Available online at www.sciencedirect.com
ScienceDirect
Molecular Immunology 44 (2007) 1516–1523
Molecular Immunology
www.elsevier.com/locate/molimm

Genomic organization, promoter characterization and expression profiles of an antiviral gene *PmAV* from the shrimp *Penaeus monodon*

Tian Luo [a], Fang Li [a,b], Kaiyu Lei [a,b], Xun Xu [a,*]

[a] *Key Laboratory of Marine Biogenetic Resources, Third Institute of Oceanography, State Oceanic Administration (SOA), No. 178, Daxue Road, Xiamen, Fujian 361005, PR China*
[b] *School of Life Science, Xiamen University, Xiamen 361005, PR China*

Received 3 July 2006; received in revised form 23 August 2006; accepted 4 September 2006
Available online 20 October 2006

Abstract

Diseases caused by viruses are the greatest challenge to worldwide shrimp aquaculture. *PmAV* is the first antiviral gene identified from the shrimp and its mRNA level is up-regulated in response to viral infection (Luo et al., 2003). The transcriptional regulation of *PmAV* is critical since the expression of this gene is regulated in response to viral stimuli. In this study, we show that the *Penaeus monodon PmAV* gene contains four exons, separated by three introns. The 5′-flanking sequence of *PmAV* gene consists of a typical TATA box, several potential regulatory elements, and a compound microsatellite repeat as well. Interestingly, a TATA box and several regulatory elements also exist in the first intron (intron 1). Both intron 1 and the microsatellite are involved in transcriptional regulation of the *PmAV* gene. *PmAV* promoter activity depends on the existence of intron 1 while the microsatellite in 5′-flanking sequence acts as a negative regulative element. In addition, using Real-time PCR, we found that *PmAV* was highly expressed in the hepatopancreas and was up-regulated from the second day post-infection with the increase of viral load. These results extend our previous findings and provide insights into the molecular regulation of *PmAV* gene expression, which will be helpful for shrimp viral disease control.

Keywords: *PmAV*; Gene structure; Shrimp; Antiviral; Promoter; Microsatellite

1. Introduction

Shrimp is one of the most important species in aquaculture, but shrimp diseases caused by viruses, especially by white spot syndrome virus (WSSV) have resulted in huge economic losses in the whole world (Lighner and Redman, 1998; Bachere, 2000). However, no effective way has so far been found to eliminate shrimp viral infection. Investigation of the antiviral mechanism of this animal will greatly promote the understanding of its disease control.

Knowledge of the shrimp defense at the molecular level has been extended rapidly in recent years. Some antimicrobial defense mechanisms have been characterized, such as the prophenoloxidase (proPO) activating system (Soderhall and Cerenius, 1998; Cerenius and Soderhall, 2004) and antimicrobial peptides (Destoumieux et al., 2000a; Bachere et al., 2004). However, only a little is known about the possible antiviral factors in the shrimp, especially at the molecular level (Pan et al., 2000; He et al., 2005), and still less is known about the regulation and control of antiviral genes in the shrimp.

In 2003 we cloned and characterized, for the first time, an antiviral gene *PmAV* from the shrimp *Penaeus monodon* by differential display (DD) (Luo et al., 2003). The *PmAV* cDNA encodes a 170 amino acid peptide with a C-type lectin-like domain (CTLD) and was found to be up-regulated in virus-resistant shrimps. Furthermore, recombinant *PmAV* protein can inhibit virus-induced cytopathic effects in cultured fish cell. The *PmAV* protein might therefore play an important role in the antiviral defense of shrimp. These findings also suggest that *PmAV* gene is inducible by infection, in contrast to the constitutively expressed shrimp penaeidin genes (Destoumieux et al.,

Abbreviations: WSSV, white spot syndrome virus; proPO, prophenoloxidase; CTLD, C-type lectin-like domain; C_T, cycle threshold value

* Corresponding author. Tel.: +86 592 2195296; fax: +86 592 2195296.
E-mail address: xxu@public.xm.fj.cn (X. Xu).

0161-5890/$ – see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.molimm.2006.09.015

1997, 2000b). Further study on the expression and regulation of the *PmAV* gene may lead to a better understanding of viral resistance in shrimp.

In this paper, we characterize the genomic structure, promoter activity of *PmAV* and the expression profile of this gene in normal and virus-challenged shrimp. These results may facilitate the overall understanding of the viral resistance mechanism of the *PmAV* gene in shrimp.

2. Materials and methods

2.1. Shrimps

P. monodon (Crustacea, Decapoda) (about 20–25 g), were purchased from a supermarket in Xiamen, China, and cultured in our laboratory in 500 L tanks (at 25 °C) filled with air-pumped circulating sea water.

