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c-Jun regulates the promoter of small subunit hemocyanin gene of *Litopenaeus vannamei*

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ABSTRACT

Hemocyanin (HMC) is a respiratory glycoprotein, which also plays multifunctional non-specific innate immune defense functions in shrimp. However, the transcriptional regulatory mechanisms of the hemocyanin gene expression have not been reported. In the present study, we cloned a 4324 bp fragment of small subunit hemocyanin (HMCs) gene of *Litopenaeus vannamei* including the 5′-flanking region, from upstream 2475 bp to downstream 1849 bp (exon 1-intron 1-exon 2) by genome walking method. Four deletion constructs were then generated and their promoter activity assessed using the luciferase reporter system. Interestingly, we identified an alternative promoter (+1516/+1849 bp) located in exon 2, which has stronger promoter activity than the full-length or the other constructs. Bioinformatics analyses revealed that the alternative promoter region contains two conserved binding sites of the transcription factor c-Jun. Mutational analysis and electrophoretic mobility shift assay showed that *Litopenaeus vannamei* c-Jun (Lvc-Jun) binds to the region +1582/+1589 bp and +1831/+1837 bp of the alternative promoter. Furthermore, overexpression of Lvc-Jun significantly increased the alternative promoter activity, while co-transfection with dsRNA-Lvc-Jun significantly reduced the alternative promoter activity of HMCs. Taken together, our present data indicate that the transcription factor Lvc-Jun is essential for the transcriptional regulation of the HMCs gene expression.

1. Introduction

Shrimp and shrimp products are a rich source of important animal proteins essential for human. According to data from the Food and Agriculture Organization, world shrimp culture expanded from 154,515 tonnes in 2000 to 3,668,681 tonnes in 2014 [1]. Despite the significant growth in shrimp farming, it is still grappled with bacterial and viral diseases, resulting in high mortality and huge economic losses [2–5]. There is therefore the urgent need of methods and/or strategies for predicting and preventing shrimp diseases [6]. Shrimp like other invertebrates do not have an adaptive immune system, but rely on their cellular and humoral innate immune system to respond to pathogens [7–9]. It is for this reason that many researchers have focused their attention on trying to understand the innate immune system of shrimp so as to be able to develop novel methods for disease prevention [10–12].

Hemocyanin is a large, copper-containing respiratory glycoprotein present in the hemolymph of both mollusks and arthropods [13–15]. Several studies have shown that apart from its respiratory function, hemocyanin is also a non-specific innate immune defense molecule [16]. It could be converted into a form with phenoloxidase activity [17,18], antimicrobial [19–21], antiviral [22,23] and hemolytic [24] properties. Given the varied and numerous functions of hemocyanin in shrimp physiology and innate immunity, researchers are interested in exploring and understanding the molecular basis behind this multifunctionality. It has been documented that *Litopenaeus vannamei* hemocyanin had extensive single nucleotide polymorphisms (SNPs) in the C-terminal and alternative splicing in the small subunit [20,25], as well as several variants sequence of the large hemocyanin subunit [26–28]. However, the transcription regulation mechanism underlying hemocyanin multifunctionality remains unknown.

The transcription factor c-Jun is a member of the AP-1 transcription factor family, a dimeric complex that includes the JUN, FOS, ATF and MAF protein families, with Fos (c-Fos, ForB, Fra1 and Fra2) and Jun (c-Jun, JunB and JunD) being the main AP-1 proteins [29]. It has been shown that c-Jun combines with c-Fos to form the AP-1 early response transcription factor [30]. For instance, Isern et al., have reported that c-Jun and c-fos were rapidly induced to activate the viral immediate-

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early genes during human cytomegalovirus (HCMV) infection [31]. In shrimp *L. vannamei*, c-fos (Lvc-fos) is reported to interact with c-Jun (Lvc-Jun), therefore playing a role in bacterial infection by activating the expression of antimicrobial peptides (AMPs) [32]. Although c-Jun forms a heterodimer with c-fos, Lvc-Jun functions as a transcription regulator in the form of a homodimer, and is phosphorylated by LvJNK in shrimp, thereby impacting on WSSV replication and viral gene expression [33]. From the foregoing, c-Jun is important in the regulation of pathogenic infections, suggesting that Lvc-Jun might also be involved in the regulation of hemocyanin.

In the present study, the 5′-flanking region of small subunit hemocyanin (HMCs) was cloned and the promoter region as well as the transcription factor binding sites (TFBSs) was characterized. It was found that HMCs gene has an alternative promoter located in exon 2. Importantly, the transcription factor Lvc-Jun could bind to the alternative promoter region thereby regulating the expression of the HMCs gene. The findings will assist in the study of the regulatory mechanism of HMCs and help to explain the molecular basis of hemocyanin's multifunctionality.

2. Materials and methods

2.1. Experimental animals

Penaeid shrimps *L. vannamei* (approximate weight of 5 g) were purchased from Shantou Huaxun Aquatic Product Corporation (Shantou, Guangdong, China). Shrimps were immediately transferred to tanks with aerated seawater at room temperature and acclimatized for at least 2 days before experiments. All animal experiments were carried out in accordance with the guidelines and approval of the Animal Research and Ethics Committees of Shantou University.

2.2. Extraction of genomic DNA

The genomic DNA of *L. vannamei* was extracted from muscle tissue with phenol-chloroform method as described by Lai et al. [34] with some modification. Briefly, shrimp muscle tissue was ground to fine powder in liquid nitrogen and lysed with lysis buffer (10 mmol/L Tris-HCl, pH 8.0, 5 mmol/L EDTA, 5% SDS, 0.5 mg/mL proteinase K). The lysate was incubated at 37 °C for 30 min, then treated successively with Tris-saturated phenol, phenol/chloroform/isopentanol (25:24:1) and chloroform/isopentanol (24:1). The genomic DNA was precipitated by adding two volumes of ethanol, washed twice with 75% ethanol, air dried and rehydrated with sterile water.

