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NOTE

Conservation of the DNA sequences encoding the major structural viral proteins of WSSV

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ABSTRACT: A cDNA library was constructed from white spot syndrome virus (WSSV)-infected penaeid shrimp tissue. cDNA clones with WSSV inserts were isolated and sequenced. By comparison with DNA sequences in GenBank, cDNA clones containing sequence identical to those of the WSSV envelope protein VP28 and nucleoprotein VP15 were identified. Poly(A) sites in the mRNAs of VP28 and VP15 were identified. Genes encoding the major viral structural proteins VP28, VP26, VP24, VP19 and VP15 of 5 WSSV isolates collected from different shrimp species and/or geographical areas were sequenced and compared with those of 4 other WSSV isolate sequences in GenBank. For each of the viral structural protein genes compared, the nucleotide sequences were 100 to 99% identical among the 9 isolates. Gene probes or PCR primers based on the gene sequences of the WSSV structural proteins can be used for diagnoses and/or detection of WSSV infection.

KEY WORDS: White spot syndrome virus · Viral structural proteins · VP28 · VP26 · VP24 · VP19 · VP15

INTRODUCTION

White spot syndrome virus (WSSV), which has been associated with major viral epizootics, was first identified in Taiwan and China between 1992 and 1993 (Chen 1995, Cai et al. 1995). WSSV has quickly spread to most shrimp-farming countries in Asia, such as Japan (Nakano et al. 1994), Thailand (Flegel et al. 1995), India (Mohan et al. 1998) and the Philippines (Magbanua et al. 2000). Although Asian in origin, WSSV was reported in the USA in 1995 in shrimp farms in Texas (Lightner et al. 1997) and subsequently in South Carolina (Nadala & Loh 1998). Since January 1999, WSSV has been detected in tissue samples of cultured shrimp from Central and South America (www.aphis.usda.gov/vs/aqua/wssv.html). In addition to cultured penaeid shrimp, WSSV has been detected in other crustaceans, including crabs and crayfish (Lo et al. 1996). The genomes of 3 WSSV isolates have been sequenced (Lo & Kou 2001, van Hulten et al. 2001, Yang et al. 2001), providing a molecular basis for proper classification and better understanding of WSSV replication and pathogenicity. Previous studies have shown that genetic variations exist among WSSV isolates (Nadala & Loh 1998, Lo et al. 1999). In this study, cDNA clones containing the genes encoding the WSSV envelope protein VP28 and nucleoprotein VP15 were isolated and the poly(A) sites in the mRNAs were identified. The genes encoding the major structural proteins of WSSV isolated from different geographic areas and from different shrimp species were sequenced and compared.

MATERIALS AND METHODS

WSSV 95C, previously named Chinese baculovirus (CBV), was originally isolated from *Penaeus japonicus* from Dalian, China (Lu et al. 1997). A cDNA library was constructed from WSSV 95C-infected shrimp tissue as

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Table 1. WSSV isolates from which structural protein genes were compared in this study

Viral isolate	Origin	Host animal	Source
WSSV 95C[a]	Dalian, China	*Penaeus japonicus*	This study
WSSV 97Indo[a]	Indonesia	*Penaeus monodon*	This study
WSSV 98Japan[a]	Japan	*Penaeus japonicus*	This study
WSSV 98SC[a]	South Carolina, USA	*Penaeus setiferus*	This study
WSSV 99C[a]	Qingdao, China	*Penaeus chinesis*	This study
WSSV Xiamen	Xiamen, China	*Penaeus japonicus*	Yang et al. (2001)
WSSV Thailand	Thailand	*Penaeus monodon*	van Hulten et al. (2001)
WSSV Taiwan	Taiwan	Not specified	Lo & Kou (2001)
WSSV Korea[b]	Korea	*Penaeus chinesis*	Moon et al. (2003)

[a]The structural protein genes (vp15, 19, 24, 26 and 28) of these isolates were sequenced in this study. The nucleotide sequences were deposited in GenBank (accession numbers: AY249434 to AY249458)

[b]Moon et al. (2003) sequenced the genes encoding the VP28, 26 and 24 proteins for the WSSV Korea isolate.

follows. Total RNA was isolated from WSSV 95C-infected shrimp gill tissues using TRL_{ZOL}®LS Reagent (GIBCO). The mRNAs were isolated from the total RNA using Oligotex™ (Qiagen). cDNA was synthesized using the TimeSaver™ cDNA Synthesis Kit (Amersham Pharmacia Biotech) with $Oligo(dT)_{12-18}$ as primer. The cDNA library was constructed by cloning cDNA into *Eco*RI-digested plasmid vector pUC19 and using this to transform competent *E. coli* XL1-Blue cells. WSSV 95C genomic DNA was isolated as previously described (You et al. 2002) and labeled with digoxigenin (DIG)-dUTP (Boehringer Mannheim). The WSSV-cDNA clones were selected from the cDNA library by hybridization with the DIG-labeled WSSV genomic DNA probe. DNA sequencing was performed with an automated DNA sequencer (Model 377, Applied Biosystems) at the Biotechnology and Molecular Biology Instrumentation Facilities, University of Hawaii.