2.2. Cloning of complete sequence of PmAV gene

The genomic DNA of *P. monodon* was isolated from abdominal muscle tissue of *P. monodon* by phenol extraction method and then partially digested by *Sau*3AI and used as the PCR template. Two primers AV1 (5′-ATGCGTCATACAATCCTA-3′) and AV2 (5′-TTAATGTGTCCTGCTTTC-3′) were designed, respectively, according to the 5′- and 3′-terminal sequence of *PmAV* cDNA ORF (GenBank accession no. AY302750). A ∼3.8 kb DNA fragment was amplified from the genomic DNA with primers AV1 and AV2, and then cloned into pMD18-T vector (Takara) for sequencing.

The upstream sequence (including promoter region) of *PmAV* gene was cloned by genomic walking method using Universal Genomewalker Kit (Clontech) following the manufacturer's instructions. In brief, *P. monodon* genomic DNA was first completely digested by *Dra*I, *Eco*RV, *Pvu*II and *Stu*I separately and the digested DNA was purified and ligated with Genomewalker adaptor to construct four genomic libraries of *P. monodon*. Secondly two gene-specific primers of *PmAV* (GSP1: 5′-CAGCCTTGGAATCATTGGC-3′; GSP2: 5′-ATGATGTTGCCACAGCCGA-3′) were designed according to the 5′ region sequence of the *PmAV* cDNA. Using four libraries' DNA as templates separately, primary PCRs were carried out with GSP1 and AP1 (Clontech adaptor primer). Secondary PCRs were performed with GSP2 and AP2 (adaptor nested primer), using primary PCR products as templates. A ∼900 bp DNA fragment was obtained and cloned into pMD18-T vector (Takara) for sequencing.

2.3. Promoter activity assay

Promoter activity was analyzed as described previously (Liu et al., 2005). In brief, pIZΔIE/EGFP was modified from pIZ/V5-His (Invitrogen) by deleting the OpMNPV IE2 promoter in front of the MCS (multiple cloning sites) and then inserting the EGFP (enhanced green fluorescence protein) gene into the MCS between *Bam*HI and *Eco*RI sites. A series of different spans of possible promoter region of the *PmAV* gene were amplified from *P. monodon* genomic DNA and then inserted into the MCS in front of the EGFP gene of the vector pIZΔIE/EGFP, respectively. The sequences of primers are summarized in Table 1. The plasmids pIZ/EGFP (constructed by inserting the EGFP gene between *Bam*HI and *Eco*RI sites of pIZ/V5-His) and pIZΔIE/EGFP were used as the positive and negative controls, respectively.

Table 1
Primers used for constructing transient expression vectors for the promoter activity assay

Primer	Sequence (5′–3′)
ProAV-N_1	GTCGGATCCAAATTGTATCTGTTATTTAGTT
ProAV-N_2	GTCGGATCCTTCTCTTCTTCGATTATATACG
ProAV-N_3	GTCGGATCCAGTCCCACACTCCATCAA
ProAV-C_1	CTGGGATCCTGTGGCATCGGCGGG
ProAV-C_2	CTGGGATCCCCTAGGATTGTATGACG
ProAV-C_3	CTGGGATCCCTGAAAGGAATATTAATATCTTG

Added restriction enzyme cutting sites (*Bam*HI) are underlined.

Since no stable shrimp cell line is available now, we used *Trichoplusia ni* High Five™ cell (Invitrogen) instead. Cells were seeded into a 24-well plate (Corning) (3×10^5 cells per well) and grown in Grace's Medium supplied with 5% fetal bovine serum (Invitrogen) overnight at 27 °C. Plasmid DNA (0.5 μg/well) was transfected into High Five™ cells with Cellfectin Reagent (Invitrogen) according to the manufacturer's instructions. EGFP fluorescence signal was observed under Nikon PE-200 inverted fluorescence microscope 72 h post-transfection, and photographically recorded using Nikon 1200F digital microscope camera.

These assays were repeated for at least three times.

2.4. WSSV challenge experiment

WSSV inoculum was prepared from virus-infected shrimp tail muscle tested positively by PCR (Wang et al., 1999). Frozen infected tissue was homogenized in 1× PBS (1:5, w/v) and centrifuged at $8000 \times g$ for 10 min. The supernatant filtered through a 0.45-μm pore-size filter was used for injection. Shrimps were maintained in 500 l tanks (at 25 °C) filled with air-pumped circulating sea water. After 6 day's of culture, healthy shrimps were selected and tested randomly to be WSSV-free by PCR (Wang et al., 1999). Each shrimp was then injected with 100 μl WSSV inoculum (corresponds to 1×10^6 copies approximately) at a point between the second and third tail tergal plates on the lateral sides by a 1-ml sterile syringe.

Different tissues including gill, heart, hemocytes, hepatopancreas, muscle, intestine and stomach of five individuals were collected prior to WSSV-infection. Hepatopancreases of five individuals were collected at different time points (0 h, 6 h, 12 h, 24 h, 2 d, 3 d, 4 d, and 5 d post-infection) for further RNA extraction.

2.5. Relatively quantitative real-time RT-PCR analysis of gene expression

Molecular beacon-based real-time RT-PCR method was adopted to analyze the relative quantification of mRNA tran-