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Expression and Assembly Mechanism of the Capsid Proteins of a Satellite Virus (XSV) Associated with *Macrobrachium rosenbergii* Nodavirus*

Jian-min WANG[1, 2], Hua-jun ZHANG[1, 2] and Zheng-li SHI[1**]

(1. State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Sciences (CAS), Wuhan, Hubei 430071, China; 2. Graduate School of CAS, Beijing 100039, China)

Abstract: The extra small virus (XSV) is a satellite virus associated with *Macrobrachium rosenbergii* nodavirus (MrNV) and its genome consists of two overlapping ORFs, CP17 and CP16. Here we demonstrate that CP16 is expressed from the second AUG of the CP17 gene and is not a proteinase cleavage result of CP17. We further expressed CP17 and several truncated CP17s (in which the N- or C-terminus or both was deleted), respectively, in *Escherichia coli*. Except for the recombinant plasmid $CP17^{\Delta C10}$, all recombinant plasmids expressed soluble protein which assembled into virus-like particles (VLPs), suggesting that the C-terminus is important for VLP formation.

Key words: Extra small virus (XSV); Satellite virus; Virus-like particle; White tail disease

White tail disease has been an important factor in the culture of giant freshwater prawn *Macrobrachium rosenbergii* in Thailand, Guadeloupe, Antilles, China and India since the early 1990's (2, 3, 8-10, 14). Two viral particles, *Macrobrachium rosenbergii* nodavirus (MrNV) and its satellite virus (extra small virus, XSV), were isolated and identified from the diseased prawns (4, 9, 13, 16). Experimental infections demonstrated that MrNV plays a key role in white tail disease and that XSV is dependent on MrNV during replication (15).

Satellite viruses are usually found in the plant kingdom. In the animal kingdom, only two satellite viruses, the adeno-associated virus (a DNA satellite virus) and the chronic bee-paralysis associated satellite virus-like (CBPSV, an RNA satellite virus) are recognized by the ICTV (International Committee on Taxonomy of Viruses) (1). Thus the XSV is the second identified RNA satellite virus associated with an animal virus. The XSV is 15 nm in diameter, icosahedral, and non-enveloped. The XSV particle consists of two structural proteins (CP17 and CP16) and encapsulates a linear single-stranded RNA genome of 0.9 kb (4, 12, 13). Previous work has indicated that the XSV genome is a monocistronic messenger in the sense orientation and has a unique ORF encoding the capsid protein CP17 (12,16). However, in the N-terminal region of the CP 17 gene, there is also a second AUG which is 33 bp downstream from the first AUG and which is in a favorable Kozak's context (13).

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* Foundation item: National Natural Science Foundation of China (30370057).
** Corresponding author. Tel: +86-27-87197240.
Fax +86-27- 87197240.
Email: zlshi@wh.iov.cn

Moreover, the N- terminus of CP17 is rich in basic amino acids and contains potential proteinase cleavage sites. Thus, a question is raised as to whether CP16 is translated independently, from the second AUG, or is a proteinase cleavage product of CP17. We also want to understand how these two proteins are assembled into the XSV particle. In this paper, we describe our recent progress concerning the expression and assembly mechanisms of the XSV capsid proteins.

