Developmental and Comparative Immunology 49 (2015) 7–18

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

journal homepage: www.elsevier.com/locate/dci

The promoter of the white spot syndrome virus immediate-early gene *WSSV108* is activated by the cellular KLF transcription factor

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ARTICLE INFO

Article history:
Received 11 August 2014
Revised 24 October 2014
Accepted 25 October 2014
Available online 6 November 2014

Keywords:
Krüppel-like factor
White spot syndrome virus
WSSV
Immediate-early gene
Litopenaeus vannamei
Penaeus monodon

ABSTRACT

A series of deletion and mutation assays of the white spot syndrome virus (WSSV) immediate-early gene *WSSV108* promoter showed that a Krüppel-like factor (KLF) binding site located from –504 to –495 (relative to the transcription start site) is important for the overall level of *WSSV108* promoter activity. Electrophoretic mobility shift assays further showed that overexpressed recombinant *Penaeus monodon KLF* (rPmKLF) formed a specific protein–DNA complex with the ^{32}P-labeled KLF binding site of the *WSSV108* promoter, and that higher levels of *Litopenaeus vannamei* KLF (LvKLF) were expressed in WSSV-infected shrimp. A transactivation assay indicated that the *WSSV108* promoter was strongly activated by rPmKLF in a dose-dependent manner. Lastly, we found that specific silencing of LvKLF expression *in vivo* by dsRNA injection dramatically reduced both *WSSV108* expression and WSSV replication. We conclude that shrimp KLF is important for WSSV genome replication and gene expression, and that it binds to the *WSSV108* promoter to enhance the expression of this immediate-early gene.

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1. Introduction

White spot syndrome virus (WSSV; family *Nimaviridae*, genus *Whispovirus*; Vlak et al., 2005) is a large (~307 kbp) double-stranded DNA virus that is the causative agent of a penaeid shrimp disease that continues to cause serious economic losses to the global shrimp farming industry (Escobedo-Bonilla et al., 2008; Lo et al., 1996, 2005; Sánchez-Paz, 2010). Like many large DNA viruses, such as the baculoviruses and herpesviruses, WSSV gene expression is temporally regulated (Liu et al., 2005). The first viral genes to be transcribed are the immediate-early (IE) genes, and their expression depends entirely on the host cellular machinery (Kovacs et al., 1991; Ross and Guarino, 1997). Most, if not all, IE genes encode transcriptional regulatory proteins that are required for the transcription, or the enhancement of transcription, of many viral genes (Carson et al., 1991; Guarino and Summers, 1986, 1987, 1988; Theilmann and Stewart, 1991). To date, at least twenty-one WSSV IE genes have been identified by cycloheximide pretreatment of WSSV-infected shrimp followed by reverse transcription-PCR (RT-PCR) analysis (Li et al., 2009; Lin et al., 2011; Liu et al., 2005). Among the known WSSV IE genes, *ie1* is the most extensively studied. WSSV *ie1* exhibits very strong promoter activity, and it is highly expressed throughout the WSSV infection cycle (Liu et al., 2005). WSSV *ie1* is activated by several host transcription factors, including STAT, NF-κB, HMGB, and Sf-PHB2 (Chen et al., 2011; Huang et al., 2010; Liu et al., 2007; Ma et al., 2012) and it was also recently shown to be activated by *Litopenaeus vannamei* Krüppel-like factor (LvKLF) (Huang et al., 2014).

