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NOTE

White spot syndrome virus down-regulates expression of histones H2A and H4 of *Penaeus vannamei* to promote viral replication

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ABSTRACT: The white spot syndrome virus (WSSV) is a highly lethal and contagious pathogen to most cultivated shrimp species. The WSSV genome contains the ICP11 gene and its encoded protein acts as a multifunctinal DNA mimic protein that disrupts the nucleosome assembly by binding to the histone proteins H2A and H3. In addition, WSSV provokes severe nuclear hypertrophy and DNA damage. However, little is known about the influence of WSSV on the expression of the host's genes encoding for histones. Therefore, we investigated the effect of WSSV infection on the expression of the genes encoding histones in shrimp *Penaeus vannamei*. An RT-qPCR assay was performed to evaluate the temporal expression of H2A and H4 transcripts in the shrimp. Significant changes were observed in the expression of these genes, which coincided with the dynamics of replication of the virus. H2A reached its maximum expression levels at 12 hpi. Thus, it may be suggested that this is a viral strategy to evade the host's immune response in order to promote viral replication.

KEY WORDS: WSSV · Histones · H2A · H4 · Immune evasion · Viral replication

1. INTRODUCTION

The eukaryotic genome is hierarchically organized into chromatin by repeating blocks of nucleosomes (Cutter & Hayes 2015), which are composed of a 'core', a linker DNA, and a linker histone. The structure of the core is relatively invariant from yeast to metazoans (White et al. 2001), and is composed of 2 copies of 4 different histone proteins (H2A, H2B, H3, and H4) (Arents & Moudrianakis 1993).

Among the pathogens affecting shrimp farming production in the world, the white spot syndrome virus (WSSV) (family *Nimaviridae*, genus *Whispovirus*) is regarded as the most lethal, highly contagious, and widespread viral agent (Sánchez-Paz 2010). Since its discovery, the virus has led to eco-

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nomic losses estimated in the range of US$ 8–15 billion yr^{-1} (Flegel et al. 2008, Stentiford et al. 2012).

Previous studies elucidating the gene expression profile of WSSV in infected hosts found that the gene encoding the non-structural protein ICP11 is highly expressed at both the transcriptional and translational levels (Wang et al. 2007). Further evidence revealed that the ICP11 protein directly interacts with the histones H2A, H2B, and H3, preventing the association of the DNA–histone complex and disrupting the assembly of the host cell nucleosome (Wang et al. 2008). Moreover, IPC11 interacts, as a DNA mimic, with the cytosolic forms of the histones H3 and H2A.x, interfering with its translocation into the nucleus, and interfering with nucleosome assembly (Wang et al. 2008). Since under normal conditions, the levels of H2A.x increase at sites of double-strand DNA breaks, its accumulation in the cytosol of WSSV-infected cells suggests that ICP11 additionally prevents DNA damage repair, which has been reported as an antiviral strategy with profound consequences on viral replication (Lilley et al. 2011, Luftig 2014). Feng et al. (2014) found that the levels of tyrosine phosphorylation of the histones H2A, H3, and H4 changed significantly during a WSSV infection in the shrimp *Fenneropenaeus chinensis*. Furthermore, DNA damage (nuclear shrinkage and DNA fragmentation) was observed in WSSV-infected hemocytes of *F. chinensis* (Feng et al. 2014). As histone tyrosine phosphorylation is a critical modification related to DNA repair and apoptosis in response to DNA damage, it seems plausible to correlate the tyrosine phosphorylation with the observed nuclear abnormalities, which may be a viral strategy to cause cell lysis, contributing to the spread of the pathogen. Although it is well established that the WSSV provokes nuclear hypertrophy and induces DNA damage (Abdel-Salam 2014), little is known about its effects on the expression profile of the histones encoding genes. Therefore, we studied the pattern of expression of the shrimp histones H2A and H4 during WSSV *in vivo* infection. This may contribute to a more comprehensive understanding of the deleterious changes occurring in the nucleus that may influence an apoptotic state of the host cell during the disease process.

2. MATERIALS AND METHODS

2.1. Shrimp samples

Specimens of the shrimp *Penaeus vannamei* (average weight: 12.5 ± 0.51 g) were sampled from a commercial shrimp farm located in Sonora, Mexico. Shrimp were maintained in 2 plastic tanks of 500 l with purified, aerated, and UV-treated seawater, and acclimated for 5 d at 28°C and 34 ppt salinity. Organisms were tested for the presence of *Penaeus stylirostris* penstyldensovirus 1 (PstDV1) and WSSV by methods described by Encinas-García et al. (2015) and Mendoza-Cano & Sánchez-Paz (2013), respectively.