All gene fragments studied in this paper are depicted in Fig. 1 and were amplified from cDNA fragments of XSV constructed in our laboratory (16). The accession number of the XSV sequence from China is DQ174318. The primer 5'-GGATCCATGAA TAAGCGCATTAATAATAA-3' (sense, the *Bam*H I site is underlined) and 5'-AAGCTTTTACTGTTCGG AGTCCCA-3' (antisense, the *Hin*d III site is underlined) were used to amplify *cp17*. The amplified fragment was digested with the *Bam*H I and *Hin*d III and cloned into pGEX-KG (Promega) and expressed in *E. coli*. The resulting fusion protein was then excised from the gel and used to produce antibody according to standard protocols. The recombinant baculovirus containing *cp17* or mutated *cp17* ($cp17^M$, in which the second translation start codon is changed into AUC and this doesn't change the polarity and amphoteric property of the amino acid) under the control of the polyhedrin promoter was generated according to the manual of Bac-To-Bac Baculovirus Expression Systems (Gibco-BRL). Briefly, the PCR products of cp17 (amplified with forward primer 5'-GGATCC ATGAATAAGCGCATTAATAATAA-3' and reverse primer 5'-AAGCTTTTACTG TTCGGAG TCCCA-3') and $cp17^M$ (amplified with forward primer 5'-GGATCCATGAGATCACGTAGGGGAC GTGGTAGGACAATCGGATCTA-3' and reverse primer 5'- AAGCTTTTACTGTTCGGAGTCCCA-3') were sequence confirmed and digested with *Bam*H I and *Hin*d III, then cloned into the donor vector pFastBacDual digested with the same enzymes. Positive clones were transformed into DH10B with AcBacmid and the helper plasmid. The recombinant Bacmids were transformed into Sf9 cells to get the recombinant baculovirus. The whole protein of the cells was then separated on SDS-PAGE. Western-blot was performed according to the standard protocol (11), with antibody against CP17 as the primary antibody (1:1 000) and alkaline phosphatase-conjugated goat anti-rabbit IgG (1:2 000, Sino-American) as the second antibody. For non-fused expression in *E. coli*, the primers 5'- TCATGAATAAGCGCATTAATAA TAA-3' and 5'-AAGCTTTTACTGTTCGGAGTCC CA-3' were used to amplify *cp17*, primers 5'-TCA TGAGATCACGTAGGGGACGTG-3' and 5'-AAGC TTTTACTGTTCGGAGTCCCA were used to amplify the truncated *cp17*, i.e., *cp16* (with deletion of the 11 amino acids at the N-terminus of CP17), primers 5'-TCATGA ATAAGCGCATTAATAATAA -3' and 5'- TTAACGAACCACTGGAACTGGA-3' were used to amplify the $cp17^{\Delta C10}$ (with deletion of the 10 amino acids at the C-terminus of CP17), primers 5'-GGATCTAATCTCATTCCTTATGC-3' and 5'-A AGCTTTTACTGTTCGGAGTCCCA-3' were used to amplify the $cp16^{\Delta N10}$ (with deletion of the 10 amino acids at the N-terminus of CP16). The *cp17* gene was cloned into pGEM-T Easy vector (Promega) and then digested with *Bsp*H I and *Hin*d III and inserted into pET-28a (+) (Noagen) which cut with *Nco*I and *Hin*d III. The *cp16* gene was cloned into pET-28a (+) (subsequently named pET-CP16) as described above.

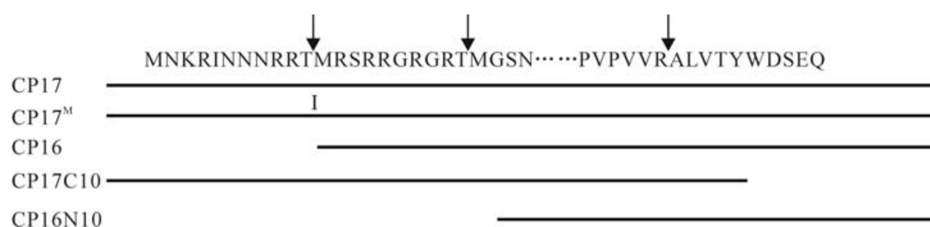

Fig. 1. Schematic maps of the N- and C-termini of capsid protein CP17 and its truncated fragment. The truncated sites are marked by arrows above the amino acid sequence and the mutated site is indicated above the line.

For truncated *cp17* (*cp17*$^{\Delta C10}$) and *cp16* (*cp16*$^{\Delta N10}$), the DNA fragment was amplified by PCR using KOD plus (Taq, Toyobo) and ligated into pET-28a (+) (subsequently named pET-CP17$^{\Delta C10}$ and pET-CP16$^{\Delta N10}$, respectively) which was cut with *Nco* I and blunt-ended using Klenow fragment (Takara). All clones were confirmed by sequencing and no encoded amino acid mutations were detected and then expressed in *E. coli*. The transformed cells were harvested after 4 h incubation at 37℃ by centri- fugation (5 000 × *g*, at 4 ℃ for 10min) and sonicated in PBS. After removing cell debris by centrifugation (16 000 × *g*, at 4℃ for 20 min), the supernatant was ultracentrifuged at 30 000 × *g* for 2 h. The pellet was resuspended in PBS and purified using 10%-40% (w/v) sucrose gradient at 30 000 × *g* for 4 h. Fractions were collected, washed and resuspended in PBS. The purified viral particles were observed under electron microscopy (HITACHT FA-7000) after staining in 2% phosphotungstic acid (pH 7.2).

In the Sf9 cells, the recombinant baculovirus containing *cp17* gave two bands which corresponded to CP17 and CP16, respectively, following Western blot with CP17 antibody, while *cp17*^{M} yielded only one band (Fig. 2). These results showed that CP16 is translated from the second AUG of CP17 and is not the result of proteinase cleavage of CP17. In *E. coli*, except the CP17$^{\Delta C10}$, all proteins were expressed in soluble form and could assemble into viral-like particles (Fig. 3B-E). The VLPs formed by CP17 and CP16 displayed a similar size (13-15 nm in diameter) and morphological structure relative to XSV native particles, while the VLPs formed by CP16$^{\Delta N10}$ were of a much smaller size (10 nm) (Fig. 3E).

