hemocytes

To determine the expression pattern of *Sp*-Hsp10, we performed quantitative real-time PCR (qPCR) on total RNA from various tissues. The PCR results indicated that the mRNA transcripts of *Sp*-Hsp10 were detected in all tissues tested, whereas the transcripts were most abundant in hemocyte, muscle, stomach, and heart, but they were comparatively low in eyestalks and the midgut (Fig. 3). qPCR was also performed to examine *Sp*-Hsp10 transcription in hemocytes of *S. paramemosain* upon exposure to heat shock. With 1 h heat shock (37 °C), expression of *Sp*-Hsp10 mRNA was upregulated approximately 1.5-fold compared with unstressed hemocytes. This level remained elevated relative to that in the control cells until after 8 h (upregulated 1.8-fold; 2.4-fold increase after 4 h) of recovery at 20 °C (Fig. 4).

Determination of the genomic organization of *Sp*-Hsp10

Using inverse PCR, the 5′- and 3′-flanking sequences of *Sp*-Hsp10 were amplified. The genomic DNA sequence of *Sp*-Hsp10 spanned 1201 bp from the initiation codon to the stop codon and included three introns, whereas the *Sp*-Hsp60 gene comprised 5913 bp from the initiation codon to the stop codon and included eight introns (JX262230) (Fig. 5A). The intron–exon boundaries in the Hsp genes conformed to the consensus 5′-GT—AG-3′ sequences for donor and acceptor RNA splicing sites (Mount 1982). The genes encoding both *Sp*-Hsp10 and *Sp*-Hsp60 were linked head to head with a 1916-bp bidirectional promoter, a sequence separating the two ATG start codons (Fig. 6). Extensive searches of the transcription factor database TRANSFAC (http://www.gene-regulation.com) reveals that many potential regulatory elements for transcription factors exist in the intergenic region. Amongst the potential sites for transcription activators were one site for SP1, NKx-2, and HLF, and four sites for AML-1a. One potential site for heat shock transcription factors, the heat shock element (HSE), which contains five

Fig. 1. (A) Nucleotide and deduced amino acid sequences of *Sp*-Hsp10 and (B) schematic representation of primer sites, PCR fragments of *Sp*-Hsp60. Highlighted nucleotides represent the primer sites of 10RP, 10R1, 10F1, and 10F2. The sites 10R2, 10F3, and 10R3 are underlined and the stop codon is marked with an asterisk.