2.2. WSSV inoculum preparation and experimental infection

A WSSV inoculum was prepared according to the method of Escobedo-Bonilla et al. (2005), and viral load was estimated by real-time PCR (qPCR) targeting a region of the VP28 encoding gene (Mendoza-Cano & Sánchez-Paz 2013). (For the kinetics of WSSV replication, see Fig. 1).

A total of 84 healthy shrimp were distributed equally into tanks of 500 l of seawater, into the following 2 treatments: the control group, inoculated with 100 µl of a 15.4 mM saline solution; and the infected group, intramuscularly injected into the third abdominal somite with 100 µl of WSSV inoculum (5.71×10^2 copies μl^{-1}). Gills and pleopods were carefully dissected from 6 shrimp from each treatment group at 0, 3, 6, 12, 24, 48, and 72 h post inoculation (hpi). Samples were fixed in 1 ml of RNAlater™ (Thermo Fisher Scientific) and stored until use. No moribund shrimp were detected during the experiment.

DNA was isolated from the pleopods using DNAzol® reagent (Invitrogen) following the manufacturer's specifications. Standard curves, based on the amplification of a fragment of the WSSV VP28 gene, were prepared for quantification of viral load in test samples. Pleopods were chosen for estimation of WSSV load because these appendages have shown a similar WSSV replication cycle as other tissues (Chen et al. 2011)

2.3. RNA isolation and cDNA synthesis

Total RNA was isolated from the gills using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Concentration of total RNA was estimated by a NanoDrop Lite spectrophotometer (Thermo Scientific) at 260 nm absorbance, and purity was assessed by the ratio of absorbance at 260/280 nm ($OD_{260/280} \geq 1.8$). RNA integrity was evaluated on 1% denaturing agarose gels following the methodology

described by Aranda et al. (2012). Genomic DNA was removed from RNA samples using DNase I recombinant RNase-free (Roche) according to the manufacturer's instructions. RNA was transcribed into cDNA using the SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen) according to the manufacturer's protocol.

2.4. Primer design and quantitative real-time PCR (qPCR)

Primers for quantitative real-time RT-PCR were designed from the H2A and H4 sequences of *P. vannamei* (GenBank accession numbers AY576483 and MH311299, respectively) using Primer3 software (http://bioinfo.ut.ee/primer3-0.4.0/primer3/input.htm), and tested for specificity using BLASTn (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul et al. 1990). Primers and amplicons are listed in Table 1.

Ten-fold dilution series of cDNA amplified with the H2A and H4 primers were used to construct a standard curve for each gene (for H2A, ranging from 2.62×10^7 to 2.62×10^3 copies μl^{-1}; and for H4, from 7.1×10^8 to 7.1×10^2 copies μl^{-1}). Samples were analyzed individually in triplicate. Quantification of the absolute mRNA copy number of the shrimp H2A and H4 genes was performed in a final reaction volume of 10 µl, consisting of 5 µl of iQ™ SYBR® Green Supermix (BioRad), 0.25 µl of each primer (10 pmol), 1 µl of cDNA template (160 ng μl^{-1}), and 3.5 µl of nuclease-free water, in 96-well plates using the LightCycler 480 system (Roche). Cycling conditions for the amplification were 1 cycle at 98°C for 3 min, followed by 35 cycles at 98°C for 15 s and 63°C for 60 s. Each PCR reaction was run in triplicate. PCR efficiencies for H2A and H4 were 96.41 and 98.48%, respectively, and the correlation coefficients (R^2) characterizing each standard curve were 0.9955 for H2A and 0.907 for H4.

2.5. Statistical analysis

The resulting data were tested for normality and homogeneity using the Shapiro-Wilk test. Since all data were normal, parametric statistics were applied, by using a 1-way ANOVA ($p \leq 0.05$). Additionally, Dunn's post hoc test for multiple comparisons was performed, to identify significant differences between the control group and the WSSV-infected group at 0, 3, 6, 12, 24, 48, and 72 hpi. Statistical analysis was performed using the software SigmaPlot 11.0.

To examine the relationship between the expression levels of the H2A and H4 genes and WSSV load, a Pearson correlation was calculated using a simple linear regression model in SigmaPlot 11.0 (n = 7 replicates).