2.3. Cloning of the 5′ -flanking region of HMCs

The Universal Genome Walker™ 2.0 Kit (Clontech, USA) was used to clone the 5′-flanking region of HMCs. Briefly, genomic DNA was digested with four restriction enzymes (Dra I, Eco RV, Pvu II and Stu I), purified and ligated to Genome Walker adaptors to Genome Walker "libraries". The PCR was performed using adaptor primers (AP1 and AP2) and specific primers (GWSP1a and GWSP1b for the primary and secondary nested PCR of first genome walking, GWSP2a and GWSP2b for primary and secondary nested PCR of second genome walking, GWSP3a and GWSP3b for primary and secondary nested PCR of third genome walking) (Table 1) according to the user manual. For HMCs 5′-flanking region cloning, the conditions for the primary round of PCR were as follows: 7 cycles of 25 s at 94 °C and 3 min at 72 °C, 32 cycles of 25 s at 94 °C and 3 min at 67 °C, and an additional 7 min extension step at 67 °C after the final cycle. The conditions for the second round of PCR were as follows: 5 cycles of 25 s at 94 °C and 3 min at 72 °C, 20 cycles of 25 s at 94 °C and 3 min at 67 °C, and an additional 7 min extension step at 67 °C after the final cycle. The PCR products were resolved on an agarose gel after which the band was excised, purified and ligated to the pMD-19T Simple vector (Takara, Dalian, China). The ligated plasmid was transformed into *Escherichia coli* DH-5*α* competent cells (TransGen Biotech, Beijing, China) and positive clones confirmed by sequencing at the commercial company, Beijing Genomics Institute (BGI, Shenzhen, China).

2.4. Prediction of potential transcription factor binding sites

To predict the potential transcription factor binding sites on the 5′-flanking region of HMCs, the free online tools Match™ −1.0 Public (http://gene-regulation.com/pub/programs.html#match), PROMO (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3), JASPAR (http://jaspar.genereg.net/), and TFBIND (http://tfbind.hgc.jp/) were used.

2.5. Construction of expression vectors containing different regions of the HMCs promoter

To investigate the promoter activity of HMCs, five recombinant plasmids with luciferase reporter gene were constructed. First, the complete potential promoter of HMCs was generated by PCR with specific primers (HsP0 and Lvhms-XR) designed from the genomic DNA sequences and contain a *Kpn* I restriction site on the forward primers and a *Xho* I restriction site on the reverse primer (Table 1). The PCR products were subcloned into the *Kpn* I/*Xho* I sites of the pGL 3-Basic firefly luciferase reporter vector (Promega, USA), and then transformed into *E. coli* DH5a. Positive clones were sequenced at BGI (Shenzhen, China) and the recombinant construct was named pGL 3-HsP0. Next, four truncated HMCs promoters (−1630/+1849 bp, +1026/+1849 bp, +1516/+1849 bp, and +1654/+1849 bp) were generated by PCR using forward primers HsP1, HsP2, HsP3, HsP4 and reverse primer Lvhms-XR (Table 1), as well as primer F (which contains a *Kpn* I site) and primer R (which contains an *Xho* I site), with pGL 3-HsP0 used as template. The PCR products were then subcloned into the *Kpn* I/*Xho* I sites of the vector pGL 3-Basic. The recombinant constructs were named pGL 3-HsP1(-1630/+1849 bp), pGL 3-HsP2 (+1026/+1849 bp), pGL 3-HsP3(+1516/+1849 bp), and pGL 3-HsP4(+1654/+1849 bp), respectively.

2.6. Dual-luciferase reporter assay

In order to determine the promoter activity of HMCs promoter and its deletion constructs, the luciferase reporter assay were perform in *Drosophila S2* cells (S2 cells). Briefly, S2 cells were cultured at 27 °C in *Schneider's Drosophila* Medium (Invitrogen, USA) supplemented with 10% fetal bovine serum (Gibco, USA). Before transfection, cells were seeded into 24-well plates and allowed to grow to 60–80% confluence. For transient transfection, the DNA plasmid constructs were transfected into S2 cells using the FuGENE HD Transfection Reagent (Promega, USA) according to the manufacturer's recommendation. Each well was transfected with 250 ng of firefly luciferase reporter construct DNA (pGL 3-HsP0, pGL 3-HsP1, pGL 3-HsP2, pGL 3-HsP3 and pGL 3-HsP4, respectively) and 2.5 ng pRL-OpIE2 renilla luciferase plasmid (*Renilla* luciferase gene driven by the basal OpIE2 promoter from the plasmid pIZ-V5/His, an internal control). For dual-luciferase reporter assays, cell lysates were prepared at 48 h after transfection, and the Firefly luciferase and Renilla luciferase activities assayed using the Dual-Luciferase® Reporter Assay System (Promega, USA) on an Infinite M200 Pro multi-detection microplate reader (Tecan, Switzerland). The relative light unit (RLU) was calculated by normalizing the firefly luciferase activity to the Renilla luciferase activity to correct for transfection efficiency. Experiments were performed in triplicates for each construct, and standard deviations calculated. Experiments were repeated at least three times.

Table 1
Primers and probes used in this study.