Apart from WSSV *ie1*, however, many of the other WSSV IE genes are still poorly understood. The transcripts of one of these genes, which corresponds to *orf108* of the WSSV-TW strain (*WSSV108*, GenBank accession no. AF440570), has expression levels that are even higher than WSSV *ie1* throughout the WSSV infection cycle (Li et al., 2009). Sequence analysis of WSSV108 shows that it shares 25% identity with the BadM/Rrf2 family transcription factors, while a yeast GAL4 DNA binding domain fusion system found that WSSV108 exhibits transcriptional activity and may function as a transcriptional activator (Li et al., 2009). Other studies have suggested that WSSV108 might exploit the host SUMOylation pathway to activate the viral transcription cascade and the expression of early and

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http://dx.doi.org/10.1016/j.dci.2014.10.015
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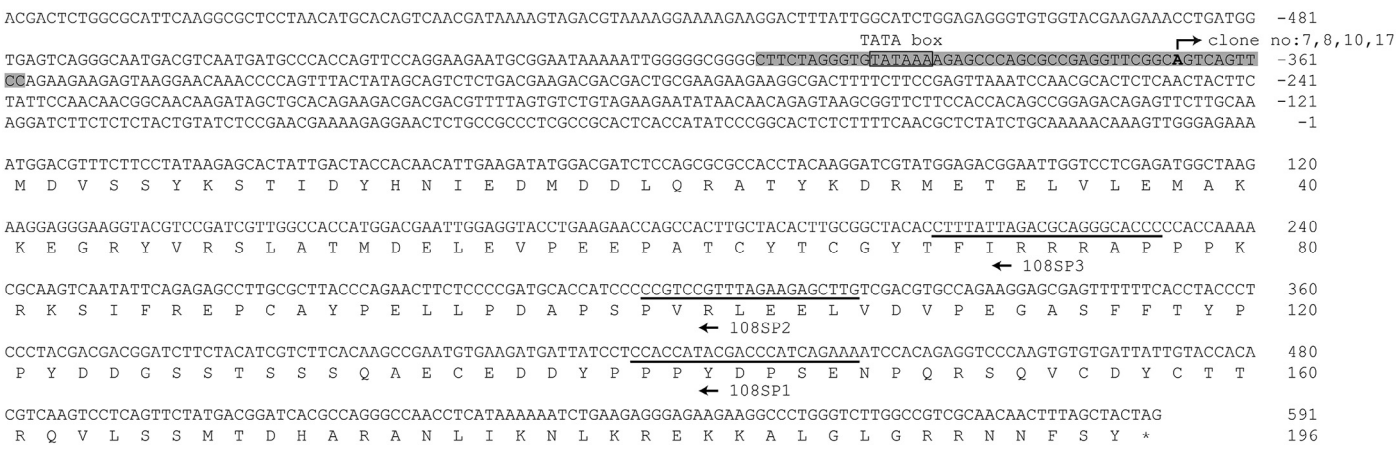

Fig. 1. Sequence of *WSSV108*. The deduced amino acid sequence is indicated below the nucleotide sequence. The primers used for 5′ RACE (108SP1, 108SP2 and 108SP3) are underlined. The shaded region between –408 and –359 nt from the translation start indicates the potential basal promoter element as predicted by the NNPP program. The bent arrow and boldface indicate the transcriptional start site as revealed by sequencing four randomly chosen 5′ RACE clones. The TATA box (TATAAA) is boxed.

late genes (Chen et al., 2013). WSSV108 therefore seems likely to play an important role in the WSSV infection cycle. In the present study, we use a series of deletions and site-directed mutations to perform a functional mapping of the *WSSV108* promoter, and we show that a DNA sequence that includes the consensus KLF binding site was critical for the promoter activity. Next, we use electrophoretic mobility shift assays (EMSA) and a transactivation analysis with a recombinant shrimp KLF to investigate the ability of PmKLF (*Penaeus monodon* KLF) to bind and activate the *WSSV108* promoter. Finally, we use *in vivo* gene silencing experiments to investigate the importance of shrimp KLF in the regulation of *WSSV108* expression and viral replication.

2. Materials and methods

2.1. WSSV inoculum preparation

The inoculum used in this study was prepared from the WSSV-TW strain, which originated from a batch of WSSV-infected *P. monodon* shrimp collected in Taiwan in 1994 (Wang et al., 1995). For WSSV inoculum preparation, we followed the methods described by Liu et al. (2007).