3. RESULTS AND DISCUSSION

As shown in Fig. 1, the viral kinetics showed an initial peak (0 hpi) (~150 WSSV copies ng^{-1} of DNA), which is associated with the injection of the inoculum. Then, as expected, the viral load gradually decreased from 3 to 24 hpi. These results are in agreement with previous studies (García-Orozco et al. 2012, Gao et al. 2014, Li et al. 2015), and this drastic decrease may be due to the host immune response. Thereafter, the viral load began to increase and entered into an exponential phase until 72 hpi, when the number of viral copies reached a peak.

Fig. 2 provides an overview of the expression levels of the H2A and H4 genes in gills of the shrimp *Penaeus vannamei* during the WSSV infection experiment. At 0 hpi, no significant differences were observed in the expression levels of H2A and H4 when compared to those of uninfected specimens. It is generally accepted that gene expression of histones is regulated by a synchronized action of transcriptional

Table 1. Oligonucleotide primers used in this study for real-time PCR (qPCR)

Primer	Target	Sequence (5′–3′)	Product size (bp)	Reference
LvH2A F2 LvH2A R2	Histone H2A	ATG CTG AAC GTG TAG GTG CT CGG GTC TTC TTG TTG TCA CG	115	GenBank acc. no. AY576483
LvH4 F LvH4 R	Histone H4	AGG TGT TGC GTG ATA ACA TCC ACA CCA CGG GTT TCT TCG TA	112	GenBank acc. no. MH311299
VP28-140Fw VP28-140Rv	WSSV VP28	AGG TGT GGA ACA ACA CAT CAA G TGC CAA CTT CAT CCT CAT CA	140	Mendoza-Cano & Sánchez-Paz (2013)
qIH-Fw qIH-Rv	PstDV1	TAA GGA AGC CGA CGT AAC ATT G CGC ATT TGT TCC ATG AAT CC	120	Encinas-García et al. (2015)

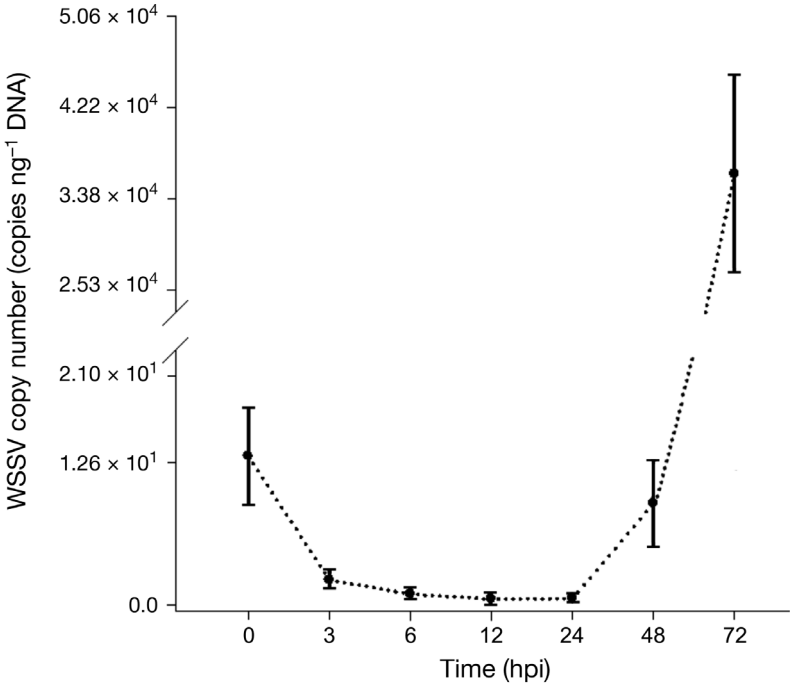

Fig. 1. Kinetics of white spot syndrome virus (WSSV) replication in whiteleg shrimp *Penaeus vannamei*. The dynamics of viral replication (copy numbers) during the course of infection was quantified by real-time PCR of viral DNA in a set of 2 pleopods from each shrimp (using the methodology of Mendoza-Cano & Sánchez-Paz 2013). Samples were analyzed individually in triplicate on the same organisms in which histone mRNAs were measured. hpi: hours post inoculation