Primer name	Sequence (5′-3′)
Genome walking adaptor primers	
AP1	GTAATACGACTCACTATAGGGC
AP2	ACTATAGGGCACGCGTGGT
Genome walking specific primers	
GWSP1a	AGACGAGTGGATCACGGCAACATA
GWSP1b	GTCGAAAAGCATGAGTGCCTCATTACG
GWSP2a	AGACGAGTGGATCACGGCAACATA
GWSP2b	CAAAAGCGAAAAATCCGATTAGCAT
GWSP3a	TGAACACAAGCACAAACACAGTAACG
GWSP3b	GATCTGAGTTACCGTAAAGGTGCCA
Primers for 5′deletion constructs[a]	
HsP0	cgg**GGTACC**CTGGTGACCTTGTTCACGTAAATGA
HsP1	cgg**GGTACC**CATGACATAAAACGGTTGAGA
HsP2	cgg**GGTACC**AACTTACCGTCCGATTTGATGCTG
HsP3	cgg**GGTACC**CATTTTCAAAGAACACAAGGACAA
HsP4	cgg**GGTACC**CGCAGTTGATGATGCTGAAGGAAT
Lvhms-XR	ccg**CTCGAG**TTCCAGCGACCTTCGTCAAATGTA
Primers for c-Jun motif mutation[b]	
P3 c-Jun mut1F	TGGAATTCTCTGGCGTGgaaGcatCAGAGCTAGCC
P3 c-Jun mut1R	atgCttcCACGCCAGAGAATTCCAAATCGGCTTTG
P3 c-Jun mut2F	GGAATCGAGTATACATTgccCaAgGGTCGCTGGAA
P3 c-Jun mut2R	cTtGggcAATGTATACTCGATTCCGTTGTTGTCTT
Primers for protein expression [c]	
V5-Lvc-Jun-F	cgg**GGTACC**ATGGAGGCAACCATGTAC
V5-Lvc-Jun-R	ccg**CTCGAG**CGCTGGTGCGTTACGAAG
Oligonucleotide sequences for EMSA[b]	
c-Jun 1 oligo	GGAATTCTCTGGCGTGtctGtcaCAGAGCTAGCCGTTG
anti c-Jun 1 oligo	CAACGGCTAGCTCTGtgaCagaCACGCCAGAGAATTCC
c-Jun 1 mut-oligo	GGAATTCTCTGGCGTGgaaGcatCAGAGCTAGCCGTTG
anti c-Jun 1 mut-oligo	CAACGGCTAGCTCTGatgCttcCACGCCAGAGAATTCC
c-Jun 2 oligo	CGGAATCGAGTATACATTtgaCgAaGGTCGCTGGAA
anti c-Jun 2 oligo	TTCCAGCGACCtTcGtcaAATGTATACTCGATTCCG
c-Jun 2 mut-oligo	CGGAATCGAGTATACATTgccCaAgGGTCGCTGGAA
anti c-Jun 2 mut-oligo	TTCCAGCGACCcTtGggcAATGTATACTCGATTCCG
Primers for dsRNA templates amplification	
dsLvc-Jun T7F[d]	GGATCCTAATACGACTCACTATAGGACCATCCTCAACAGCAACACG
dsLvc-Jun F[d]	ACCATCCTCAACAGCAACACG
dsLvc-Jun T7R[d]	GGATCCTAATACGACTCACTATAGGCGCTCCTGGCACTCCATATC
dsLvc-Jun R[d]	CGCTCCTGGCACTCCATATC
dsEGFPR	TACGGCGTGCAGTGCTTCAG
dsEGFPF	CTTCACCTCGGCGCGGGTCTTG
dsEGFP T7F	GGATCCTAATACGACTCACTATAGGCTTCACCTCGGCGCGGGTCTTG
dsEGFP T7R	GGATCCTAATACGACTCACTATAGGTACGGCGTGCAGTGCTTCAG

[a] Restriction sites for cloning are in bold font.
[b] Wild-type or mutated nucleotide are underlined and in lower-case letters.
[c] Primers according to reference [33].
[d] Primers according to reference [32].

2.7. Construction of the HsP3 mutants

Mutations of two c-Jun binding sites on the HMCs core promoter (+1516/+1849 bp) region were carried out using overlapping PCR with P3 c-Jun mut1F/R and P3 c-Jun mut2F/R primers (Table 1), following the protocol of Fast Mutagensis System (TransGen Biotech, Beijing, China). For HsP3mut1, the wild-type sequence "TCTGTCA" located at +1582/+1589 bp (designed as c-Jun 1 binding site) was mutated to "GAAGCAT"; for HsP3mut2, the wild-type sequence "TGA CGAA" located at +1831/+1837 bp (designed as c-Jun 2 binding site) was mutated to "GCCCAAG"; for HsP3mut3, both of the c-Jun 1 and c-Jun 2 binding sites were converted as above. The mutated promoter fragments were cloned into the pGL 3-Basic Vector (Promega, USA) to generate pGL 3-HsP3mut1, pGL 3-HsP3mut2 and pGL 3-Hsp3mut3 reporter gene plasmids in the same conditions as described in Section 2.5.

2.8. Construction of pIZ-LvcJun-V5/His

To examine if *L. vannamei* c-Jun (Lvc-Jun) regulated the HMCs promoter, Lvc-Jun expression plasmids were constructed. Briefly, the forward primer V5-Lvc-Jun-F and reverse primer V5-Lvc-Jun-R (Table 1) were used to cloned the open reading frame (ORF) of Lvc-Jun (GeneBank no. KM401573) by PCR. Next, the PCR products were digested and ligated into the pIZ-V5/His vector (Invitrogen,USA) to produce V5-tagged Lvc-Jun expression plasmid pIZ-Lvc-Jun-V5/His using the same conditions as described in Section 2.5.

2.9. Co-transfection assay

To investigate whether Lvc-Jun binds to the c-Jun motif located in the core promoter region of HMCs promoter, co-transfection assay was perform in S2 cells. The wild-type construct (pGL 3-HsP3) or mutant constructs (pGL 3-HsP3mut1, pGL 3-HsP3mut2 and pGL 3-Hsp3mut3) were co-transfected with pIZ-Lvc-Jun-V5/His (pIZ-V5/His as a control), and pRL-OpIE2 renilla luciferase plasmid was used as an internal control, pGL 3-Basic as a negative control. The protocols used for cell culture, transient transfection and dual-luciferase reporter assay are as describe above.

