Identification and localization of a prawn white spot syndrome virus gene that encodes an envelope protein

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Among the important challenges to shrimp aquaculture worldwide are the diseases caused by viruses, in particular by white spot syndrome virus (WSSV), which has a genome estimated to contain 305 kb. By analysis and comparison of the WSSV genomic DNA and cDNA libraries, an ORF (vp28 gene) was identified. The gene, encoding a novel 204-amino-acid protein, was expressed in Escherichia coli and purified. A specific antibody was raised using the purified VP28 protein. After inoculation of healthy adult Penaeus monodon shrimp with WSSV, the gene transcript and VP28 protein were first detected at low levels at 6 and 18 h post-infection, respectively. These experiments suggest that it might be a late gene. Immuno-electron microscopy with gold-labelled antibody revealed that the gold particles were distributed in the outer envelope of WSSV virions and showed that vp28 encodes a virus envelope protein.

Introduction

In 1993, an epizootic broke out in the cultured prawn population in South-east Asia. This epizootic has spread and has now become one of the most serious diseases known to the shrimp farming industry all over the world, causing heavy economic losses. A non-occluded virus, prawn white spot syndrome virus (WSSV), was found to be the major pathogen of the disease (Chen et al., 1997; Chou et al., 1995; Wongteerasupaya et al., 1995). Electron microscopy studies revealed that virions of WSSV are enveloped, non-occluded and rod-shaped (Chang et al., 1996; Chen et al., 1997; Chou et al., 1995; Wongteerasupaya et al., 1995). The virion morphology and morphogenesis are reminiscent of insect baculoviruses (Durand et al., 1997), but the family that WSSV belongs to is still unknown. WSSV has not yet been assigned to a virus family (Chen et al., 1997). In 1997, WSSV genomic DNA was successfully purified from Penaeus japonicus (Yang et al., 1997) and genomic DNA and cDNA libraries were constructed. Sequencing of WSSV genomic DNA revealed that it consists of a 305 kb double-stranded circular DNA (Yang et al., 2001). Studies on WSSV gene structure and regulation will be helpful for diagnosis and control of the virus infection. However, only a few reports have been published because of the difficulties of culturing shrimp viruses in cell lines and the purification of WSSV (Tsai et al., 2000; van Hulten et al., 2000a, b; Zhang et al., 2001a). The present study aimed to identify WSSV genes from our cDNA library and their localization and function. The vp28 gene is one of the genes that we have characterized.

Methods

Screening, sequencing and computer analysis of the vp28 gene. mRNA from WSSV-infected tissues of P. japonicus was isolated and reverse-transcribed into cDNA. Double-stranded cDNA fragments were blunt-ended, cloned into pBluescript SK+ (Stratagene) with restriction enzyme EcoRI/HindIII. The corresponding plasmids were prepared and purified with resin (Sambrook et al., 1989). Clones containing 800–2000 bp inserts were selected from the cDNA library of WSSV by digestion with EcoRI + HindIII. The corresponding plasmids were prepared and purified with resin (Sambrook et al., 1989).
Subsequently, sequencing was performed using pBluescript KS and sk primers in an automatic DNA sequencer. The DNA sequences generated and the deduced amino acid sequences were respectively analysed using DNAsis and pross software (Hitachi Software Engineering, version 4). The deduced amino acid sequences were subjected to BLAST analysis in the GenBank and SWISS-PROT databases.