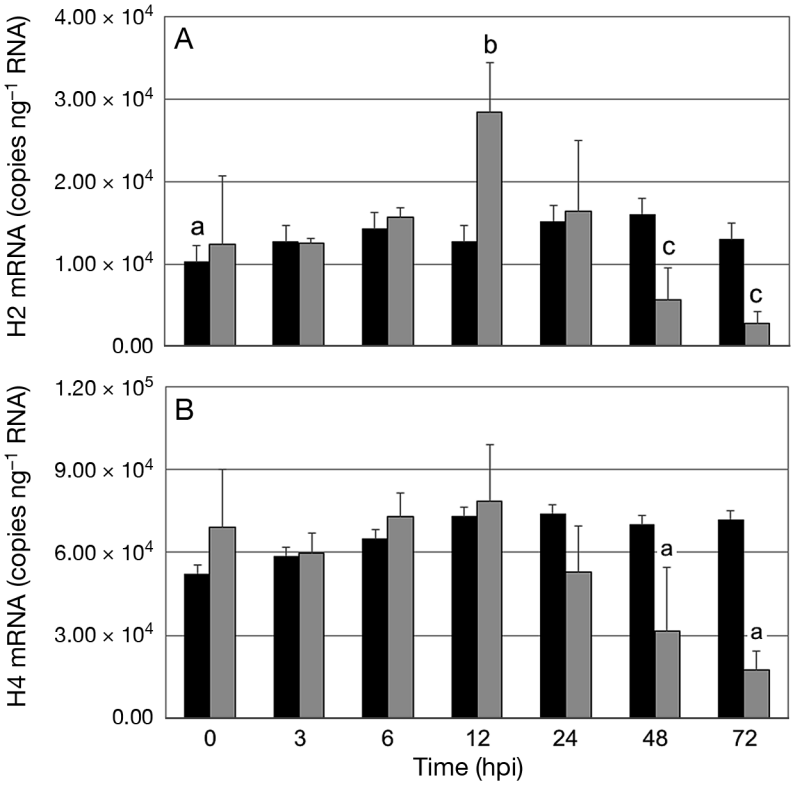

Fig. 2. Absolute expression of the *Penaeus vannamei* encoding genes for histones (A) H2A and (B) H4 in gills following white spot syndrome virus (WSSV) infection. Black bars: uninfected shrimp (control treatment); grey bars: WSSV-infected shrimp. Samples (n = 6 per treatment group per timepoint) were analyzed individually in triplicate. Error bars: SD. Different letters above the bars: significant differences between control and infected shrimp at each timepoint ($p < 0.05$). hpi: hours post inoculation

factors, histone chaperones, and chromatin-bound proteins (Zunder & Rine 2012), and their expression is strongly confined to the S-phase of the cell cycle, which is tightly regulated to ensure genomic stability (Lygerou & Nurse 2000). Thus, the initial expression levels of H2A and H4 in infected shrimp may be controlled by transcription repressors, histone chaperones, chromatin-bound proteins, or acetylation levels (Mei et al. 2017) to maintain as much as possible normal cell cycle progression, genome stability, and gene transcription.

It is worth mentioning that the steady-state levels of both genes were regulated coordinately and changed more abruptly after 12 hpi. In both cases (Fig. 2), maximum levels of expression were achieved at 12 hpi, after which the expression declined sharply, reaching its lowest levels at 72 hpi, which coincides with the WSSV replication dynamics in Fig. 1. No significant changes were observed in H2A expression levels in the uninfected controls. The amount of H2A mRNA gradually increased to reach maximum levels at 12 hpi, and subsequently, the number of transcripts of this gene dropped abruptly until 72 hpi (Fig. 2A). Similarly, significant changes were detected in the abundance of H4 mRNA transcripts at 48 and 72 hpi, when H4 mRNA expression was drastically reduced; however, the transcription levels of this gene remained fairly constant during the first 12 hpi. No significant changes were observed in H4 expression levels in uninfected controls (Fig. 2B).

