Heat shock cognate 70 gene in *Haliotis diversicolor*: responses to pathogen infection and environmental stresses and its transcriptional regulation analysis

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Received: 9 August 2017 / Accepted: 13 September 2017 / Published online: 23 September 2017
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Abstract Heat shock cognate 70 (HSC70) is a class of highly conserved proteins which functions as a molecular chaperon, participates in tolerance processes, and is involved in protein folding, degradation, targeting, translocation, and protein complex remodeling. In this study, the mRNA expression level of the *Haliotis diversicolor* HSC70 (*HdHSC70*) gene was detected by quantitative real-time PCR in different tissues and under different stresses. The results showed that the *HdHSC70* gene was ubiquitously expressed in seven selected tissues. The highest expression level was detected in gills ($P < 0.05$). The expression level of the *HdHSC70* gene was significantly upregulated by thermal stress, hypoxia stress, *Vibrio parahaemolyticus* infection, and combined thermal and hypoxia stress. The upregulation occurred at the early stage of stress. These results indicated that the *HdHSC70* is an important component in the immune system of *H. diversicolor* and is involved in the early stress response. Meanwhile, 5'-flanking region sequence (2013 bp) of the *HdHSC70* gene was cloned; it contains a putative core promoter region, heat shock element, CpG, and transcription elements including NF-1, Sp1, Oct-1, interferon consensus sequence binding protein (ICSBP), etc. In HEK 293T cells, the 5'-flanking region sequence is able to drive expression of the enhanced green fluorescent protein (EGFP), proving its

promoter function. The promoter activity increased after high-temperature treatment, which may be the immediate reason why the expression of the *HdHSC70* gene was significantly upregulated by thermal stress. After the ICSBP-binding site was mutated, we found the luciferase activity significantly reduced, which suggested that the ICSBP-binding site has a certain enhancement effect on the activity of the *HdHSC70* promoter.

Keywords *HSC70* · Pathogen · Hypoxia · Thermal · Transcriptional regulation · *Haliotis diversicolor*

Introduction

As a species of marine gastropod mollusks, the small abalone *Haliotis diversicolor* is one of the most important cultured abalones in the coastal provinces of southern China (Ke and You 2011). However, due to the deterioration of its living environment, the natural resources of the abalone are greatly reduced (Fujino et al. 1992). Although this species has a higher tolerance to high temperatures compared to other species of abalone (Ke et al. 2000; Liu et al. 2008), water temperature is still one of the important factors that affects the growth of *H. diversicolor*. High temperature in seawater has resulted in changes in the respiration and metabolic rates in marine benthic organisms (Wang et al. 2012). In addition, *Vibrio parahaemolyticus* has become a main pathogen of cultured *H. diversicolor* in summer, which causes the outbreak of mass mortality of cultured abalone (Cheng et al. 2004; Liu et al. 2000).

Heat shock proteins (HSPs) are a group of highly conserved proteins that act as molecular chaperones in almost all living organisms by preventing protein aggregation and promoting orthometric refolding of denatured proteins during

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environmental stress (Cheng et al. 2015; Shen et al. 2016). These proteins represent a predominant self-protecting mechanism adapting to extreme changes in temperature and can also be induced by various stress factors, such as starvation (Jiang et al. 2012), chemical exposure (Shu et al. 2011), and heavy metal pollution (Shi et al. 2016). Based on sequence similarity and molecular size, five major HSP families have been identified (HSP100, HSP90, HSP70, HSP60, and the small HSPs) (Gu et al. 2012; Shen et al. 2016). Among them, the HSP70 family is one of the most highly conserved proteins and participates in various cellular processes, including protein translocation, cell cycle, apoptosis, and degradation of denatured proteins (Daugaard et al. 2007; Thanaphum and Haymer 1998). This family is expressed in two different patterns (Boorstein et al. 1994; Karlin and Brocchieri 1998). The first group is heat shock protein 70 (*HSP70*), which expresses itself with a very low level under non-pressure conditions (Bahar et al. 2013), but its transcription and translation are quickly induced by a hundred-fold under many kinds of stress (Choi et al. 2014; Mahroof et al. 2005). In contrast, another group, heat shock cognate70 (*HSC70*), is constitutively expressed at high levels in normal conditions. It has been reported that the *HSC70* gene does not change under exposure to stress such as hot or cold shocks, but it has also been reported that the *HSC70* gene expression patterns vary in response to different kinds of stress conditions, such as thermal, starvation, and heavy metal exposure (Chuang et al. 2007; Sun et al. 2016a, b). However, to date, it has not been reported whether the expression of the *HSC70* gene in abalone is induced by various types of stress or not.