Expression of the vp28 gene in E. coli. The vp28 gene was cloned and expressed in pBV220–DH5α (Zhang et al., 1990). PCR was performed using primers synthesized (Shanghai Beckman Life Science and Technology Laboratory) for amplification of the vp28 gene. The forward primer used was 5’ AGAGAATTCATGATCTTCTTCGCAG 3’ (EcoRI site in italics) and the reverse primer was 5’ CAGGTCGACTTACTCG-GTCTCAGTGC 3’ (SalI site in italics). The amplified DNA and plasmid vector pBV220 were digested with EcoRI + SalI. After purification and ligation of DNA fragments, the vp28 gene was inserted into pBV220 vector downstream of its promoter. The resulting plasmid was named pBV28. Competent cells of E. coli DH5α were transformed with pBV28 and colonies containing transformants were screened by PCR. The presence of pBV28 was verified by digestion with EcoRI + SalI and DNA sequencing. The following treatments were conducted for the expression of vp28:

A. pBV28–DH5α (containing the vp28 gene) induced;
B. pBV28–DH5α non-induced;
C. pBV220–DH5α (the vector only as a control) induced;
D. pBV220–DH5α non-induced.

After incubation at 30 °C overnight, pBV28–DH5α and pBV220–DH5α were inoculated into new medium at a ratio of 1:100. The cultures were grown to an OD600 of 0:5 at 30 °C and then transferred immediately for induction for an additional 6 h at 42 °C. The induced and non-induced bacteria were analysed by SDS–PAGE.

Purification of expressed protein. The recombinant pBV28–DH5α was inoculated and cultured in LB medium at 30 °C overnight. Cells were transferred into 1000 ml fresh LB medium and incubated at 30 °C. When the OD600 was 0:5, the culture was moved immediately to 42 °C for 6 h. The induced bacteria were spun down (5000 r.p.m.) at 4 °C, followed by suspension in lysis buffer (50 mM Tris–HCl, 1 mM EDTA, 100 mM NaCl, 2% Triton X-100, 2 M urea, 1 mM PMSF, pH 8:0) and sonication for 30 s on ice. The sonicated sample was treated with DNase I at room temperature for 1 h and then spun down at 30000 g for 30 min. The pellet was resuspended in Milli-Q water. After addition of an equal volume of TE buffer (10 mM Tris–HCl, 1 mM EDTA, 1 M NaCl, 8 M urea, 1% SDS, pH 8:0), the mixture was placed at room temperature for 1 h and then centrifuged at 100000 g for 10 min. The supernatant was dialysed against 0:05 M Tris–HCl (pH 8:0) and subjected to SDS–PAGE.

After running the extract on SDS–PAGE, a section of the gel was stained in 0:3 M CuSO4 to determine the position of the expressed VP28 protein. The remaining, non-stained portion of the gel at the position corresponding to VP28 was then excised and transferred into dialysis tubing. After electrophoresis in SDS–PAGE electrode buffer for 1 h, the eluate in the tubing was dialysed against 0:05 M Tris–HCl, pH 8:0.

Preparation of antibody. Mice were immunized by intradermal injection of the purified VP28 protein fortnightly over an 8-week period. Five µg antigen (VP28) was mixed with an equal volume of Freund's complete adjuvant (Sigma) for the first injection. Subsequent injections were conducted using 5 µg antigen mixed with an equal volume of Freund's incomplete adjuvant (Sigma). Four days after the last injection, mice were exsanguinated and antisera were collected. The titre of anti-VP28 sera were 1:20000, as determined by ELISA. The IgG fraction was purified with protein A–Sepharose (Bio-Rad) (Sambrook et al., 1989) and stored at −70 °C. The optimal dilution of purified IgG, after serial dilutions, was 1:1000 as determined by ELISA. Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG was obtained from Sigma. Antigen was replaced by PBS in control assays.

Transcriptional analysis of vp28 gene

Shrimp infection with WSSV. Infected tissue from a Penaeus monodon shrimp with a pathologically confirmed infection was homogenized in TN buffer (20 mM Tris–HCl, 400 mM NaCl, pH 7:4) at 0:1 g/ml. After centrifugation at 2000 g for 10 min, the supernatant was diluted to 1:100 with 0:9% NaCl and filtered (0:22 µm filter). Aliquots (0:2 ml) of the filtrate were injected intramuscularly into healthy shrimps (as determined by PCR) in the lateral area of the fourth abdominal segment. At various times post-infection (p.i.), four specimens were selected at random and their haemolymph was collected. The collected haemolymph samples were frozen immediately and stored at −70 °C.