The expression profile of the H2A and H4 genes coincides with the WSSV replication kinetics. Remarkably, the expression of H2A gave a medium negative Pearson correlation value ($r = -0.566$, $p = 0.185$), while the expression of H4 gave a very high negative correlation ($r = -0.726$, $p = 0.0648$). At 12 hpi, when the WSSV load was almost undetectable (due to the immune response of the shrimp), the abundance of H2A and H4 mRNAs were at maximal levels. However, as the WSSV infection progressed to its maximum levels at 72 hpi, the number of transcripts of these genes decreased markedly. Arockiaraj et al. (2013) found that mRNA levels of a gene encoding a histone of the freshwater prawn *Macrobrachium rosenbergii* (*Mr*His), highly similar to H2A, increased almost 19-fold at 12 h after the specimens were challenged with WSSV, and subsequently decreased until 48 hpi. Our results revealed that the H2A expression in *P. vannamei* infected with WSSV showed a similar trend to that reported by Arockiaraj et al. (2013), but less conspicuous. The observed differences between our findings and those of Arockiaraj et al. (2013) may be a species-specific response to the virus. The increase in H2A expression at 12 hpi observed in the current study may be a pivotal process to inhibit viral replication. New evidence has highlighted the participation of histones in the host's immune response (Parseghian & Luhrs 2006, Sathyan et al. 2012). Several studies have demonstrated that fragments of the histone H2A possess antimicrobial activity (Kim et al. 1996, Park et al. 1998, Arockiaraj et al. 2013), and that a peptide derived from the N-terminus of the H2A histone of *P. vannamei* possesses antimicrobial activity (Patat et al. 2004), and Sruthy et al. (2019) demonstrated the antimicrobial activity of an H2A-derived histone peptide (Fi-Histin) from the Indian white shrimp *Fenneropenaeus indicus* against Gram-negative (*Vibrio vulnificus* and *Pseudomonas aeruginosa*) and Gram-positive bacteria (*Staphylococcus aureus*). Thus, the overexpression of H2A to generate peptides with antiviral activity may represent a novel mechanism to block viral replication. However, this possibility still remains speculative, and further research is needed to elucidate a possible role of the histone-derived peptides against WSSV.

Histones are proteins that condense eukaryotic nuclear DNA into chromatin, and their structure and regulation are highly important in transcription and DNA replication (Wolffe & Hayes 1999). Histone H2A is responsible for the packaging and compactation of DNA in the nucleus. In addition, H2A displays a high diversity of variants that are involved in apoptosis, DNA repair, gene regulation, and genome integrity (González-Romero et al. 2012). Histone H4, a nucleosome subunit in eukaryotes, when acetylated, plays a crucial role in chromatin decompaction during DNA replication (Ruan et al. 2015). Thus, it seems plausible that WSSV down-regulates the expression of the histones H2A and H4, both involved in regulatory switches within the transcriptome, in order to reduce the number of relevant host transcripts, to evade the host's immune system. A similar strategy to sequester the host's immune system by controlling its gene expression through interfering with chromatin functionality has been reported in the influenza A virus (Marazzi et al. 2012). Hepat & Kim (2011) reported that transient expression of the viral histone H4, a histone mimic encoded by the *Cotesia plutellae* bracovirus (CpBV), in late-instar larvae of the red flour beetle *Tribolium castaneum* affected the normal epigenetic control of the expression of 12 genes encoding antimicrobial peptides, suppressing the host's immune response. In a subsequent study, Hepat et al. (2013) injected an expression vector containing the open reading frame encoding for an H4 viral histone of CpBV (CpBV-H4) into the hemocoel of late-instar larvae of *T. castaneum*, and total RNAs were extracted and read through a next-generation sequencing technique. It was found that the viral histone H4 alters host gene expression by interacting with the host nucleosome, and inhibiting its immune response. Finally, Avgousti et al. (2016) reported that protein VII of adenoviruses, which resembles cellular histones, forms complexes with nucleosomes and interferes with DNA accessibility, a mechanism to blunt the host immune response.

Zhu et al. (2018) found that the expression of the histones H2A, H2B, H3, and H4 in hemocytes of the Chinese shrimp *F. chinensis* was down-regulated upon WSSV infection. This is in agreement with our findings of the reduction of the expression of the histone H2A of shrimp. Feng et al. (2014) found an upregulation of H4 in hemocytes of *F. chinensis* infected with WSSV from 0 to 6 hpi, which was followed by a sharp decline at 12 and 18 hpi. That was a similar transcriptional profile of H4 to that observed in the current study. Thus, this may be a strategy of WSSV to suppress the synthesis of proteins involved in the immune response. However, further studies are still required to clarify this point.

Another reasonable explanation for the down-regulation of H2A and H4 may be related to cell death induced by the WSSV protein ICP11. As we mentioned above (Section 1), ICP11 is the most highly

expressed WSSV nonstructural protein. The expression of ICP11 induces cell death either by a direct induction of nucleosome disorder or through nucleosome destabilization by binding to histones in the cytoplasm, which prevents its translocation into the nucleus (Wang et al. 2008). Thus, as viral load increases with disease progression, cell death becomes common, and the expression of H2A and H4 steeply declines with time.

The present study reinforces the notion that the multiple pathogenic effects of WSSV involve viral products that induce complex alterations in cellular functions. The down-regulation of the host's histones is a highly sophisticated strategy of WSSV to evade shrimp immunity, thus favoring viral perpetuation.

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