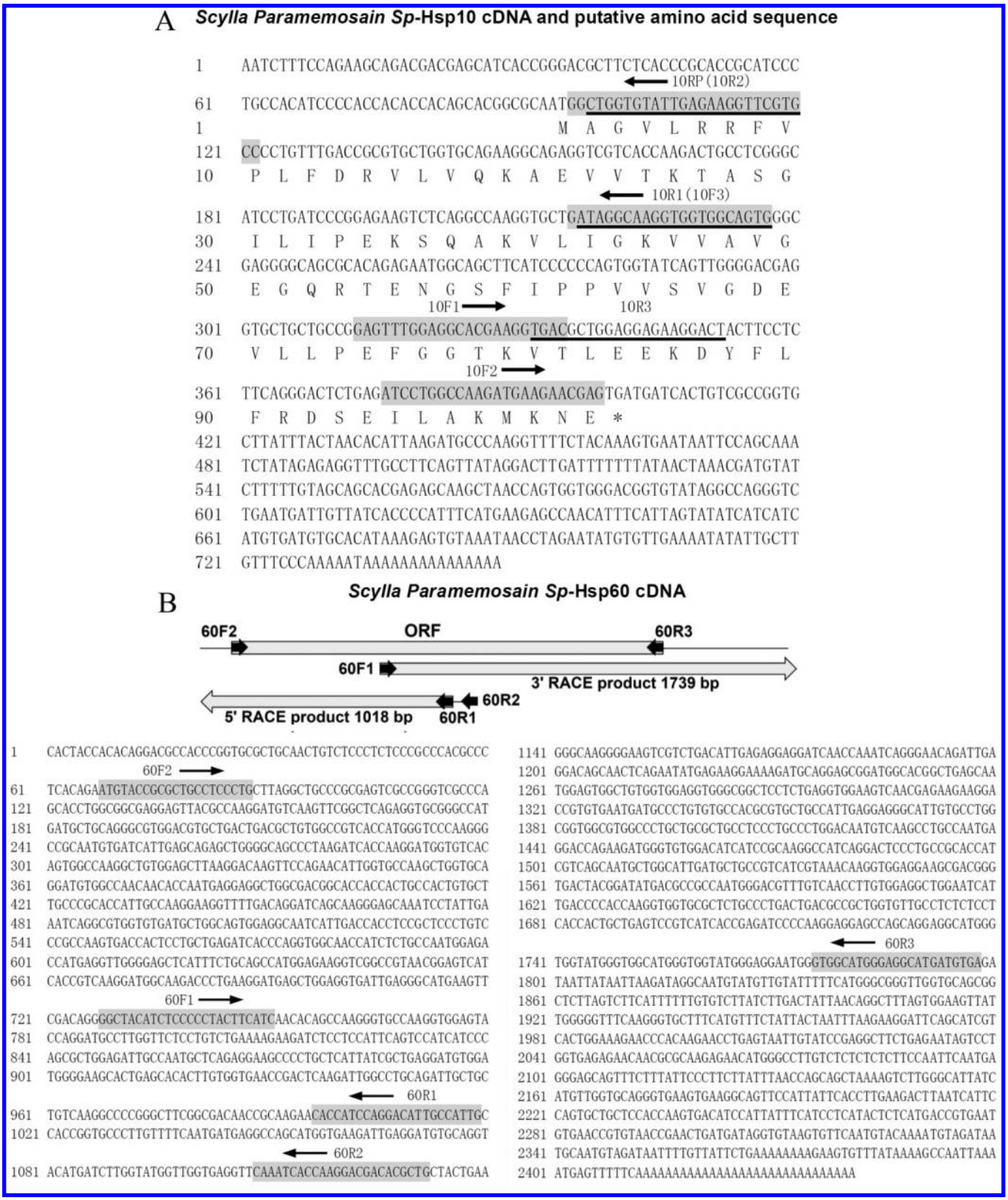


Fig. 2. Phylogenetic analysis of *Sp*-Hsp10 relative to other Hsp10 proteins from different species using MEGA 5.05. All the sequences in the tree are labeled as organism type and GenBank accession number. The scale bar indicates 0.05 substitutions per site. Two thousand bootstraps were performed for the neighbor-joining trees to check for the repeatability of the results. *Sp*-Hsp10 in the cluster is marked with ♦.