Five WSSV isolates were used in this study (Table 1). Specific pathogen-free (SPF) *Penaeus vannamei* were used for WSSV propagation. WSSV propagation and purification methods have been described by Nadala et al. (1998).

For sequence comparison of the genes encoding the major structural proteins of WSSV isolated from different shrimp species and/or geographic areas, 5 pairs of primers (Table 2) were designed, based on the DNA sequences of VP28, 26, 24,19 and 15 of the WSSV Thailand isolate (van Hulten et al. 2001). Genomic DNA of 5 WSSV isolates (Table 1) was purified as described previously (You et al. 2002). The PCR reactions were performed using the GeneAmp PCR System 9600 (Perkin Elmer). Standard PCR reactions (final volume of 50 µl) contained 10 ng of WSSV DNA, 200 µM each of dATP, dTTP, dCTP and dGTP, 0.2 µM of each specific primer, 2.5 mM $MgCl_2$ and 1.25 units *Pfu* DNA polymerase (Stratagene). The PCR was run for 30 cycles of 30 s at 94°C, 30 s at 60°C and 30 s at 72°C. The PCR products were purified using a PCR purification kit (Qiagen) and sequenced in both directions.

cDNA sequences were analyzed using the BLAST® program. Homologous sequences were aligned using ClustalW (Thompson et al. 1994) (clustalw.genome.ad.jp) and the alignments were printed out with the BOXSHADE program (bioweb.pasteur.fr/seqanal/interfaces/boxshade.html).

RESULTS AND DISCUSSION

Among the WSSV cDNA clones which were identified and sequenced, clone 95C-C6 had an insert of ~750 base pairs (bp). An open reading frame (ORF) of 615 bp was identified in this insert. Nucleic acid

Table 2. Primers used for PCR and sequencing of genes encoding the WSSV major structural proteins

Gene	Primers	Size of PCR product (bp)
vp28	vp28F 5′ CGACATCTTAATAACCAAGCAACG 3′ vp28R 5′ AAAAGCACGATTTATTTACTCGG 3′	677
vp26	vp26F 5′ ATCCAACCAACACGTAAAGG 3′ vp26R 5′ CAATTCCCACTTTACTTCTTCTTG 3′	673
vp24	vp24F 5′ AATAAATCTCTCCCTAACAATGAAAGG 3′ vp24R 5′ TTTTCTCTCATGACCTTTGTACAACTT 3′	762
vp19	vp19F 5′ GTCTTTACGTTACATTGACGTAC 3′ vp19R 5′ GTTTTAATTATAATTTTTGTCCC 3′	456
vp15	vp15F 5′ CTTAACCACAGTTGCACTCAC 3′ vp15R 5′ GTACCCTTAAACTTTTATACCAC 3′	468

sequence comparison showed that the ORF was identical to that of ORF1 from the WSSV Thailand isolate (van Hulten et al. 2001), which encodes an envelope protein VP28 (van Hulten et al. 2000). VP28 is equivalent to the envelope protein VP27.5 identified previously from CBV (Nadala et al. 1998). The ORF in clone 95C-C6 was identical to the following WSSV DNA sequences in GenBank: ORF wssv480 of the WSSV Taiwan isolate (Lo & Kou 2001) and ORF wsv421 of the WSSV Xiamen isolate, China (Yang et al. 2001). A putative poly(A) signal AATAAA was found 54 bp downstream of the ORF stop codon TAA in the WSSV cDNA insert in clone 95C-C6. The poly(A) tail was 15 bp downstream of the poly(A) signal (Fig. 1). The position of the poly(A) site was confirmed by sequencing another cDNA clone, 95C-C8, which has an insert containing vp28 gene sequences and a poly(A) tail.