2.10. Electrophoretic mobility shift assay (EMSA)

To further determine whether Lvc-Jun could bind to the c-Jun

Fig. 1. The 5′-flanking sequence of small subunit *L. vannamei* hemocyanin gene (HMCs). Nucleotide sequence of HMCs 5′ upstream and downstream regions with potential transcription factor binding sites. Putative transcription start site (+1) is indicated with the nucleotide sequence numbered above. The potential transcription binding sites are underlined and labeled. The start codons (ATG) are depicted by boxed. Sequences of introns are shown in lowercase letters.

binding motif of HMCs core promoter region, the EMSA was perform and by using the Chemiluminescent EMSA kit (Beyotime, Beijing, China). Briefly, four wild type oligonucleotides, c-Jun1oligo and anti c-Jun1oligo, c-Jun2oligo and anti c-Jun2oligo (Table 1), which correspond to the c-Jun 1 (+1582/+1589 bp) and c-Jun 2 (+1831/+1837 bp) binding site in the promoter of HMCs gene were synthesized. At the same time, four mutated type oligonucleotides for the two c-Jun binding site, viz. c-Jun1mutoligo and anti c-Jun1mutoligo, c-Jun2mutoligo and anti c-Jun2mutoligo, which were mutated as described above for the construction of HsP3mut1 and HsP3mut2, were also synthesized. The oligonucleotides c-Jun 1 oligo and anti c-Jun 1 oligo, c-Jun 2 oligo and anti c-Jun 2 oligo were labeled with biotin at the 5′-end using the EMSA Probe Biotin Labeling Kit (Beyotime, Beijing, China), respectively. And then annealed into biotin-labeled probe as Bio-c-Jun 1 probe and Bio-c-Jun 2 probe according to the manufacturer's instruction. The unlabeled probe Unbio-c-Jun 1, Unbio-c-Jun 2, Mut-unbio-c-Jun 1 and Mut-unbio-c-Jun 2 were made by annealing c-Jun 1 oligo and anti c-Jun 1 oligo, c-Jun 2 oligo and anti c-Jun 2 oligo, c-Jun 1 mut-oligo and anti c-Jun 1 mut-oligo, c-Jun 2 mut-oligo and anti c-Jun 2 mut-oligo, respectively. The reaction system contained EMSA binding buffer (5×), whole cell extracts from S2 cells transfected with pIZ-Lvc-Jun-V5/His or pIZ-V5/His and biotin-labeled probe. A cold competition experiment was carried out using an excess unlabeled double-stranded probe (25, 50 and 100-fold higher than biotin-labeled probe), which was added to the reaction mixture prior to adding the labeled probe. DNA-protein complex was electrophoresed on a 4% polyacrylamide gel in 0.5×TBE electrophoresis buffer (pH 8.3) at 80 V and then transferred onto a positive nylon membrane, UV-cross-linked with the membrane, probed with streptavidin-horseradish peroxidase (HRP) conjugate, and incubated with the biotin substrate. Photos were captured using the GE Amersham Imager 600 imaging system (GE, USA).

2.11. Overexpression of Lvc-Jun

To investigate whether or not Lvc-Jun regulated the HMCs promoter, pGL 3-HsP3 was co-transfected with different amounts of pIZ-Lvc-Jun-V5/His (0, 125, 250, 500 ng) into S2 cells. The protocols for cell culture, transient transfection and dual-luciferase reporter assay are as described above.

2.12. RNA interference (RNAi)

The RNAi method used was a modification of the method by Natesampillai et al. [35]. Briefly, the dsRNA of Lvc-Jun (dsLvc-Jun T7F and dsLvc-Jun R, dsLvc-Jun F and dsLvc-Jun T7R) and EGFP (dsEGFP T7F and dsEGFP R, dsEGFP R and dsEGFP T7R) using specific primers (Table 1) were synthesized using the T7 RiboMAX™ Express RNAi System (Promega, USA). Then, S2 cells were transfected with dsRNA-Lvc-Jun or dsRNA- EGFP (as a negative control) using FuGENE HD

Transfection Reagent (Promega, USA) according to the manufacturer's recommendation to achieve 400 ng dsRNA per well (24-well plate). The cells were also transfected with pGL 3-HsP3, pIZ-Lvc-Jun-V5/His or pIZ-V5/His (as a negative control). At 48 h post transfection, cells were lysed and lysate used to assay for luciferase activity. At the same time, the cell lysate was analyzed by Western blot so as to ascertain the knockdown of Lvc-Jun expression. In brief, it was boiled in 5 × SDS sample buffer (250 mM Tris, 10% SDS, 50% glycerol, 10% β-mercaptoethanol, 1% bromophenol blue, pH 6.8) for 10 min. Proteins were separated on 10% SDS-PAGE gels and then transferred onto PVDF membranes (Millipore, USA). The membranes were blocked for 1 h at room temperature in 5% skim milk dissolved in TBST buffer (20 mM Tris, 150 mM NaCl, 0.1% Tween 20, pH 7.6), followed by incubation with rabbit anti-V5 (1:2000, Millipore, USA) or mouse anti-tubulin (1:1000, Sigma-Aldrich, USA) antibodies diluted in SignalBoost™ Immunoreaction Enhancer antibody diluent (Millipore, USA) with gentle rocking for 1 h at room temperature. After being washed three times with TBST buffer, membranes were incubated with goat anti-rabbit-IgG-HRP (1:1000) or goat anti-mouse-IgG-HRP secondary antibodies (1:1000 or 1:4000, Sigma-Aldrich, USA) for 1 h at room temperature. Signals were visualized using Millipore Immobilon™ Western Chemiluminescent HRP Substrate (Millipore, USA) detection system.

3. Results

3.1. Cloning of the 5′-flanking region of HMCs

A 4324 bp fragment of the 5′-flanking region of the HMCs of *L. vannamei* was obtained from genomic DNA by three rounds of nested PCR. This fragment spans from upstream 2475 bp to downstream 1849 bp relative to the transcription initiation site of HMCs gene, predicted at the 12 bp upstream of the ATG (Fig. 1). Using the online freeware Match™ −1.0, PROMO, JASPAR, and TFBIND to analyze the 4324 bp fragment revealed that it contained the following putative transcription factors binding sites: C/EBP, c-Jun, IRF-1, GATA-1, v-Myb, SOX-10, STAT, EcR/usp, TBP, KLF5, KLF4, KLF12 and NF-κB (Fig. 1), therefore suggesting that the 5′-flanking region of the HMCs gene might be a putative promoter region.