RT–PCR. Total RNA was isolated from WSSV-infected shrimp haemolymph according to the manufacturer’s instruction (Macherey-Nagel). RT–PCR was performed with primers 5’ AGAGAATTCATGATCTTCTTCGCAG 3’ and 5’ CAGGTCGACTTACTCG-GTCTCAGTGC 3’ by using the TITANIUM One-step RT–PCR kit (Clontech). The RT–PCR program was as follows: 50 °C for 1 h followed by 94 °C for 5 min; 30 cycles of 94 °C for 30 s, 65 °C for 30 s and 68 °C for 1 min and, finally, 68 °C for 2 min.

Western blot. Infected shrimp haemolymph samples from various time-points were analysed in 12% SDS–PAGE gel. Proteins were visualized by Coomassie brilliant blue staining. The proteins were transferred onto nitrocellulose membrane (Bio-Rad) in electroblotting buffer (25 mM Tris–HCl, 190 mM glycine, 20% methanol) for 3 h. The membrane was immersed in blocking buffer (3% BSA, 20 mM Tris–HCl, 0:9% NaCl, 0:1% Tween 20, pH 7:2) at 4 °C overnight followed by incubation with polyclonal mouse anti-VP28 IgG for 3 h. Subsequently, the membrane was incubated in HRP-conjugated goat anti-mouse IgG (Sigma) for 1 h and detected with substrate solution (4-chloro-1-naphthol, Sigma).

WSSV and immuno-electron microscopy (IEM). Infected tissue from P. monodon shrimp was homogenized and centrifuged as above and the supernatant was injected (1:100 dilution in 0:9% NaCl) intramuscularly into healthy Cambareus clarkii crayfish from Singapore in the lateral area of the fourth abdominal segment. Four days later, haemolymph, freshly extracted from infected crayfish, was layered on the top of a 10–40% (w/v) continuous sodium bromide gradient and centrifuged at 110000 g using an RP40–T rotor in a Prespin ultracentrifuge (Shimadzu Model MSE-75) for 2 h at 4 °C. Virus bands were collected by side puncture, diluted 1:10 in TNE buffer (50 mM Tris–HCl, 100 mM NaCl and 1 mM EDTA, pH 7:4) and pelleted at 119000 g for 1 h at 4 °C. The resulting pellets were resuspended in TNE as intact WSSV virions (Huang et al., 2001). Some intact WSSV particles were treated with 0:5–10% Triton X-100 for 30 min at room temperature and then centrifuged at 119000 g using an SW41-Ti rotor (Beckman). The pellet was resuspended in 0:1 x TNE buffer and centrifuged at 119000 g. After several repeats to remove the Triton X-100 completely, the resulting WSSV nucleocapsids were resuspended in TNE. Virus samples were examined under transmission electron microscope (JEOL 100 cx II) for purity.

The purified WSSV virion suspension and nucleocapsids were mounted on carbon-coated nickel grids (200 mesh) and incubated for 1 h at room temperature. After washing with PBS, the grids were blocked in 3% BSA for 1 h. The grids were rinsed with PBS and incubated in anti-VP28 IgG or anti-grouper iridovirus IgG (kindly provided by Qiwei Qin, Tropical Marine Science Institute, National University of Singapore) as a
Results

Structure of the WSSV vp28 gene

Many genes of insect baculoviruses encoding structural proteins are slightly more than 1000 bp in length. From this, it was inferred that the sizes of the transcripts for the envelope and nucleocapsid protein genes of WSSV are probably 1000 nt or more. Therefore, clones with 800–2000 bp inserts were screened and sequenced from a cDNA library of WSSV constructed in our laboratory. An ORF of 612 bp was identified in clone C37 (Fig. 1). The nucleotide sequence of the insert was compared with the genomic DNA sequence of WSSV. This showed that the sequence of C37 was identical with that of a fragment of genomic DNA except for its poly(A) tail (Fig. 1). This confirmed that C37 was transcribed from the genomic DNA of WSSV.