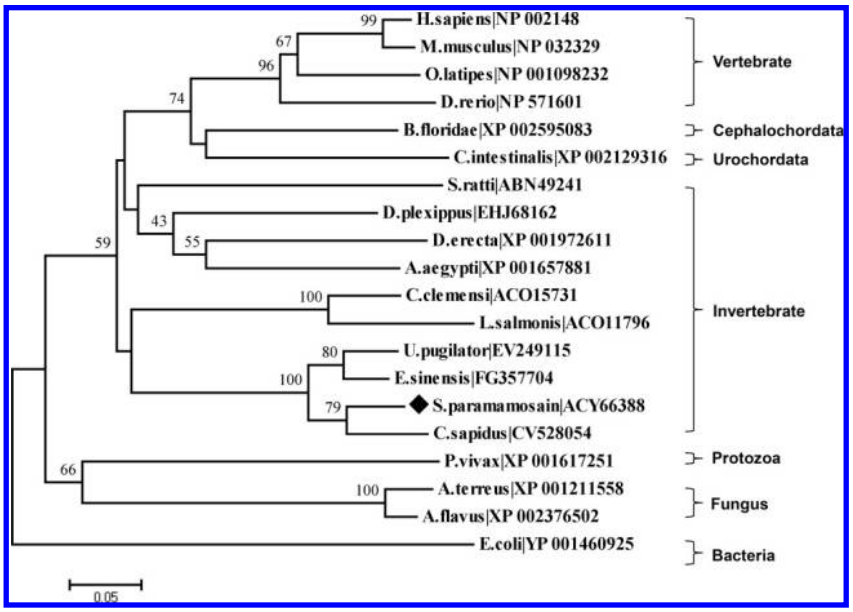


Fig. 3. Gene distribution of *Sp*-Hsp10 mRNA in the tested tissues revealed using qRT-PCR. Ey, eyestalk; Gi, gills; Hc, hemocyte; Md, midgut gland; Dm, dermis; St, stomach; Mu, muscle; and Ht, heart.

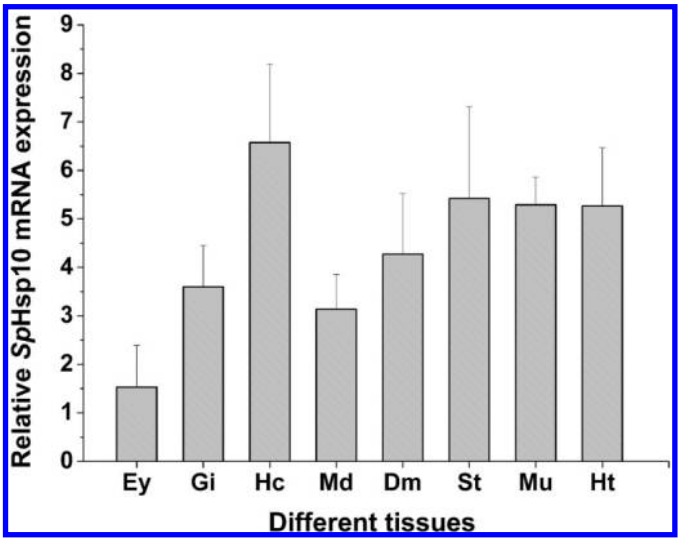
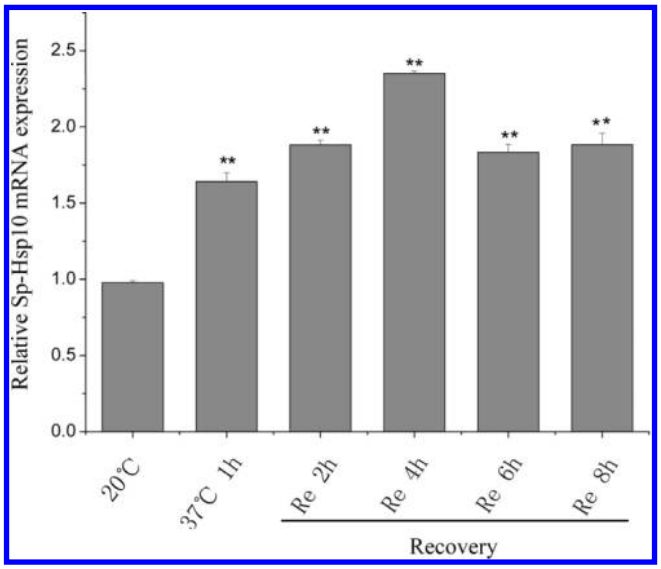


Fig. 4. qRT-PCR analysis of *Sp*-Hsp10 mRNA expression in hemocytes. Expression of *Sp*-Hsp10 is heat inductable. Shown are hemocytes maintained at 20 °C; hemocytes that were exposed to 1 h of heat shock at 37 °C; the heat-shocked hemocytes that were allowed to recover (Re) at 20 °C for 2, 4, 6, and 8 h. The data are representative of three independent experiments, and error bars are ± standard deviation. ** indicates significant difference at $p < 0.01$.