As the most important link in the process of gene expression, the transcriptional regulation of eukaryotes mainly regulates the initiation of gene transcription and the intensity of gene expression through the interaction of the promoter with the corresponding transcription factors (Bansal et al. 2014; Grünberg and Hahn 2013). At present, the transcriptional regulation of HSPs in mammals (Deb et al. 2013), plants (Gong et al. 2009), fish (Li et al. 2012), and many other species has been studied. However, the transcriptional regulation of *HSC70* has not been well characterized, especially in animal cells. In aquatic animals, Chuang et al. (2007) studied the *HSC70* promoter of tiger shrimp and confirmed the essential role of the core promoter region, the perfect heat shock elements (HSE) and the near CAAT box in the transcription. However, that article has not reported any transcription factors related to the promoter. Zhao et al. (2013) studied the *Litopenaeus vannamei* *HSC70* promoter and found that deletion of the NF-kappaB binding site resulted in a significant increase in the expression of the reporter gene. However, to date, no studies have been done on the transcriptional activation or regulation of *HdHSC70*. In general, the presence of transcription factor recognition sequences in the 5' untranslated region (5'-UTR) of the target messenger RNA (mRNA) is

responsible for regulation of transcription of the target gene. Defining the *cis*- and/or reactive components of 5'-UTR is a necessary first step in understanding the mechanism of molecular transcriptional control (Li et al. 2012).

In our preliminary experiments, we observed that the expression level of *HdHSC70* was upregulated by heat shock. Based on current knowledge and our observations, we hypothesized that (1) the expression of the *HdHSC70* gene may be induced not only by high temperature but also by hypoxia and infection of *V. parahaemolyticus*, (2) single stress and two stresses may stimulate different expression of *HdHSC70*, and (3) the change of *HdHSC70* expression levels is related to the transcriptional regulation. Testing these hypotheses will lead to a better understanding of the *HdHSC70* gene expression pattern and its regulatory mechanisms in abalone.

Materials and methods

Animals and preparation of samples

Adult small abalones (body length 5.18 ± 0.50 cm, weight 15.7 ± 2.50 g) were purchased from the Peiyang abalone farm (Xiamen, China) in August 2015. These abalones were fed with sea tangle once a day and maintained in recycling systems at a temperature of 25 °C and dissolved oxygen (DO) of 6.2 mg/L. They were held for 8 days to be ready for the experiment.

At least six healthy small abalones were dissected for different tissues. Hemocytes (He) were collected by cutting off the foot quickly and were isolated by centrifugation at $2000 \times g$ at 4 °C for 10 min from hemolymph. Soon afterwards, hemocytes were immediately stored in liquid nitrogen until being used for RNA isolation and quantitative real-time PCR (qRT-PCR). Hepatopancreas (Hp) and gills (Gi), mucous gland (Mg), digestive tract (Di), mantle (Ma), and muscle (Mu) were immediately stored in liquid nitrogen until being used for RNA isolation and qRT-PCR.

Animals were randomly divided into four groups: (1) thermal stress group: according to the method of Huang et al. (2013a, b), the stress condition was set to temperature (T_m) = 31 °C. The temperature of the control group was set at 25 °C. The temperature of the experimental group was set at 31 °C which was achieved by increasing from 25 °C by 1 °C per hour. When the temperature reached 28 °C, it was set as the first phase and 31 °C as the second phase. Four, 24, 96, and 192 h at 31 °C were set as the 3,4,5,6 phase, respectively. (2) Hypoxia stress group: according to the method of Cai et al. (2014), the stress condition was set to dissolved oxygen (DO) = 2 mg/L. Samples were taken at 4, 24, 96, and 192 h, respectively. (3) Bacterial challenge group: the injection concentration was determined according to the method of Sun

et al. (2016a, b). All individuals were injected with 50 mL live *V. parahaemolyticus* in 0.9% NaCl (1.1×10^8 cfu/mL) into the foot muscle, with the control injected with 50 mL of 0.9% NaCl. Samples were taken at 0, 4, 12, and 24 h. (4) Thermal and hypoxia combined stress group: according to the method of Zhang et al. (2014), the stress conditions were set to $T_m = 30^\circ\text{C}$ and $\text{DO} = 4$ mg/L. At the beginning of the joint stress experiment, DO content of water was 4 mg/L, and 30°C was achieved by increasing from 25°C with 1°C per hour. The temperature of 30°C was maintained and gills were sampled at 0, 4, 24, 96, and 192 h. At least six abalone gills were sampled at different time phases in all four group experiments.

Determination of *HdHSC70* expression after stresses

Total RNA was extracted using total RNA Kit II (Omega, USA) according to the manufacturer's protocol from different tissues of all samples. The complementary DNA (cDNA) was synthesized in a system including 1 μg total RNA and 2 μL 10 mM random primers by M-MLV reverse transcriptase (Promega, USA). The synthesized cDNA was stored at -20°C until use.

qRT-PCR was carried out in LightCycler 480 Roche real-time thermal cycler in accordance with the manual with a 20- μL reaction volume containing 9 μL of 1:100 diluted original cDNA, 1 μL of $10\times$ SYBR Green Master Mix (Promega, USA), and 0.5 μL of each primer (10 mM). The comparative CT method ($\Delta\text{CT} = \text{CT of the target gene minus CT of the } \beta\text{-actin gene}$ and $\Delta\Delta\text{CT} = \Delta\text{CT of any sample minus calibrator sample}$) for the relative quantification of gene expression was used to calculate the relative expression level of all these genes. Six biological replicates were tested, and each sample was assayed in triplication. The *t* test was used to determine the difference in the mean values among the treatments. The difference was considered as significant when $P < 0.05$.