The ORF in C37 encoded a 204 aa protein (VP28) with a theoretical molecular mass of 23 kDa, and it was termed the vp28 gene (GenBank accession no. AF227911). Sequencing has shown that the WSSV genome is a 305 kb double-stranded circular DNA, and a guanine residue at the start of the largest BamHI fragment has been designated as the starting point of the physical map of the genome (Yang et al., 2001). The vp28 gene was located on a 27 277 bp BamHI fragment of the WSSV genome. A typical TATA box (TATAT) was found 14 bp upstream of the transcription initiation site of C37. Two putative polyadenylation signals (AATAAA) were present, 212 and 14 bp upstream of the poly(A) (Fig. 1). Its 5’ and 3’ non-coding regions were longer than the corresponding regions of genes from baculoviruses and WSSV, respectively 482 and 293 bp. In the leading sequence of the vp28 gene, a

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**Fig. 1.** Nucleotide sequence of the genomic region containing the C37 clone and the deduced amino acid sequence (single-letter code) of vp28. The transcription initiation site of C37 and position of the poly(A) tail are respectively indicated by solid and dotted arrows. The putative TATA box and polyadenylation signal are marked by solid and dotted lines, respectively. The primers used for the amplification of vp28 gene are shown by thick bars.
minicistron could be found in-frame with \( vp28 \) (Fig. 1). The nucleotide sequence surrounding the methionine start codon (GTCATGG) of \( vp28 \) was consistent with Kozak’s rule for efficient eukaryotic translation initiation (PuNNATGPu) (Kozak, 1987). The amino acid sequence of the VP28 protein was subjected to BLAST analysis in GenBank. It was identical to a presumptive envelope protein of WSSV (van Hulten et al., 2000a). Computer analysis (PROSIS) of VP28 showed that there was a strong hydrophobic region at its N terminus. At its C terminus, another hydrophobic region was found.

**Expression and purification of the \( vp28 \) gene product**

The \( vp28 \) gene was cloned into the pBV220 vector containing the promoter P\(_R\)P\(_L\). The recombinant plasmid was named pBV28. pBV28 and pBV220 were transformed into DH5\( \alpha \). After induction at 42°C, induced and non-induced pBV28–DH5\( \alpha \) and pBV220–DH5\( \alpha \) were analysed by SDS–PAGE (Fig. 2). A band corresponding to the VP28 protein was observed in the induced pBV28–DH5\( \alpha \) (Fig. 2, lane 4). No protein was found at the same positions in the non-induced

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**Fig. 3.** Temporal analysis of \( vp28 \) gene transcription. (a) RT–PCR with \( vp28 \) gene-specific primers using total RNA extracted from the haemolymph of healthy and infected shrimp. (b) Western blot with anti-VP28 antibody using the haemolymph of healthy and infected shrimp. Lane headings show times post-infection (in hours). Lanes M: DNA (a) or protein (b) markers.

**Fig. 4.** IEM of purified WSSV virions and nucleocapsids with anti-VP28 IgG or anti-grouper iridovirus IgG followed by gold-labelled secondary antibody. (a) Intact WSSV virions. (b) WSSV virions labelled with anti-VP28 IgG and gold. (c) WSSV nucleocapsids labelled with anti-VP28 IgG and gold. (d) WSSV virions labelled with anti-grouper iridovirus IgG and gold. Arrows indicate gold particles. Bars, 92.6 (b) or 185 (a, c, d) nm.
pBV28–DH5x (Fig. 2, lane 2) or in induced and non-induced pBV220 cells (Fig. 2, lanes 3 and 5). This showed that the vp28 gene was expressed. The VP28 protein was extracted and purified from the inclusion bodies by SDS–PAGE (Fig. 2, lane 6).