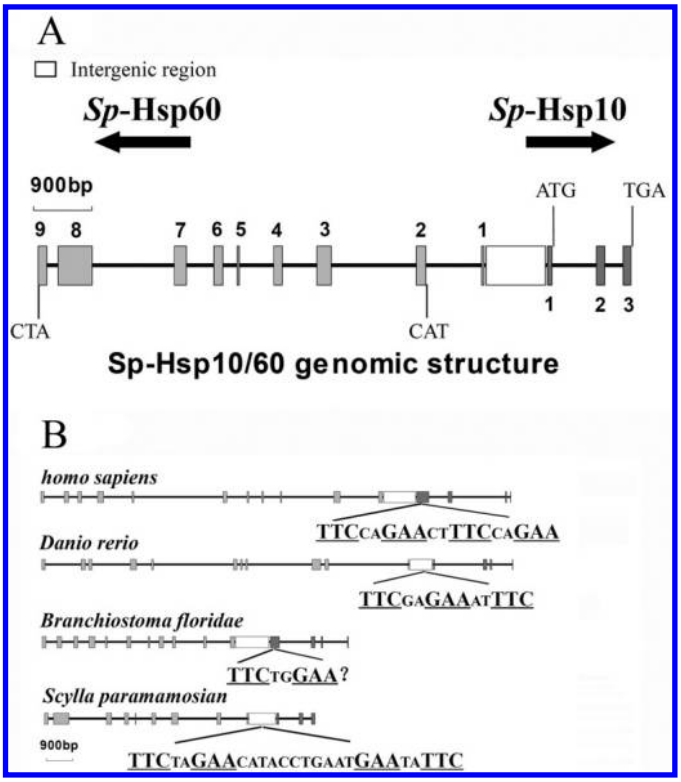


inverted *n*GAA*n* repeats (5′-TTCtaGAAcaTACctGAAtGAAtaTTC-3′), was also identified. Besides, there was a putative HSE with the consensus sequence 5′-*n*TTC*nn*GAA*n*-3′ present in exon-1 of the *Sp*-Hsp10 gene. No consensus TATA-box or CCAAT-box was detected within −80 bp upstream of the transcription start site (TSS) of the *Sp*-Hsp10 and *Sp*-Hsp60 genes.

Determination of the promoter region within the overlapping sequence of *Sp*-Hsp10 and *Sp*-Hsp60

A series of vectors (designated 10P1, 10P2, 10P3, 10P4, and 10P5) were constructed by insertion of the *Sp*-Hsp60–*Sp*-Hsp10 intergenic promoter region into the promoterless pGL3-basic plasmid. The vector 10P1 contains a 1916-bp fragment spanning the entire region between the two ATG start codons of *Sp*-Hsp10 and *Sp*-Hsp60. Four progressive deletions from the position of −889 to +93 constructs (10P2–10P5 vectors) were then generated to identify the minimal promoter region necessary to drive the *Sp*-Hsp10 gene transcription (see Fig. 7A). Endothelial progenitor cells were transiently transfected with the promoter constructs and the luciferase activity present in the resulting cell extracts were measured under both normal growth conditions (25 °C) and in cells subjected to heat shock (37 °C, 1 h). The different promoter constructs exhibited different promoter activity when measured in the luciferase reporter assay (Fig. 7B). All constructs, 10P1–10P5, showed increased activities compared with the basal luciferase activity of the pGL3-basic vector. The construct 10P2 (−889/+93) exhibited the highest promoter activity, and the sequence between −889 and −379 seemed critical for promoter activity of the

Fig. 5. (A) Schematic map of the genomic structure of *Scylla paramemosain* (JX262230) Hsp10 and Hsp60 genes. (B) Diagram of the genomic organization of *Homo sapiens* (NG_008915.1), *Danio rerio* (BX571722.18), *Branchiostoma floridae* (XM_002595038.1), and *S. paramemosain* (JX262230) Hsp10 and Hsp60 genes. The positions of the start and stop codons of *Sp*-Hsp10 and *Sp*-Hsp60 are indicated. The bidirectional promoter region is between the coding exons. The heat shock element of the bidirectional promoter region between these two genes is highlighted with its nucleotide sequences.