3.2. Identification of the HMCs core promoter region

In order to investigate the promoter activity of the HMCs gene and to identify its core promoter region, we generated five constructs (Fig. 2A) with luciferase reporter of the 5′-flanking region of the HMCs gene (pGL 3-HsP0) and its four truncations (pGL 3-HsP1, pGL 3-HsP2, pGL 3-HsP3 and pGL 3-HsP4). These were transiently transfected into S2 cells and their luciferase activities were determined. The results showed that all five constructs were active and that the pGL 3-HsP3 (+1516/+1849 bp) construct retained the highest activity (Fig. 2B), suggesting that the DNA fragment from +1516/+1849 bp might be the core promoter region of HMCs. It also suggests that this fragment was important and sufficient for HMCs basal transcriptional activity.

3.3. The HMCs core promoter region contains c-Jun binding sites

To delineate the potential transcription factor binding sites, we further analyzed the HMCs core promoter region (+1516/+1849 bp) using the online tools Match™ −1.0 Public and PROMO. The binding sites for the following transcription factors were found (Fig. 1): c-Jun (+1582/+1589 bp and +1831/+1837 bp), STAT (+1516/+1530 bp, +1567/+1572 bp and +1621/+1631 bp) and Krupple like factor 5 (+1540/+1549 bp). Notably, the activity of the construct pGL 3-HsP4 (+1654/+1849 bp), which contains only one c-Jun binding site, also remained by about 57.3% compared to the construct pGL 3-HsP3, suggesting that the HMCs core promoter region seems to contain c-Jun binding sites, which might play an important role in regulating the transcription of HMCs.

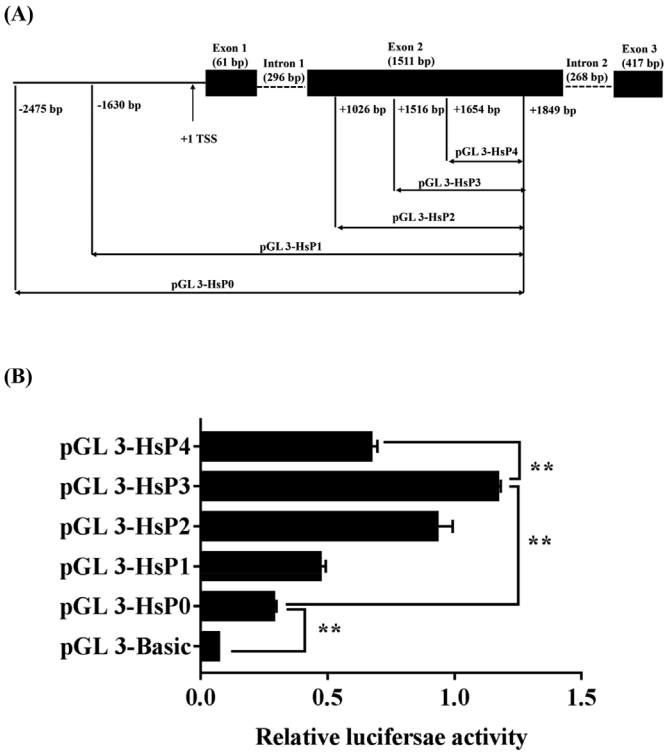

Fig. 2. Analysis of the core promoter region of the HMCs gene. (A) A schematic representation of the deletion mutant constructs of the putative HMCs promoter region. (B) Luciferase activity assay of the HMCs promoter plasmids and plasmids of deletion constructs derived from the HMCs gene promoter region. The results are expressed as the means ± S.D in arbitrary units based on the firefly luciferase activity normalized against the Renilla luciferase activity for triplicate transfections. The error bars denote the standard deviation. Paired Student's t-test was used to detect significant differences, $^{**}p < 0.01$.

To determine whether the two putative c-Jun binding sites, located at +1582/+1589 bp (designed as c-Jun1 binding site) and +1831/+1837 bp (designed as c-Jun2 binding site), respectively (Fig. 3A), were essential for HMCs promoter activity, three mutations (designated as pGL 3-HsPmut1, pGL 3-HsPmut2, and pGL 3-HsP3mut3), were constructed at these sites based on the pGL 3HsP3 construct (wild-type). The mutants and wild-type constructs were co-transfected with pIZ-Lvc-Jun-V5/His into S2 cells. As shown in Fig. 3B, compared with the control (pIZ-V5/His), over-expression of Lvc-Jun could increase the activity of the HsP3 promoter by about 32.4-fold, with that of the HsP3mut1 promoter increased by 10.7-fold, while that of HsP3mut2 promoter was by 11.8-fold, and the HsP3mut3 promoter by 7.8-fold. Thus, mutation of the c-Jun binding motif on the core promoter region attenuates its activation by Lvc-Jun. To further determine whether Lvc-Jun could bind to the c-Jun 1 and c-Jun 2 binding motifs, two biotin-labeled probes, Bio-c-Jun 1 probe and Bio-c-Jun 2 probe, containing the two predicted c-Jun binding motifs, were prepared and used for EMSA. Shift bands of DNA-protein complex were detected when the Bio-c-Jun 1/2 probes were incubated with cell lysates of pIZ-Lvc-Jun-V5/His transfected S2 cells (Fig. 3C and D lane 3). Notably, the shift bands were competitively reduced by the unlabeled wild cold probes at increasing (25×, 50× or 100×) excess molar concentrations (Fig. 3C and D lanes 4–6), but were not affected by the unlabeled mutant cold probes at 100× the molar concentration (Fig. 3C and D lane 7). These results imply that Lvc-Jun associates with the HMCs gene promoter and therefore suggest a role of these factors in the regulation of HMCs gene transcription.