Cloning of the 5'-flanking regions of the *HdHSC70* gene

The 5'-flanking regions of the *HdHSC70* gene (GenBank accession number FJ812176.1) were isolated using the Tail-PCR and Universal Genome Walker Kit (TaKaRa, Japan). The primer sequences used in this study are listed in Table 1. PCR products were then purified and cloned into the pMD19-T simple vector (TaKaRa, Japan) and sent for sequencing at Sangon (Shanghai, China). The sequences were analyzed using the online transcription element search software Alibaba2 (<http://www.gene-regulation.com/pub/programs/alibaba2/index.html>).

Generation of reporter plasmid constructs

To analyze the *HdHSC70* basal promoter activities, multiple promoter fragments of the *HdHSC70* gene were generated by

PCR and cloned into the pGL3-Basic luciferase reporter vector. First, the universal reverse primers were used in combination with different forward specific primers to generate DNA fragment and cloned into pMD19-T simple vector (TaKaRa, Japan). Second, the promoter fragment constructs were digested with Kpn I and Xho I and subcloned into Kpn I/Xho I-cut pGL3-Basic reporter vector. Finally, all plasmid constructs were verified by sequencing and purified with an E.Z.N.A.TM Endo-free Plasmid Mini Kit (OMEGA, USA) for transfection.

After determining the transcription factor that may play an important role, the binding site of this transcription factor is mutated and the plasmid is constructed. The method is consistent with the previous method.

Transfection and luciferase assays

HEK 293T cells were routinely cultured in DMEM high glucose medium. Transfection experiments were performed in 48-well culture plates. Briefly, 24 h before transfection, recipient cells were seeded into wells at a density of $1\text{--}3 \times 10^5$ cells/well. The cells were transfected with 1 μg of reporter construct DNA and internal reference plasmid in 50 μL medium without FBS per well using 1 μL Lipofectin 2000 (Invitrogen) according to the manufacturer's recommendations. At 24 h post transfection, EGFP (enhanced green fluorescent protein) expression was observed using an inverted fluorescence microscope.

After transfection, each cell sample was suspended in 60 μL of $1\times$ Passive Lysis Buffer (PLB). After centrifugation, the supernatant of each sample was taken as 15 μL . The firefly and Renilla luciferase activities were measured by the Dual-Luciferase[®] Reporter Assay System (Promega, Shanghai, China) with a tube luminescence meter (Berthold Technologies, Germany) reader. The ratio of luciferase activity and the luciferase relative activity were calculated. All the data were obtained from three independent transfection experiments performed in triplicate.

Results

Expression of the *HdHSC70* gene

The expression of *HdHSC70* was assessed by qRT-PCR in different tissues from healthy small abalone. *HdHSC70* was expressed in all examined tissues with high levels in Gi, He, and Hp ($P < 0.05$) (Fig. 1). Because the gill is a very important site of pathogen entry and the highest expression level of *HdHSC70* was detected in Gi, the Gi was set as the experimental tissue in a separate stress group.

For the thermal stress group, the expression level of *HdHSC70* in the Gi was significantly higher (~ 15 -fold) than the control group at the temperature of 31°C (0 h) ($P < 0.05$), and then, the expression decreased rapidly (Fig. 2).

Table 1 Primers used in this study

Primer name	Primer sequence (5' → 3')	Used for
hsc70-r1	TCCATCGGCTGAGAAGACTG	Genome Walking and Tail-PCR
hsc70-r2	CAGGAAGGTCAAGGCGGTTC	
hsc70-r3	CGATAACGAGATTTGCTAAACTGG	
hsc70-r4	ACATCATCGGCTACACACGG	
hsc70-r5	GCAAGTCAAAAGTCATTCCG	
c-70-1	CGGGGTACCATAAGTGATAAAGCCGTTGTTGC	Hsc70 promoter activity
c-70-2	CGGGGTACCGTCTGACCCATTGTTGATGCGA	
c-70-3	CGGGGTACCTCACGAGATGTGTACGGGAGT	
c-70-4	CGGGGTACCTTATTGCTGTGAGAGCGGCGTTTG	
c-70-5	CGGGGTACCTGACTCCAAACAAGTGACTACCT	
c-70-6	CGGGGTACCCACAACCTGAAGAGAAACTGAA	
c-70-7	CGGGGTACCCATCGGCTGAGAAGACTGT	
c-70-r	CCGCTCGAGTCTTCTCTGCCCTCCGCTTC	
c-70-r1	CCGCTCGAGCAGTCTTCTCAGCCGATGGA	
c-rt-f	TTGATCTTGGCGGTGGTACA	Real-time quantitative PCR
c-rt-r	CTGGGTGCTGGAGGAAAAGTG	
b-actin F	CCGTGACCTTACAGACTACCT	
b-actin R	TACCAGCGGATTCCATAC	

For the hypoxia stress group, the expression of *HdHSC70* in the Gi was significantly higher (~ 10-fold) than the control group at 4 h after stress ($P < 0.05$), and then, the expression level of *HdHSC70* decreased rapidly (Fig. 3).