Temporal analysis of vp28 gene transcription

RT–PCR was used to detect the vp28 gene-specific transcript from haemolymph of adult P. monodon shrimp at various stages (0, 6, 18, 24, 30, 36 and 48 h p.i.) of infection with WSSV. The vp28 gene transcript was first detected at a low level at 6 h p.i. and continued to be detected up to 48 h p.i. (Fig. 3a). This suggested that vp28 might be a late gene.

Haemolymph samples taken both before and after (at 6, 18, 24, 30, 36 and 48 h) artificial infection with WSSV were transferred onto nitrocellulose membrane for the detection of VP28 protein from WSSV with Western blot. The VP28 protein was first detected at 18 h p.i. (Fig. 3b), which was later than the mRNA transcript. The VP28 protein was produced maximally at 24 h p.i.

Transmission electron microscopy studies

Crayfish (Cambarus clarkii) were infected with WSSV by intramuscular injection of a virus preparation from P. monodon shrimp. Four days after infection, the virus was isolated and purified from the haemolymph. As a negative control, haemolymph was also taken from healthy crayfish. These preparations were observed under transmission electron microscopy for the presence and purity of WSSV virions. No virus particles were found in the healthy crayfish samples, but, in the infected samples, numerous enveloped and rod-shaped virions were found (Fig. 4a).

Purified WSSV virions and nucleocapsids were incubated with anti-VP28 IgG followed by incubation with gold-labelled secondary antibody on carbon-coated nickel grids. After hybridization, gold particles could be seen clearly surrounding the envelopes of WSSV virions (Fig. 4b), but no gold particles could be observed in the non-enveloped nucleocapsid (Fig. 4c) or outside the virions. When an anti-grouper iridovirus IgG was used instead of anti-VP28 IgG, no gold particles were detected (Fig. 4d). This showed that the VP28 protein was distributed in the outer envelope of WSSV virions.

Discussion

In this study, we have successfully cloned a novel gene (vp28) from WSSV by using a cDNA library. The gene possibly encoded a structural protein. In order to locate the vp28 gene product by IEM, the gene was expressed in E. coli and the purified VP28 protein was used as an antigen to prepare a specific antibody. While our studies were in progress, homology searches with the VP28 protein against GenBank using FASTA and BLASTA showed that vp28 was identical to a gene reported by van Hulten et al. (2000a). The product of the latter gene was identified from SDS–PAGE of purified WSSV and N-terminal amino acid sequencing and was inferred from the difference between protein bands of purified WSSV virions and nucleocapsids to be a virus envelope protein. However, there was no direct information regarding its intracellular localization. In the present study, IEM revealed that gold particles were present along the outer envelope of WSSV virions, but not non-enveloped nucleocapsids. This provided visual evidence that the vp28 gene encodes one of the envelope proteins of WSSV. However, the VP28 protein was not glycosylated, as shown by SDS–PAGE of purified WSSV followed by glycoprotein detection (Immun-Blot kit for glycoprotein detection, Bio-Rad; data not shown). vp28 is one of the structural genes that we have located in WSSV.

Temporal analysis revealed that vp28 might be a late gene of WSSV, but the conserved motif (ATAAG) present in late genes of insect baculoviruses could not be found in the DNA sequence of the vp28 gene. This suggests a difference between WSSV and baculoviruses. Analysis showed that the 5’ non-coding region of the gene was longer than the corresponding region of genes from baculoviruses and WSSV. A minicistron was found in the leading sequence of vp28 (Fig. 1), which might affect translation of the vp28 gene. The significance of this observation is presently unknown.

Previously, anti-VP28 IgG served successfully as a specific diagnostic reagent in the detection of WSSV in shrimp by ELISA (Zhang et al., 2001b). The IEM result confirmed the immunoassay detection. Moreover, because the VP28 protein is one of the major envelope proteins of WSSV, it could be used for further analysis, in particular, to study its relatedness to structural proteins of other viruses, including baculoviruses. It could also be used to examine the infection process of WSSV in shrimp by hybridization in situ and to study whether the VP28 protein has an effect on WSSV infection. Such studies would be helpful to reveal the function of the gene.

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References


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