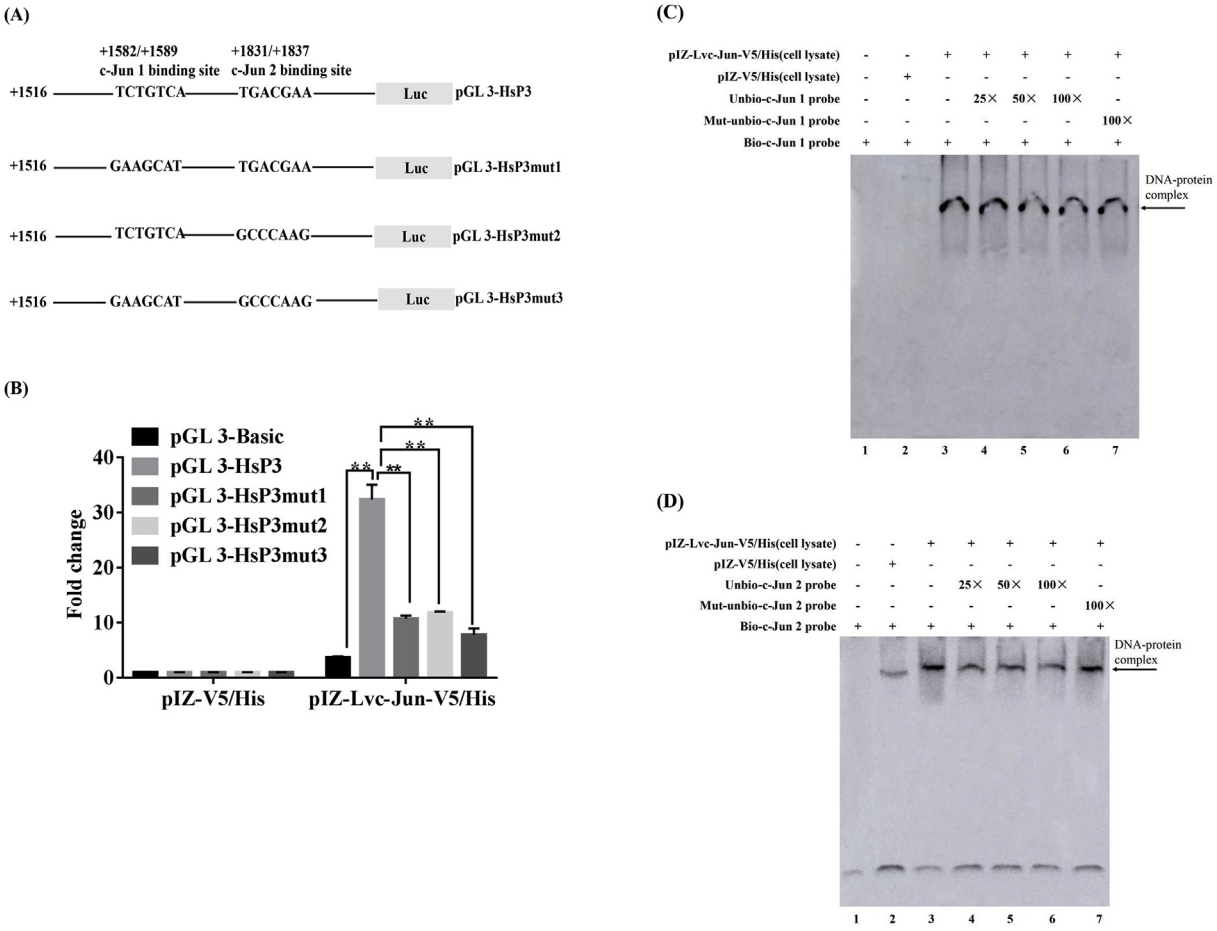

Fig. 3. Identification and analysis of c-Jun binding sites on the *L. vannamei* HMCs gene promoter. (A) Schematic diagram of the wild type and mutant constructs. (B) Dual luciferase activities of the pGL 3-HsP3 plasmid and mutant plasmids pGL3-HsP3mut1/2/3. A Renilla luciferase reporter plasmid pRL-OpIE2 was used as an internal control. Cells transfected with the control pIZ-V5/His and PGL3-Basic vectors were used as control and set as 1.0. The bars indicate mean values ± S.D. of the luciferase activity (n = 3). $^{**}p < 0.01$. (C–D) EMSA analysis of Lvc-Jun binding to the putative c-Jun 1 (C) and c-Jun 2 (D) binding motifs on the HMCs promoter. Bio-c-Jun 1/2 probes were incubated with the Lvc-Jun protein extracts from *Drosophila* S2 transfected cells (lane 3). Various concentrations of unlabeled wild cold probes (lanes 4 to 6), and mutant cold probes (lane 7), were added for competition assays.

3.4. Lvc-Jun transactivates the promoter of HMCs

In order to investigate the effect of Lvc-Jun over-expression on HMCs transcriptional activity, different amounts of the Lvc-Jun constructs were transfected and used in transactivity assays. The results showed that over-expression of the transcription factor Lvc-Jun could increase the promoter activities of HMCs gene in a dose-dependent manner (Fig. 4A). Furthermore, the technique of RNAi was used to investigate the role of Lvc-Jun in the regulation of the HMCs gene. There was a significant decrease in the HMCs promoter activity when S2 cells were co-transfected with dsRNA-Lvc-Jun, pGL 3-HsP3 and pIZ-Lvc-Jun-V5/His, as compared co-transfection with dsRNA-EGFP, pGL 3-HsP3 and pIZ-Lvc-Jun-V5/His. The knockdown efficiency was ascertained using Western blotting (Fig. 4B). The results thus suggest that Lvc-Jun could promote HMCs gene expression.

4. Discussion

Shrimps defend and protect themselves against the vast sea of microbial pathogens and infections relying only on their efficient innate immune system. There is therefore a growing research interest in trying to understand how the immune-related genes and immune signaling pathways in shrimp are able to accomplish this without an adaptive immune system [10,12,36]. However, only a few promoters of these innate immune-related genes, such as penaeidin [37], C-type lectin-like protein [38], anti-lipopolysaccharide factor [39,40], and so on, have been reported. Although hemocyanin is the main hemolymph protein and a non-specific innate defense molecule in shrimp [16,20], the regulatory mechanism of its transcription has not been explored. Moreover, as a multifunctional protein important in shrimp immunity, it is important to better understand how the hemocyanin gene is regulated and the regulatory elements involved. The current study focused on the HMCs of *L.vannamei*, seeking to explore and understand its transcription regulatory mechanism in innate immune response.