For the *V. parahaemolyticus* infection group, the expression of *HdHSC70* in the Gi was significantly higher (~ 4-fold) than the control group at 4 h after injection ($P < 0.05$), and then, the expression level of *HdHSC70* decreased rapidly (Fig. 4).

For the thermal and hypoxia combined stress group, the expression of *HdHSC70* in the Gi was significantly higher than the control group at 4 and 96 h after stress ($P < 0.05$) (Fig. 5).

methods. The core promoter, transcription initiation site, CpG island, and potential transcription factor binding sites were predicted using the software NNPP, TFSEARCH, and MethPrimer. The position of the transcription initiation site (TSS) was defined as one, in the region of -40 ± 10 bp containing the putative core promoter region. The promoter of the *HdHSC70* gene has a TATA box between -30 and -32 bp upstream of the transcription initiation site (A) and has a CpG island with a length of 108 bp and has many transcription factor binding sites, such as ATF, NF-1, TBP, Sp1, Oct-1, C/EBPalp, and many HSE.

5' upstream sequences of the *HdHSC70* gene

The 5' flanking sequence (2013 bp) of the *HdHSC70* gene was obtained (Fig. 6) by Tail-PCR and Genome Walker

Activity of the *HdHSC70* promoter in vitro

To identify the promoter activity of the *HdHSC70* gene, the complete 2013 bp 5'-upstream region was inserted into the

Fig. 1 Expression level of *HdHSC70* in different tissues of *H. diversicolor*. He hemocytes, Ma mantle, Gi gills, Mu muscle, Hp hepatopancreas, Mg mucous gland, Di digestive tract (The different letters on the error bars represent significant differences, $P < 0.05$)

Fig. 2 Expression level of the *HdHSC70* gene after thermal stress in Gi. 1 (28 °C), 2 (31 °C, 0 h), 3 (31 °C, 4 h), 4 (31 °C, 24 h), 5 (31 °C, 96 h), 6 (31 °C, 192 h). The significant difference between the challenged and the control group is indicated by a (*) at $P < 0.05$

pEGFP-1 vector (pEGFP-Hsc70) and used to drive expression of the EGFP gene in HEK293T cells. Under a fluorescence microscope, EGFP fluorescent signals could be observed (Fig. 7).

To identify the core promoter region of the *HdHSC70* gene, two constructed reporter plasmids were prepared and transfected into HEK293T cells; the one containing the complete 2013 bp 5'-upstream region was named pGL-c70-1r, and the one removing the core promoter region was named pGL-c70-1r1. The activity of pGL-c70-1r was significantly higher than pGL-c70-1r1 and negative control (pGL3-Basic, plasmid without inserts) ($P < 0.05$). The pEGFP-N1 promoter was used as a positive control; a high fluorescence activity as expected was detected. No green fluorescence protein

Fig. 3 Expression level of the *HdHSC70* gene after hypoxia stress in Gi. The significant difference between the challenged and the control group is indicated by a (*) at $P < 0.05$

Fig. 4 Expression level of *HdHSC70* after injection of *V. parahaemolyticus* in Gi. The significant difference between the challenged and the control group is indicated by a (*) at $P < 0.05$

expression was detected in pEGFP-1 which was the negative control (Fig. 8).

To determine if expression of the luciferase reporter driven by the *HdHSC70* promoter was also induced by heat shock, HEK293T cells were exposed under high temperature stress. Thirty-seven, 39, and 42 °C were selected as the induction temperature; the treatment time was 40 min. We found that under the induction of 39 and 42 °C, luciferase activity was significantly increased ($P < 0.05$), and the activity in 39 °C was the highest, and significantly higher than in 37 and in 42 °C ($P < 0.05$) (Fig. 9).

In order to identify important transcription factor binding sites in the *HdHSC70* promoter region, we amplified a continuous truncated promoter fragment of the *HdHSC70* gene by PCR. The truncated 5' flank regions of *HdHSC70* were cloned into luciferase reporter vectors (named as pGL-c70-1r, pGL-c70-2r, pGL-c70-3r, pGL-c70-4r, pGL-c70-5r, and pGL-c70-6r). The constructs were used to transfect into HEK293T cells. All truncated promoters had detectable activities compared with the control (pGL3-Basic, plasmid without inserts). There was a significant difference between pGL-c70-5r and pGL-c70-6r ($P < 0.05$) (Fig. 10).