Sp-Hsp10 gene. In addition, the deletion from –1823 to –888 had obvious effects on enhancing the promoter activity. Exposure to heat shock (37 °C, 1 h) significantly increased luciferase activity in all transfected cells of constructs in comparison with those maintained at 25 °C (Fig. 7B). Moreover, heat shock was able to increase luciferase activity markedly in construct 10P2, resulting in the most significant increase relative to other constructs.

Discussion

Hsp10 was originally described solely as an essential partner of Hsp60 in the Hsp10/60 mitochondrial protein folding machine. However, besides being located in the mitochondria, Hsp10 is also found at a variety of specific extramitochondrial sites in normal rat tissue, such as zymogene granules, hormone granules, secretory granules, and mature red blood cells (Sadacharan et al. 2001). The expression of Hsp10 in extramitochondrial sites is associated with a variety of activities including immunomodulation, cell proliferation, and differentiation (Jia et al. 2011). Recently, it was discovered that Hsp10 was also a critical gene in response to heavy metal stress and H_2O_2 injury (Lee et al. 2002; Lin et al. 2004).

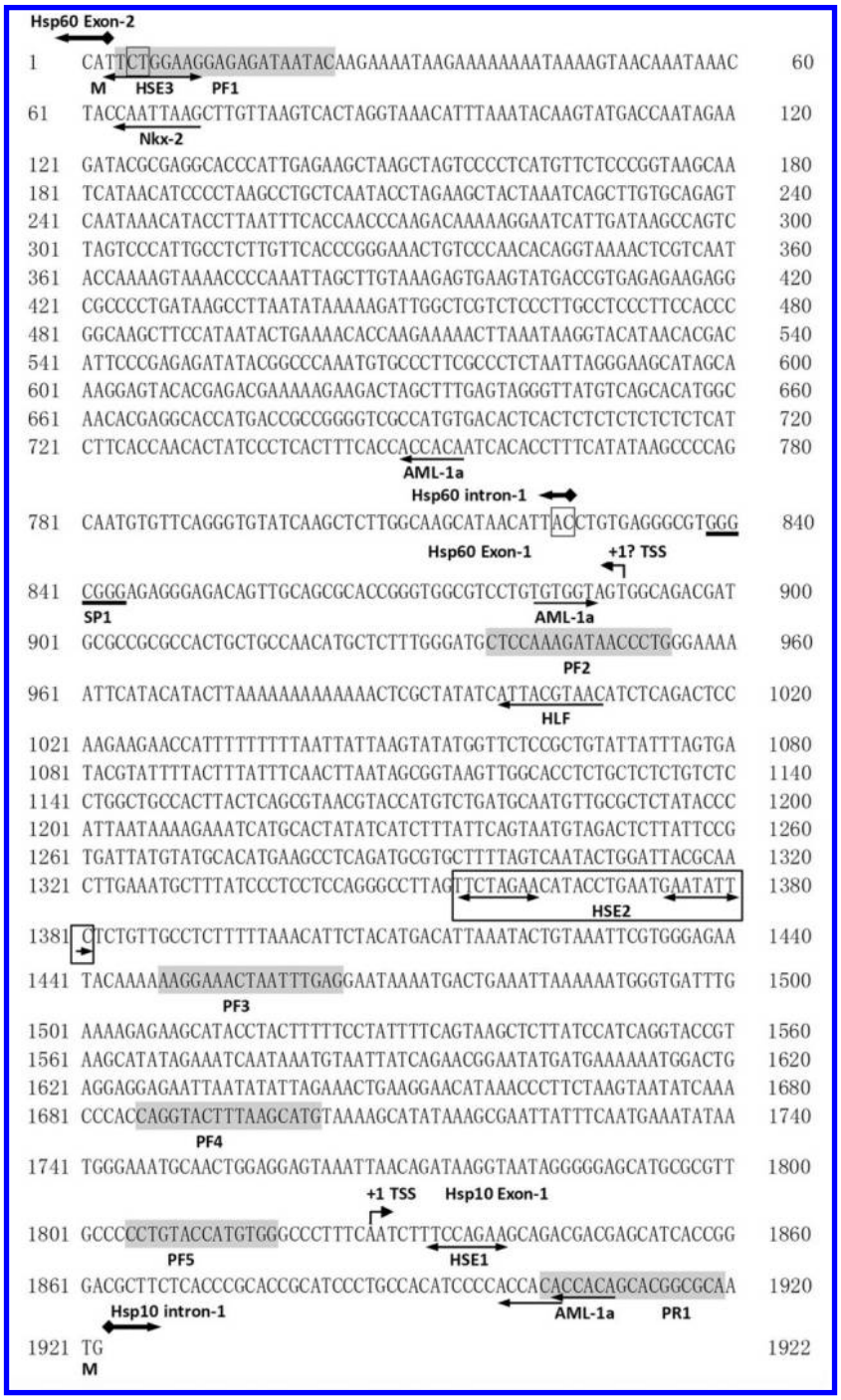
To elucidate the potential roles of Hsp10 in *S. paramemosain*, the full-length cDNA and genomic DNA sequences of *Sp*-Hsp10 and *Sp*-Hsp60 were, respectively, determined. Amino acid alignments revealed *Sp*-Hsp10 had a significant homology