To examine the transcription regulation of HMCs, we first cloned its 5′-flanking region. After obtaining a 4324 bp amplicon of HMCs by nested PCR, we went on to identify the core promoter region by generating a series of deletion constructs. The core promoter region (pGL 3-HsP3) was found to be located in exon 2 (+1516/+1847 bp), as pGL 3-HsP3 had a higher activity than the full-length HMCs promoter (pGL 3-HsP0) and the other three deletion constructs (pGL 3-HsP1, pGL 3-HsP2 and pGL 3-HsP3). This observation is synonymous with a previous study on the human D-amino acid oxidase gene (hDAO) promoter, which has an alternative promoter in the proximal upstream region of exon 2, and that was shown to have a much stronger activity than the others [41]. Whether the core promoter of HMCs gene is located in exon 2 with enhancer activity still need more evidence. However, based on the current study, we supposed that the core promoter of the HMCs gene located in exon 2 is the alternative promoter. This is not far from true because alternative promoters were widespread in mammalian

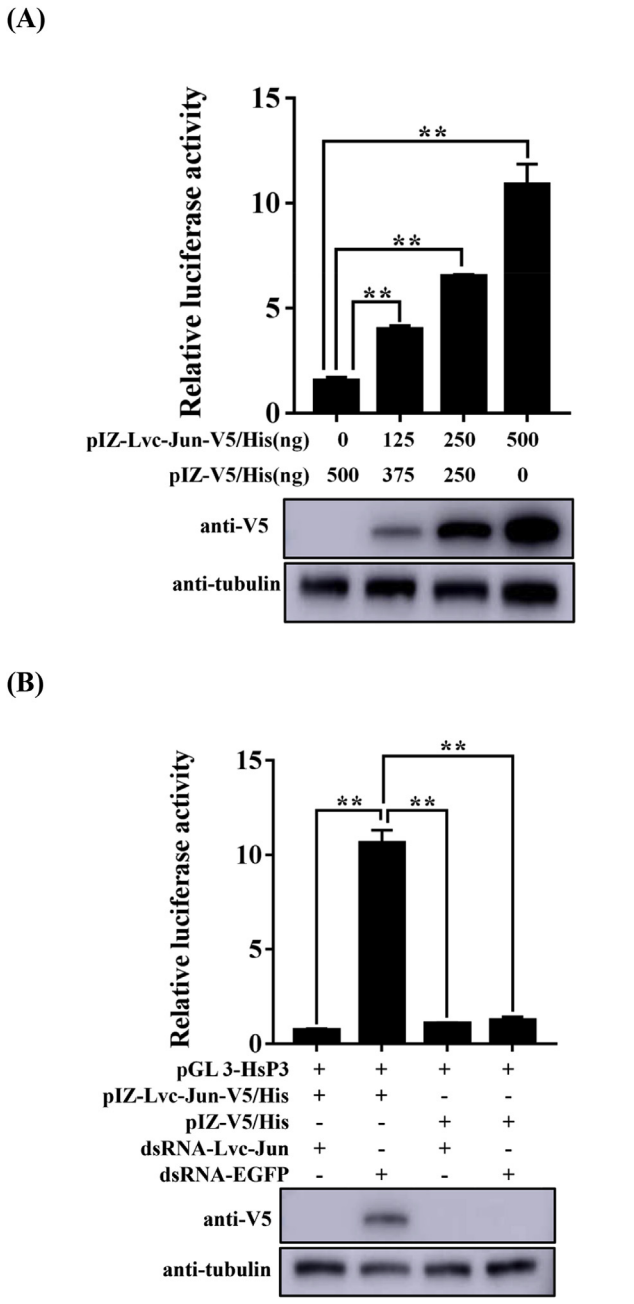

Fig. 4. Transactivation of HMCs promoter by Lvc-Jun. (A) Over-expression of Lvc-Jun transactivates the HMCs promoter activity. *Drosophila* S2 cells were co-transfected with various amounts of effector plasmids, reporter plasmids together with the internal plasmid pRL-OpIE2. (B) Silencing of Lv-cJun attenuates the activity of the HMCs promoter. The luciferase activity of *Drosophila* S2 cells co-transfected with construct pGL 3-HsP3 and pIZ-Lvc-Jun-V5/His or pIZ-V5/His (as control) and dsRNA-Lvc-Jun or negative control dsRNA-EGFP was determined. The Renilla luciferase reporter plasmid pRL-OpIE2 was used as an internal control. Data plotted are means with standard deviations from three independent experiments and was analyzed using Student's t-test and significant differences indicated by asterisks (**$p < 0.01$) compared with pIZ-V5/His group. To control for expression of Lvc-Jun, Western blot analysis was performed using rabbit anti-V5 antibody, demonstrating that Lvc-Jun was over-expressed in increasing amounts. Tubulin was used as loading control.

genomes and exist in 30–50% of human genes [42,43]. Of these, many promoters are on exons. For instance, the hDAO possess an alternative promoter in exon 2 [41], and the alternative promoter of the microtubule-associated protein tau gene (MAPT) is located in exon 1 [44], while the Wnt family member 5A gene (WNT5A) has an alternative promoter B located in exon 2 [45].

The existence of alternative promoters has not only been used to explain differential tissue specificity and differential response to transcription, but also transcription of shorter transcripts. For instance, 14 short transcripts are derived from the alternative promoter located in exon 1 of the MAPT under pathological conditions [44]. It has also been revealed that the p53 gene, which has an internal promoter located in intron-4, encodes different p53 transcripts [42,46]. Interestingly, we previously reported on an 18.4 kDa antimicrobial truncation from *L.vannamei* hemocyanin [47], which had its coding region located downstream of the alternative promoter region. Thus, we think that probably the previously observed 18.4 kDa truncation of small subunit hemocyanin was generated by the alternative promoter transcription, rather than by post-translational modification.