The difference between pGL-c70-5r and pGL-c70-6r is the part of -252 to -103 bp. There are many predicted transcription factor binding sites in this region, such as interferon consensus sequence binding protein (ICSBP), Oct-1, Cos, and Sp1. Since there is only one ICSBP binding site in the promoter fragment of *HdHSC70*, we hypothesized that ICSBP was an important transcriptional binding factor for *HdHSC70* and decided to mutate it. The binding site of the transcription factor ICSBP is AAGAGAACT, and the recombinant plasmid was named as pGL-wt. When we mutated the AG into TC, the sequence becomes AAGTCAAAC, and the mutant recombinant plasmid was named as pGL-mut. The

Fig. 5 Expression level of *HdHSC70* after both thermal and hypoxia stress in Gi. The significant difference between the challenged and the control group is indicated by a (*) at $P < 0.05$

results showed that after the ICSBP-binding site was mutated, the luciferase activity was significantly reduced ($P < 0.05$) (Fig. 11).

Discussion

HdHSC70 mRNA expression in tissues

Studies of the heat shock protein 70 family have confirmed its importance in protein metabolism under normal physiological conditions and in cellular protection under stress conditions (Wang et al. 2013a, b), indicating that the study of the distribution of *HdHSC70* mRNA in different tissues would help us understand the mechanism of its response to stress. qRT-PCR results showed that the *HdHSC70* gene was expressed in all examined tissues with highest expression in Gi, He, Hp, especially in Gi (Fig. 1). Gi is the main structure of the suspension filter, and as a respiratory organ, it is directly stimulated by a variety of environmental stresses. It has been reported in mollusks that He and Hp play an important role in the innate immune system (Gueguen et al. 2003; Song et al. 2011). Therefore, compared with other tissues, Gi, He and Hp may be more sensitive to environmental stresses. Many of the immune-related genes such as *AbNF-kB* and *SaAkin2* reported in the *H. diversicolor* also have higher expression levels in Gi, He, and Hp (Zhang et al. 2014).

HdHSC70 mRNA expression after stresses

The general pattern of the *HSC70* gene expression is actively expressed in normal cells, but is not influenced by stress. Zhang et al. (2010) and Yoshimi et al. (2009) have reported that expression of *HSC70* in corn earworm and Chironomidae did not increase under cold, heat stress, or exposure to heavy metals. However, some researchers have

reported that it can also be induced. Sun et al. (2016) and Chuang et al. (2007) found that in *Apolygus lucorum* and *Penaeus monodon*, the *HSC70* expression was significantly higher under high temperature stress. Interestingly, Shim et al. found the expression of *HSC70* in *Tetranychus urticae* was not increased under cold or heat stress, but significantly increased in response to starvation (Shim et al. 2006). These different results indicate that the expression of *HSC70* is plastic, which may depend on the type, intensity, and duration of environmental stresses. The *HdHSC70* also appears to possess a stress-associated role. Our experimental results showed that whether after hypoxia stress, *V. parahaemolyticus* injection, or combined thermal and hypoxia exposure, the *HdHSC70* was expressed at a significantly higher level compared to the control group at some time phases. Based on the function of the *HdHSC70* gene, the transcriptional upregulation related to stresses suggests that *HdHSC70* is an important factor in cell protection and survival in *H. diversicolor*.

How organisms cope with rapid changes in their environment is a major question in biology. We found that elevated expression of *HdHSC70* was detected at the early stage of stresses exposure, so we assume that the *HdHSC70* was involved in the early stress response. Li et al. obtained a similar result that *HSC70* mRNA expression level in most tissues was rapidly upregulated after bacterial infection in *Schizothorax prenanti* (Li et al. 2015). During stress, large amounts of *HSC70* mRNA might be synthesized instantly to protect against damage, resulting in a big increase in *HSC70* mRNA level compared to the basal expression. Additionally, *HSC70* is commonly accepted as a biomarker for different classes of environmental stresses assessments in various animal species (Mukhopadhyay et al. 2010). Based on this fast and sensitive response, *HdHSC70* is appropriate to be a biomarker for environmental stresses in *H. diversicolor*.

Fig. 6 Nucleotide sequence of the 5' flanking region of the *HdHSC70* gene. Intron regions are shown in italic. Positions of potential binding sites for transcription factors are underlined. The overlapping binding sites are shaded. The transcriptional site and start codon are blacked