with the Hsp10 gene reported from the crabs *C. sapidus* and *U. pugilator*. This suggested that Hsp10 proteins are highly conserved across species. Thus, the *Sp*-Hsp10 gene is 57.8% and 58.0% identical to that of humans and *Drosophila*, respectively. Similar to the molecular characterization of Hsp10 from *S. ratti* and rat, no signal peptide sequence has been identified in the *Sp*-Hsp10 ORF, suggesting that its extracellular exportation might proceed through a nonclassical endoplasmic reticulum – Golgi independent pathway. Instead, an amphipathic alpha helix region was present within the first N-terminal 30 residues of *Sp*-Hsp10, which might be accessible to target and cross the mitochondrial membrane (Jarvis et al. 1995; Tazir et al. 2009).

The *Sp*-Hsp10/*Sp*-Hsp60 genomic structure was determined using overlapping PCR with specific nested primers. The entire genomic structure of *Sp*-Hsp10/*Sp*-Hsp60 genes covered approximately 9 kb (JX262230). Both *Sp*-Hsp10 and *Sp*-Hsp60 genes were linked by a bidirectional promoter region of a 1916-bp sequence, which separated two ATG start codons of both genes. In the bidirectional promoter region, the initiator-like sequence relative to the adjacent TSS fits the consensus initiator sequence of PyPyANT/APyPy (Py stands for a pyrimidine; N stands for any base; A is the TSS) (Xi et al. 2007), with the only minor difference being a Y→A variation at position +4 of the *Sp*-Hsp60 initiator element.