In any case, alternative promoters are associated with diversification of transcriptional regulation and therefore play an essential role in various cell lineages, tissues and developmental stages [48]. Thus, to find out the transcription factor(s) that might be involved in HMCs gene expression, the bioinformatics tools PROMO and Match were used to predict two c-Jun motifs in the alternative promoter region of HMCs. Intriguingly, our previous transcriptome data revealed that the expression of HMCs was significantly increased in shrimp hemocytes stimulated with acute hepatopancreas necrosis disease (AHPND) *V. parahaemolyticus* (with *Pir*) or non-AHPND *V. parahaemolyticus* (without *Pir*) compared to control. A similar pattern in the expression level of c-Jun was also observed in the same transcriptome data [49]. It thus seems to suggest that c-Jun might be involved in the regulation of HMCs gene expression.

To further delineate if Lvc-Jun did play a role in modulating the transcription of HMCs, we generated c-Jun binding site mutants and used EMSA to confirm that indeed Lvc-Jun could bind to the c-Jun binding motif in the core promoter region of HMCs, significantly attenuating the promoter activity. Our observation is similar to a previous study where a mutation in the AP-1 binding site reduced the activity of the metastasis-associated in colon cancer 1 (MACC1) gene promoter [50]. RNAi and overexpression of Lvc-Jun further revealed that Lvc-Jun could transactivate the alternative promoter of HMCs gene, which is synonymous to a previous study where KLF13 repressed the low density lipoprotein receptor (LDLR) gene promoter when siRNAs and luciferase construct and/or KLF13 construct were co-transient into granulosa-luteal cells [35]. These results thus indicate that Lvc-Jun could regulate the transcription of the HMCs gene via the alternative promoter.

It has previously been reported that c-Jun was a downstream effector of JNK-c-Jun signaling pathway and plays a critical role in innate immune response during pathogen invasion [51,52]. Musti et al. indicated that the phosphorylation of c-Jun by JNK enhances its transcriptional activity and increases its stability by decreasing its ubiquitin-dependent degradation [53]. Turjanski et al. found that the mitogen-activated protein kinases (MAPKs) modulate the activity of c-Jun and c-fos (which dimerize to form the AP-1 complexes), allowing them to bind the promoter region [54]. Notably, apart from c-Jun, other members of the MAP kinase family including JNK and p38 were also identified in our transcriptome data, although their differential expression differed from that of c-Jun [49]. Since the current study revealed that Lvc-Jun enhanced the transcription activity of the alternative promoter of HMCs gene, it is therefore plausible to hypothesize that hemocyanin might participate in shrimp innate immune response by triggering the MAPK-c-Jun signaling pathway. In addition, the MAP kinases might cross-talk with the Toll pathway, as it has been revealed that the Toll pathway acts as a downstream modulator for JNK-dependent cell death in *Dorsophila*, in addition to the fact that JNK signaling also results in Toll pathway activation [55]. Moreover, it has been reported that on the TLR3, TLR7, TLR9 pathway, the TAK1 (TGF-β activated kinase 1) activates MAPKK3/6, resulting in phosphorylation of JNKs, p38 and culminating in activation of AP1 [56]. Thus, it seems

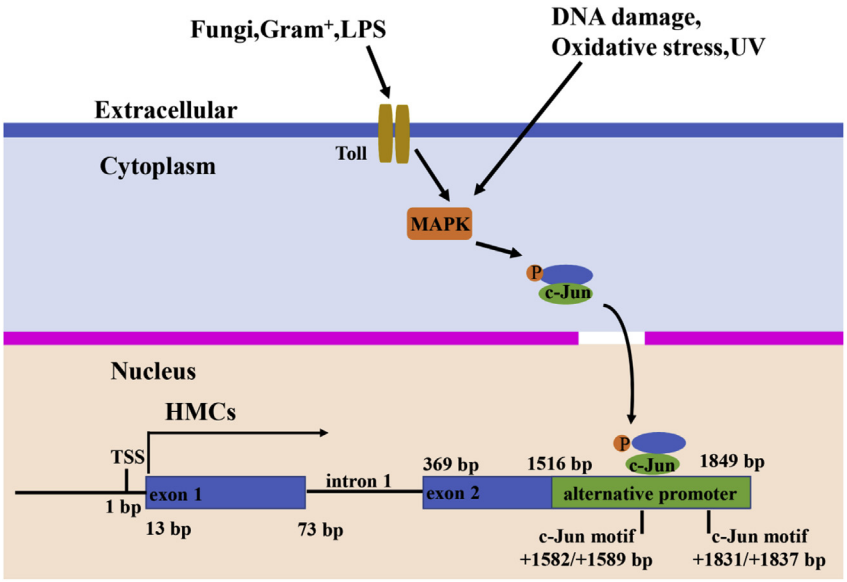

Fig. 5. Propose model for the Lvc-Jun regulation the alternative promoter of HMCs gene. Lvc-Jun might be regulated by MAP kinase through phosphorylation, which allows the activated or phosphorylated c-Jun to enter the nucleus to bind to the c-Jun motif located on the alternative promoter region of the HMCs gene, thereby driving the transcription of the gene.

to suggest that there is a possible association between Toll signaling and the transcription of hemocyanin. Collectively, these details and our experimental results support a proposed model and cascade showing the binding sites of Lvc-Jun and the gene regulation events that might be involved when Lvc-Jun binds to the promoter region of the HMCs gene (Fig. 5).

In conclusion, the present study was the first to clone and characterize the promoter of *L. vannamei* HMCs gene, as well as demonstrated that an alternative promoter located in exon 2 could be activated by the transcription factor Lvc-Jun. With these novel and interesting results, future studies would leverage the use of bioinformatics to predict the presence of other factors as well as employ DNA pull-down assays to explore other important factors that might also be involved in regulating the expression of the HMCs gene.

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