ATAGTGATAAAGCCGTTGTTGCCAAGCAACAGATCACTTGTCTTAATCAGCATTGAGAAACAGATGCTTTGAAACAAATGCAGC -1914
 CAGATTAGGTTTGTGAATGTTGAAAGATAAGACCGCCATTGACTCGGCGTTTATTACAGCAATCTAATCTTAATTAATGAATT -1829
 CCTTTATTAATATCAAAAGGATGGCTAATGTCTTATGGCAGACATTACGTCATTGCTGACCCATTGTTGATGCGAACGGAT -1744
 TAGTGAGTACTGAGCCCTGGATACATGTAGAAATTCCTTAATTAATCAATCTGGAGATGATCGGTCGTTATTCAAGAGAATAACT -1659
 NF-kappaB
 ATTGACAGACCTGTTTGAACGGTTTTCAGAGAGTTGGGAGTGGTGATAAAGGGGGTGGGGTGGCGGGGTGTGTCTTATT -1574
 TTGTGAAAAATTGACTAAGCCACCCACTAACCCTACCCCGCTACTTCCACCACTTTTCAATTTATCTCTATGACAGC -1489
 TGGACATGCCCTGCATGATTTTCATAAATGCAAGTCAAAAGTCATTCCGAAGTCACGAGATGTGTACGGGAGTGACAACGCATA -1404
 CTAATACACATCATCGGCTACACACGGAAATTCCTCAATCACTCGGTCATTCCGGGTGATGCTCGGAGAAAGCGACGATAA -1319
 NF-kappaB HSE
 CGAGATTGCTAACTGGAATCTGTGTAGTATGCGCAAAATATTGGGACGACAGTGTCCACAAAGTCTTGGGTAATGGTCATGCG -1234
 CTGAAGACTCTGACCTGTTCTAAAAATAGAAATGGCGGGAATCAGACAAAGACCGGTGCACCTTGGCCCGGAGAAAACTGTTGG -1149
 NF-kappaB
 TGAAGCTTCATATATTCAAGCAGTGTACCGCTAGTCACAGGACACAGTTCGATTATCAACACCATCTGGGGAATGCCATTA -1064
 HSE
 CACACACATTAATATATAGTACTGTTTACTTATATGGTCTCTGTTATAATCATTTCAAATCATATCAACACCTTTCAAATGTT -979
 TTGAATATTTGAATACACATACACAATGCAGAGATCGATCTATGAATGAAAGTATGACAATATATGGATGAACAAGTCTTTAT -894
 TGCTGTGAGAGCGGCGTTTGTATAGTGTGTTGACGTGAAATCGTACCGAGGAATTTCACTTTAAACAATAAATTTAAAGTG -809
 Sp1 Oct-1 NF-kappaB
 AATCATATAAAGTGAAATATAGATTGCGAATCCTCTAGGTTTACCTTGCCAAATGAAATGTAATATCAGAAATAAATTA -724
 Oct-1 NF-1 Oct-1
 ATTAGGAAAAAAGTTATCACAATTACTGGTTGTATTGCAAGGGAGTAAGCACATTAATGACCACCGAGAACAGGACGGTTG -639
 GATA-1
 TTCAAAATTTAGTTAACGCTCATCAGAACTACAAGTGCATTGTTCTCTGTTCACAGAGACCATGTAATATTAATTAATTC -554
 GATA-1
 GTAGGTCATTTACGTCGACATGAGTATCTATAGGATCACTTCTGGTATATAAATATTACAAAGAAATATAGTTTCGTTAGAGGT -469
 Oct-1
 TTCTTTGTGCGAGTGTTTAGAGAACTCACCTTTCTAACAAAAAGTTTCTACTACATTACGTGCTCGATCACTCTAAACTGTCTC -384
 GCTTTATCATAAATTTCTAGAAATGTTTACTCCAAACAAGTACTACCTTTAGTCTCTTAATATCCGTAATATTGACAACAA -299
 GATA-1 HSE
 TAATTCTCATTTAAATGAAAGGAGCCCAAGTATGATCAGAGATTCACAACCTGAGAGAAATCTGAACCTGGGGAATCTAAAA -214
 OCT2.1 HSE ICSBP Oct-1
 CATTAGAATTGCAGGAAGGTCAAGGCGGTTTCAGCGCATGTGACATGCAACCGAAGCTGAATGGTATTAAACAACCAATCGCTGC -129
 CoS Sp1 Oct-1 Cp1
 GTGTTTGTCTTCGACCAATTAAATCCATCGGCTGAGAAGACTGTTACCTAGTTTCAGGGTTCTCGAACGTTCTAAATGACGTC -44
 Cp1 HSE Hsf
 ACATCCGCAATTATATATAAGCTGCACCACGCTAAACCCGGTAATAACGACAGGTCATCAAGTTGGAAGCGGAGGGCAGAGAAG -42
 TATA box Core promoter region
 ACGTTCAAGCTTCCTTTAAGACTCTTACAGACAACTTACTCAAGG TATGTAACATAAAGCTTCTGGTGTGAAATTTGTTCTTA 127
 AATGCAAAAGACCTACCGTGGAAATCTGAATTTGCGTTTTCATTGATGATACCGGTCCTCGGCGCACACGCTGGTGTGACTGC 212
 AACATGGCGAATTTCTCTTTTGGAGGGTGAATATGCTCTTTATGACCGATCTCGATAGTTAAGCGTTCAGATTGCTTGAACCT 297
 GAAATGTAAAGAGTATCAGGAACATTTGATGGTTTGTATCGTTTACGTTTAAAGATGATAGTGAACCTGTGAAACACAAAA 382
 GATTCCCGATCGATTTTCTTTCTTCCGCTTCCATTTCGTATCCGACAGGTTAATATTTTGTACTGGGTTTACCGTGATGATA 467
 TGAACCTCTGCTTAACCTATAAGTAAATCCCGCTTAAATTTGGCGATAACCGATCTTTGATCGTTTCAAAATTTCAACGA 552
 AACGTGACGGGCGGTGTCACATTATCAGTGGTGGCGGATTCGGGATAATGAGGAGTCGATTGTAGACTACTAGTGTGCTTAA 637
 ATGAATACCTAATCATTCAATTTGTTAAAGTGTACCTCTCGAGAATTTTACAAACGACGATTTTGTCTCTCAAGAGACCGAGAT 772
 TTTTCAAAATGAAAGTAGCTTAGAAGCTTCCATTAGTCTAAGCGGAAGCTAAACATAAAGCTGACTGTGATGTAATCTATT 807
 TTCCATTATCAGGACatgccaaggcaccagctattggtatagatctgggg 860
 M A K A P A I G I D L G