Bidirectional promoters direct the transcription of approximately 10% of genes in the human genome (Anno et al. 2011). There has been no clear explanation yet of the biological and evolutionary advantages of this distinct type of divergent gene pairs. Theoretically, only when two genes are divergently organized in bidirectional gene pairs can they be co-expressed and co-regulated by a shared intergenic bidirectional promoter (Huang and Chang 2009). Mammalian Hsp10 is well characterized, and, in humans, Hsp10 and Hsp60 are localized head to head on chromosome 2 (Hansen et al. 2003). We identified the bidirectional promoter region shared by both *Sp*-Hsp10 and *Sp*-Hsp60 genes in *S. paramemosain*. And no consensus TATA-box or CCAAT-box was detected in the *Sp*-Hsp10/60 intergenic region. A TATA-less promoter is often found to direct the expression of house-keeping genes, oncogenes, growth factors, and transcription factors (Zhang 1998). To confirm the promoter region of *Sp*-Hsp10, several constructs were designed for luciferase reporter assay. All the constructs tested were able to drive the expression of the luciferase gene, but the construct 10P2 (–889/+93) exhibited the highest promoter activity, which suggested that the region from –1823 to –888 had a negative regulatory domain and that the region from –889 to –379 had a positive regulatory domain. Moreover, heat shock was able to increase luciferase activity markedly in all constructs compared with those maintained at 25 °C. In brief, we supposed that the DNA sequence encompassing the TSS of a TATA-less promoter contained enough information to drive *Sp*-Hsp10 gene transcription. The resulting data also demonstrated that the *Sp*-Hsp10 promoter could be activated by heat shock.

Bioinformatics analysis revealed an HSE (5′-TTCtaGAAcaTACct GAAtGAAtaTTC-3′) consisting of five inverted *n*GAA*n* motifs in the *Sp*-Hsp10 promoter region. There were two conserved HSE sites in the forward (5′-TTC*nn*GAA-3′) and in the reverse direction (5′-GAA*nn*TTC-3′). The sequence of this HSE is different from the other Hsp10/60 HSEs that are reported in rats (Ryan et al. 1997) and humans (Hansen et al. 2003) with the consensus sequence 5′-*n*TTC*nn*GAA*n*-3′, and also in zebrafish (5′-GAA*nn*TCC*nn*GAA-3′) (Martin et al. 2002). It was interesting to note that the level of luciferase activity of the promoter construct 10P2 containing the HSE was higher than that of other HSE-absent constructs in *Sp*-Hsp10 under normal and heat shock conditions. This suggested that the HSE was likely to be the core transcription factor for the basal and inducible expression of *Sp*-Hsp10. Deletions of this element caused a reduction of transcription. A single HSE conserved in rat and human is also present in the Hsp10/60 intergenic region. Because of the reverse complement sequence of the HSE (5′-GAAtaTTCaTTCagGTAtgTTCtaGAA-3′), which consists of a complete conserved HSE motif in the forward direction (GAA*nn*TTC) and in the reverse direction (TTC*nn*GAA), it is postulated that those palindromic motifs might also play an important role in the regulation of *Sp*-Hsp60. The presumption is supported by the pre-