Zhang et al. (2002) isolated a cDNA clone C37 from a cDNA library of WSSV-infected *Penaeus japonicus* tissue from Xiamen, China. Clone C37 was found to contain the gene encoding the VP28 protein. Two putative poly(A) signals (54 and 201 bp downstream of the vp28 gene stop codon, respectively) and a poly(A) site (221 bp downstream of the vp28 gene stop codon)

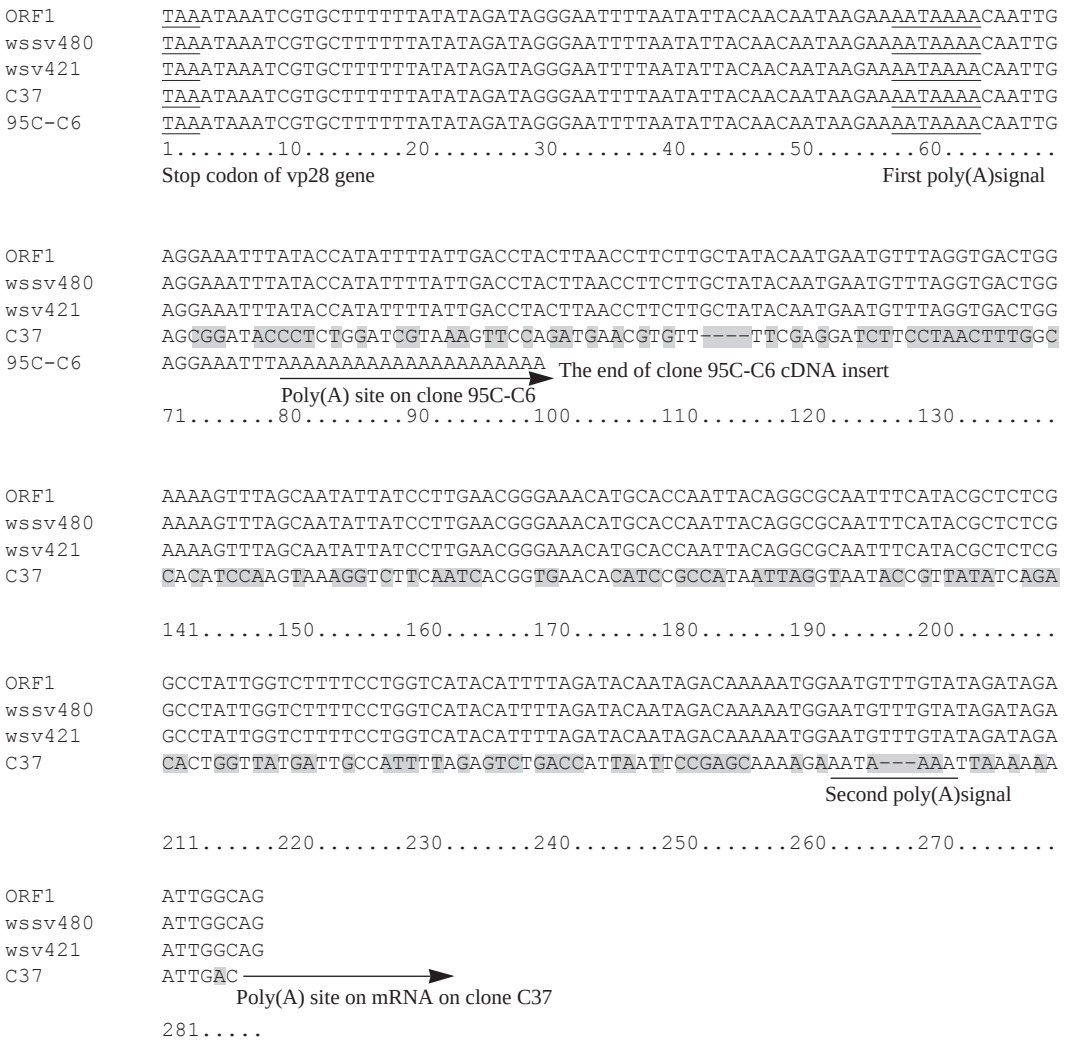

Fig. 1. Boxshade of multiple-aligned WSSV genomic DNA and mRNA (cDNA) sequences, starting from the stop codon (TAA) of the gene encoding the VP28 kDa protein. ORF1: DNA sequence of the WSSV Thailand isolate (van Hulten et al. 2001, GenBank accession no. AF369029: from 613 to 900 bp). wssv480: DNA sequence of the WSSV Taiwan isolate (Lo & Kou 2001, GenBank accession no. AF440570: from 279 477 to 279 764 bp). wsv421: DNA sequence of the WSSV Xiamen isolate (Yang et al. 2001, GenBank accession no. AF332093: from 244 854 to 245 141 bp). C37: mRNA sequence (clone C37) of the WSSV Xiamen isolate (Zhang et al. 2002, GenBank accession no. AF227911: from 1095 to 1373 bp). 95C-C6: cDNA clone sequence of WSSV95C (this study, GenBank accession no. AY249434). Grey shading shows the differences between the C37 insert sequence (mRNA) and the corresponding genomic DNA sequence of the WSSV Xiamen isolate and the other isolates