In the high temperature and hypoxia combined stress group, the expression level of *HdHSC70* gene was significantly increased at 4 h, then decreased rapidly at 24 h, and then increased significantly again at 96 h. The expression of HSP in the organism after stress enables the cells, tissues, and even the whole organism to obtain a higher resistance to stress. However, as the organism is unable to continue to cope with the stress, it will once again start the expression of heat shock-related genes (And and Robertson 2003; Wang et al. 2006). We speculate that the abalone cannot adapt to double stresses for a long time, since *HdHSC70* has a significant increase at 96 h.

The *HdHSC70* 5' upstream sequence

The heat shock element (HSE) is necessary for transcriptional activation of eukaryotic HSP genes, and it has some characteristics of enhancers (Karn et al. 2011). The sequence of HSE is highly conserved, which is based on the core sequence containing five nucleotides (-nGAAn-), where n represents nucleotides that are conservatively low but very important in evolution. Usually, HSE contains at least two (-nGAAn-), and the monomer is attached in the form of -nGAAn nGAAn- or -nTTCnnGAAn- (Zhao et al. 2011).

Fig. 7 EGFP expression of the *HdHSC70* promoter in HEK293T cells at 24 h post transfection with pEGFP-Hsc70 which used the *HdHSC70* full-length promoter (A and a), pEGFP-N1 as a positive control (B and b), and the promoter-less pEGFP-1 as a negative control (C, c). Fluorescent fields are shown in (A, B, and C), and bright fields are observed in (a, b, and c) separately

The 5' regulatory region of the *P. monodon HSC70* gene contains a long HSE with three -nGAAn-, and the HSC70 mRNA expression was induced after the heat shock by about 8-fold (Chuang et al. 2007). The 5' regulatory region of the *Crassostrea hongkongensis HSC70* gene contains six HSEs, and the HSC70 mRNA expression can be induced about 20-fold after heat shock (Zhang 2010). There is no canonical correlative rule between the number of HSE and the degree of induced expression by a heat shock. However, increasing the number of HSE more or less may enhance the expression of the heat shock protein (Amin et al. 1987; Brade et al. 2000). The 5' regulatory region of the *HdHSC70* gene contains five HSE elements; this may be related to the *HdHSC70* mRNA expression that was induced after the heat shock by about 15-fold. On the other hand, the results from Amin et al. (1987) showed that the intensity of *HSP70* initiation depends on the spatial arrangement of HSE and TATA boxes, the number of HSEs, and the location of HSE in *Drosophila melanogaster*. Therefore, although the number of HSEs can influence the activity of the promoter, it is not the sole factor that determines the activity of the promoter. More experiments are needed to explore the relationship between HSE and *HSC70* gene expression.

The TATA box is one of the most important binding sites in the eukaryotic polymerase II promoter and is one

of the most common motifs in the promoter (Wang et al. 2004). In transcription initiation, transcription factors TF2 and TATA frames form stable complexes and then bind to DNA in a certain order by other transcription factors to form a transcription initiation complex and then start transcription (Zhang et al. 2010). The TATA box is an important component in the core promoter region. *Branchiostoma belcheri HSP70a* (Li et al. 2012) and the human *HSP70* gene (Hunt and Morimoto 1985) core promoter region contained a TATA box. We found that the *HdHSC70* core promoter region also contained a TATA box. When we deleted the core promoter region, the promoter activity was significantly reduced, inextricably linked to the absence of the TATA box.

In vitro expression assay of the *HdHSC70* promoter

The HEK293T cell line has been widely used in vertebrate promoter functional analysis. For example, Kuang et al. (2015) and Wang et al. (2013) used the cell lines to study the human miR-1908 promoter and goldfish two kiss promoters. In invertebrates, Huang et al. used 293 cell lines to study the *Pinctada fucata* IRF-2 promoter (Huang et al. 2013). Because there is no stable *H. diversicolor* cell line

Fig. 8 Analysis of the relative activity of the *HdHSC70* gene containing and removing the predicted core promoter region of the plasmid. The significant difference is indicated by an asterisk at $P < 0.05$ as compared with the control (basic)

Fig. 9 Changes of *HdHSC70* promoter activity in HEK293T cells (The different letters on the error bars represent significant differences, $P < 0.05$)

available now, the HEK293T cell line was used for the promoter assay in this study.