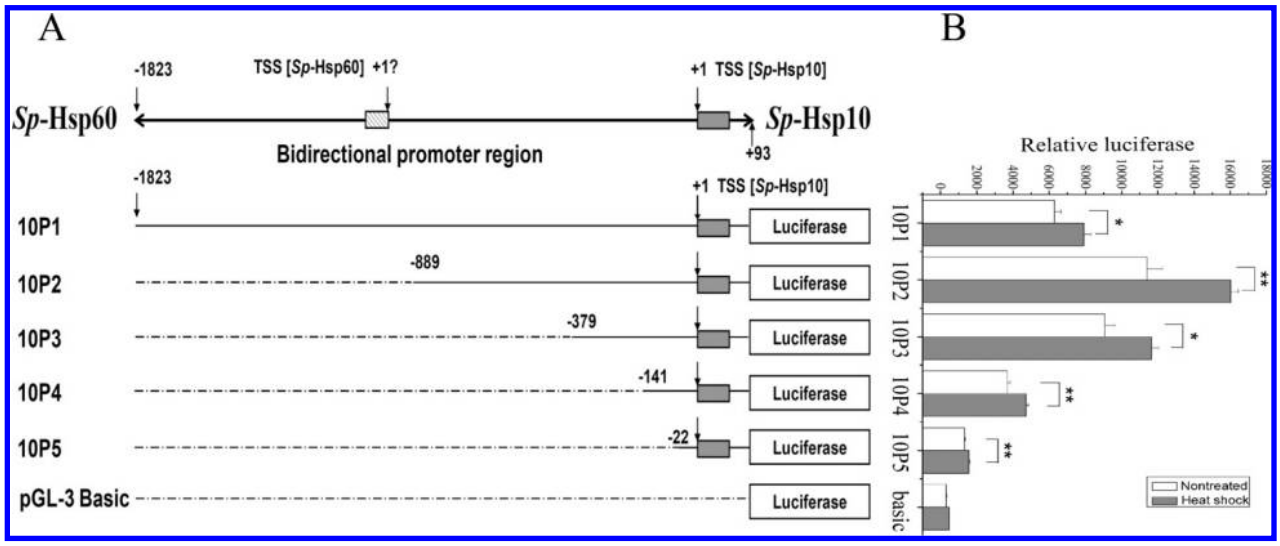
Fig. 6. Nucleotide sequence of the intergenic region between the *Sp*-Hsp10 and *Sp*-Hsp60 genes spanning from the last nucleotide in intron-1 of *Sp*-Hsp60 to the last nucleotide in exon-1 of *Sp*-Hsp10. Potential regulatory elements are indicated with arrows, and the SP1 transcription factor binding site is underlined. *Sp*-Hsp10 and *Sp*-Hsp60 5′ UTR transcription start sites (TSSs) are depicted by an angled arrow and numbered as nucleotide +1. Highlighted nucleotides represent the primer sites used for cloning the constructs. The donor and acceptor splice site of *Sp*-Hsp60 intron-1 and the heat shock element located in intergenic region is boxed.



vious report that the regulation of Hsp10 and Hsp60 genes was found to be simultaneous in mammalian cells under normal conditions and stress such as a heat shock stimulus (Hansen et al. 2003), or during carcinogenesis (Cappello et al. 2003). An additional HSE in the first exon was identified with a 10-bp palindrome (5′-*n*TTC*nn*GAA*n*-3′), and data showed that the promoter construct 10P5 presented low promoter activity and also could be induced by heat shock. So we supposed that the HSE played an important role in the regulation of *Sp*-Hsp10 gene expression as a useful cofactor of the initiator.

The hemocytes were subjected to heat shock in which the mRNA levels of *Sp*-Hsp10 increased significantly. These results indicated that the induction of the *Sp*-Hsp10 gene was associated with the increase of temperature as a member of the small Hsp family. Therefore, *Sp*-Hsp10 might play an essential role in maintaining cellular homeostasis under heat shock in *S. paramemosain* hemocytes. Finally,

Fig. 7. Analysis of promoter activity of the intergenic region in endothelial progenitor cells. (A) The directions of *Sp*-Hsp10 and *Sp*-Hsp60 gene transcription are indicated by arrows. A series of promoter constructs was used in a transient transfection experiment. All constructs (10P1–10P5) in the *Sp*-Hsp10 direction contain *Sp*-Hsp10 exon-1 but without the ATG start codon. (B) Heat-inducibility of *Sp*-Hsp10 promoter luciferase constructs. Twenty four hours after transfection, the luciferase activity present in the resulting cell extracts were measured under both normal growth conditions (25 °C, nontreated group) and in cells subjected to heat shock (37 °C, 1 h heat shock group). The pSV-β-galactosidase plasmid was used as an internal control for transfection efficiencies and cell viability. The experiments were performed in triplicate. The data are representative of three independent experiments, and error bars are ± standard deviation. * indicates significant difference at $p < 0.05$. ** indicates significant difference at $p < 0.01$.



for the first time, we characterized a new *Sp*-Hsp10 in a crustacean and showed that the *Sp*-Hsp10 identified could be the first step towards elucidating its role in *S. paramemosain*.

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