2.2. Experimental animals

Batches of *L. vannamei* (~3 g body weight) were used for the WSSV challenge and gene knockdown experiments. The *L. vannamei* used in this study were purchased from the Aquatic Animal Center at the National Taiwan Ocean University. All shrimp were acclimatized in the laboratory in water tanks with a salinity of 33 ± 1 ppt at 25 ± 1 °C for at least 3–5 days before the experiments.

2.3. Mapping the 5′ terminus of the WSSV108 transcripts

The 5′ end of the *WSSV108* mRNA was mapped by using the protocol described in the manufacturer's 5′ RACE (rapid amplification of the cDNA end) system manual (5′/3′ RACE kit, 2nd generation, Roche). First, the total RNA was isolated from the *L. vannamei* at 12 h after WSSV infection and treated with DNase I (Invitrogen). The three *WSSV108* gene-specific primers used for rapid amplification of the cDNA 5′ ends were designed based on the underlined sequences in Fig. 1 and are listed in Table 1. The final amplification products were cloned into pGEM-T Easy vector (Promega) and sequenced. The sequences of the inserts were checked against the WSSV genomic DNA sequence.

2.4. Construction of the series deletion WSSV108 promoter plasmids by PCR

To analyze the behavior of the *WSSV108* basal promoter and regulatory regions, progressive 5′ deletion plasmids were constructed from the promoter region by PCR cloning using genomic DNA from the hepatopancreas of WSSV-infected *L. vannamei* as the template. PCR with a universal reverse primer (Rev1) and six different forward primers (Table 2) was used to generate fragments that started at different positions and ended at nucleotide (nt) +368 relative to the transcription start site (+1). These DNA fragments, which contained the *WSSV108* promoter region and had XhoI and HindIII

Table 1
Primer sequences used for 5′ RACE.

Name	Primer sequences (5′–3′)
108SP1	TTTCTGATGGGTCGTATGGTGG
108SP2	CAAGCTCTTCTAAACGGACGG
108SP3	GGGTGCCCTGCGTCTAATAAAG

Table 2
Primers used for generating series deletion and mutant plasmids for *WSSV108* promoter activity assays. The restriction enzyme cutting sites are underlined.

Plasmid	Primer sequences (5′–3′)
p(–677/+368)	Fwd1 (CGACTCGAGGCTTGAACAACGCATTCTTC)
	Rev1 (GCTAAGCTTATTTCTCCCAACTTTGTTTTTGC)
p(–603/+368)	Fwd2 (CGACTCGAGTGTGCAACTCTCCACCTCC)/Rev1
p(–507/+368)	Fwd3 (CGACTCGAGTTGTGGAGGGAATAAGGGC)/Rev1
p(–410/+368)	Fwd4 (CGACTCGAGATTCTTCTTCCCCGTCATCC)/Rev1
p(–256/+368)	Fwd5 (CGACTCGAGAT AAGACGTGTTTAATGATGC)/Rev1
p(–100/+368)	Fwd6 (CGACTCGAGATGACGTCAATGATGCCCA)/Rev1
p(–507/+368)	KLF mut-F (ATTATGGAGTACGTTGTGGATT)
KLF-mut	KLF mut-R (CGCCCTTATTCCCTAAACAAG)
p(–507/+368)	GATA-1 mut-F (ACGTTGTGGATTGAGTATAAG)
GATA-1-mut	GATA-1 mut-R (ACTCCATAAGAGCCCTTATTC)
p(–507/+368)	C/EBP mut-F (AGCAAGCGTGGTATAACCCTG)
C/EBP-mut	C/EBP mut-R (TATACTCAATCCATTACGTAC)
p(–507/+368)	c-Myc mut-F (TTCTTCTTCCCCGTCATCCTC)
c-Myc-mut	c-Myc mut-R (TTAGTCATCGCTGCAGTCGGTG)
p(–507/+368)	AP-1 mut-F (CCTGCAGCGATCTCTAATTCTT)
AP-1-mut	AP-1 mut-R (TGGTGGTTTCAGGGTTATAC)

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