In general, in order to further characterize the function of the gene promoter, the detection of the promoter activity and the determination of the transcription initiation site are carried out. The activity of the complete 2013 bp promoter in this study was verified using the fluorescent expression on the transfected cells with the promoter-EGFP vector (Fig. 7). The luciferase activity with the promoter-luciferase vector was used to identify the core promoter region of the *HdHSC70* gene (Fig. 8). In previous studies, many researchers used the same method to study gene promoters, for example, the research of the *P. monodon* and *L. vannamei* *HSC70* promoter (Chuang et al. 2007; Zhao et al. 2013).

The *HdHSC70* promoter was regulated by high temperature stress

The heat shock protein gene promoter is an inducible promoter. Under high temperature or other stresses, the activity of the promoter will increase and can significantly increase the transcription level of the gene (Pei et al. 2007; Yi et al. 2006). Chuang et al. used Sf21 cells to study the *HSC70* promoter of tiger shrimp (*P. monodon*) and found that the activity of luciferase increased significantly after virus infection (Chuang et al. 2007). Zhuang et al. (2011) used the BmN cell line to study the *Bombyx mori* *HSP70* promoter and found that the activity of luciferase was significantly increased after heat treatment. Our result was consistent with their reports: a significant increase in luciferase activity of *HdHSC70* after

Fig. 10 Promoter activity analysis of the serial deletion constructs from the 5'-flanking region of the *HdHSC70* gene. The relative luciferase activity is presented as the average firefly luciferase activity normalized

to the Renilla luciferase activity. Six plasmids showed positive but variable promoter activities ($P < 0.05$). The significant difference between pGL-c70-6r and pGL-c70-5r is indicated by a (**) at $P < 0.01$

Fig. 11 Activity analysis of the site-directed mutation plasmid pGL-mut-ic. Plasmid pGL-mut-ic was constructed by mutating the putative ICSBP-binding site from pGL-c70-5r. The pGL3-Basic plasmid was served as a

negative control (The different letters on the error bars represent significant differences, $P < 0.05$)

treatment of HEK 293T cells at 39 and 42 °C (Fig. 9). Our findings indicated that the activity of *HdHSC70* promoter can be increased by high temperature stress, which is consistent with the increase of *HdHSC70* mRNA expression under stress.

However, our result showed that the *HdHSC70* promoter activity at 42 °C was lower than at 39 °C (Fig. 9). In the study of human *HSP70B* promoter, Li et al. (2014) explored the thermal induction conditions in human breast cancer MCF-7 cells and found that cell viability decreased significantly when the cells were induced at 42 °C for 40 min. This may explain why the temperature is higher but the activity is reduced.

Serial deletions and mutation on the activity of the *HdHSC70* promoter

During gene transcriptional regulation, the promoter controls downstream gene expression under the synergistic effect of transcription factors, which contain positive and negative regulation. Shao et al. studied the proximal promoter activities of the ajarginase gene in *Apostichopus japonicus* and found that the transcription factor NF- κ B/Rel and STAT5 could significantly inhibit the ajarginase promoter activity (Shao et al. 2016). Zhao et al. have studied the *HSC70* gene of *L. vannamei* and found that NF- κ B could significantly inhibit the expression of *HSC70* (Zhao et al. 2013). By studying these transcription factors that play a key role in regulation, we can provide a theoretical basis for further understanding of the promoter regulatory mechanism. In our study, the pGL-c70-6r plasmid decreased the promoter activity to a significantly low level ($P < 0.05$) (Fig. 10). It indicated that the deleted site (– 252 to – 103 bp) has an important role in the basic transcription of *HdHSC70* promoter and this location may contain an important transcription factor. After the ICSBP-binding site was mutated, we found the ICSBP-binding site in – 252 to – 103 bp has a certain enhancement effect on the activity of the *HdHSC70* promoter (Fig. 11).

Interferon consensus sequence binding protein (ICSBP) is a member of the interferon regulatory factor (IRF) family of

proteins that is expressed exclusively in cells of the immune system and plays an important role in cellular immunity (Wang et al. 2000). Some reports have provided evidence that ICSBP plays a critical role in modulating the immune response by influencing the differentiation and maturation of immune cells and by affecting the cytokine expression (Scheller et al. 1999; Tamura and Ozato 2002). *HSC70* can participate in immune function and help the body to stimulate the immune response to the environment. Therefore, we speculate that ICSBP is also involved in the body's immune response by enhancing the expression of *HdHSC70*. Interleukin-18 (IL-18) is an important cytokine in the regulation of immune responses. Studies have shown that there is an ICSBP-binding site located on the murine *IL-18* promoter, and it is involved in the upregulation of the *IL-18* gene transcription in murine macrophages (Kim et al. 2000). The result is similar to the effect of ICSBP on *HdHSC70*. However, the specific regulatory mechanism of ICSBP on *HdHSC70* remains to be studied in depth.

Funding information The work was supported by the Natural Science Foundation of China (No. 41176152, No. 41006105).

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