were identified in the insert of clone C37 by Zhang et al. (2002). However, we analyzed the genome sequence of WSSV isolated from infected *P. japonicus* tissue from Xiamen, China (Yang et al. 2001) and found only one putative poly(A) signal (54 bp downstream of the vp28 gene stop codon) for the vp28 gene (ORF wsv421) (Fig. 1). Analysis of the genomic sequence downstream of vp28 genes of WSSV from Thailand (van Hulten et al. 2001) and Taiwan (Lo & Kou 2001) also showed only one putative poly(A) signal, which is consistent with our sequencing results. Further study is needed to find out why the second poly(A) signal in clone C37 is not present in the WSSV genome. We have also noted that the 3′-nontranslated region of the vp28 mRNA (C37) reported by Zhang et al. (2002) showed significant base changes when compared to its genomic counterpart in the WSSV isolate from Xiamen, China (Yang et al. 2001) and 2 other WSSV isolates (Lo & Kou 2001, van Hulten et al. 2001) (Fig. 1). Generally, precursor RNAs made in the nuclei of eukaryotic cells undergo 3 types of modification. These include methylation of the 2′-hydroxyl group of the ribose near the co-transcriptionally added cap, removal of introns and polyadenylation before transport into the cytoplasm for translation. Many genes have been characterized as having alternative polyadenylation sites at the 3′-end of their mRNAs, according to the cellular environment. Multiple poly(A) sites have been observed in the genes of herpes simplex virus and adenovirus. Apart from the observations of Zhang et al. (2002), significant base alterations in the 3′-nontranslated region have not been reported.

Another WSSV cDNA clone, 95C-C958, had a ~313 bp insert (GenBank accession no. AY245783). The ORF in the cDNA insert encoded a 61 amino acid peptide, which was identical to the peptide sequence of the WSSV structural protein VP15 (van Hulten et al. 2002) and WSSV basic peptide P6.8 (Zhang et al. 2001). A poly(A) site was found 15 bp downstream of a putative poly(A) signal (AATAAA), which was consistent with the finding of Zhang et al. (2001).

In this study, genes encoding 5 viral structural proteins (VP15, 19, 24, 26 and 28) of 5 different geographical WSSV isolates (Table 1) were sequenced. Sequence comparison indicated that, as in 4 other WSSV isolates reported (Table 1), the sequences of each gene were all identical among the 9 isolates (Table 1). The only exception was the vp28 gene of the Korean isolate which has 1 base difference from that of other WSSV isolates, but this alteration does not cause a change in the amino acid sequence (Moon et al. 2003). These results strongly suggest that the genes of WSSV structural proteins are highly conserved.

Though highly conserved, variations have been observed in WSSV structural protein sequences. Wang et al. (2000) sequenced the NH_2-terminal sequence of a 14.5 kDa nucleocore protein of WSSV isolated from *Litopenaeus setiferus* from South Carolina, USA, in 1998 as VARGGKTKGRRG (Swiss-Prot Protein Data Bank accession no. P82006). Assuming the 14.5 kDa protein is equivalent to the VP15 protein, it is only 74% identical to this protein (VARSSKTKSRRG) in the other 8 WSSV isolates. Wang et al. (2000) also showed that the 19 kDa envelope protein of WSSV isolated from crayfish appeared slightly larger in size than that of WSSV isolated from shrimp. Protein sequencing is needed to confirm this difference.

The conservation of the DNA and amino acid sequences of the structural proteins of different WSSV isolates provides the molecular basis for the development of highly specific and sensitive nucleic acid and immuno-based detection methodologies for WSSV. For example, polyclonal antibodies developed against the complete virion (Nadala et al. 1997, 1998) or the immuno-dominant structural protein VP27.5 (You et al. 2002) have been successfully employed in the detection of different geographical WSSV isolates by Western blot (Nadala et al. 1997, Magbanua et al. 2000) and immuno-dot procedures (Nadala & Loh 2000). Furthermore, as shown in the present study, PCR primers prepared from the gene sequence of the structural viral proteins can be used for WSSV diagnosis/detection. However, it is epidemiologically important to be able to distinguish the different geographical isolates of WSSV. Studies have shown that in hosts such as *Litopenaeus vannamei* and *Farfantepenaeus duorarum*, the WSSV isolated from different geographical areas can show variation in virulence (Wang et al. 1999). Although restriction fragment length polymorphism (RFLP) and genomic sequence analyses may show differences, more studies are needed to identify other genetic markers which could also help to track the WSSV involved in an outbreak.

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