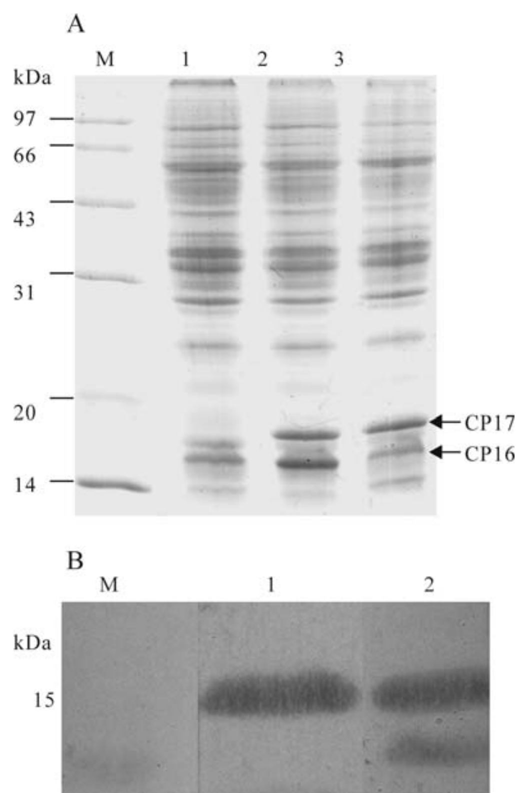

Fig. 2. SDS-PAGE and Western blot detection of CP17 and CP17^{M} expressed in insect cells. A: SDS-PAGE, M: protein marker, Lane 1: mock-infected Sf9 cells; Lanes 2, 3: expression of the CP17 and CP17^{M} genes, respectively; B: Western blot with antibody against the CP17 protein. M: protein marker; Lane 1: expression of the CP17^{M} gene; Lane 2: expression of the CP17 gene.

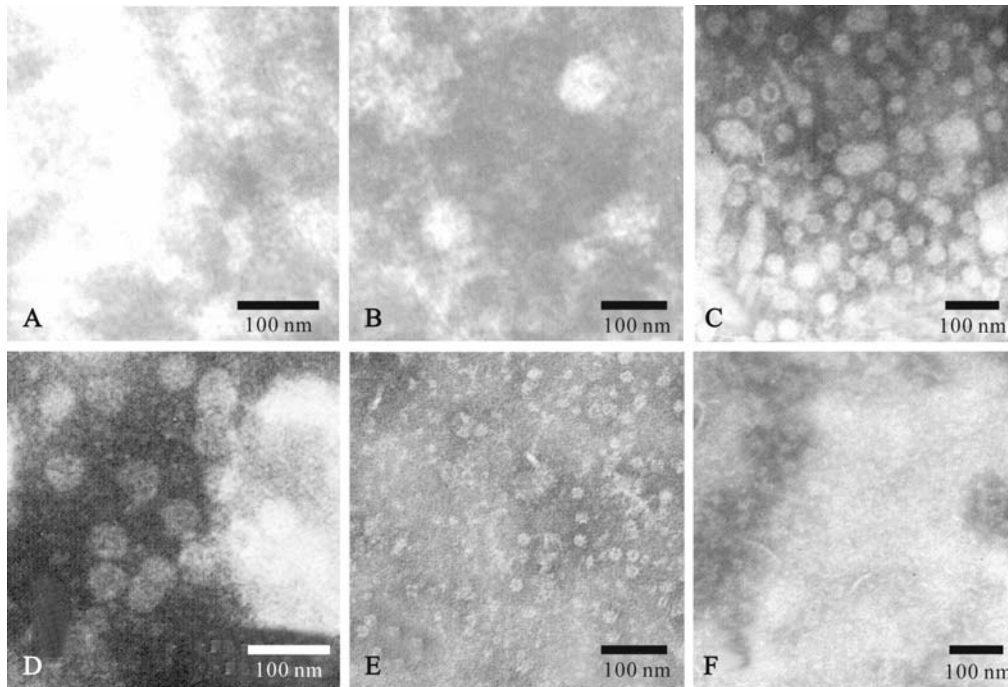

Fig. 3. Electron microscopic observation of VLPs from CP17 expressed in insect cells (B), and from CP17, CP16, CP16$^{\Delta N10}$ and CP17$^{\Delta C10}$ expressed, respectively, in *E. coli* (C-F). A: The supernatant of mock infected insect cells under EM.

Because of the limited size of viral genomes, the translation of several proteins from the same mRNA usually occurs during virus protein translation (5). Our previous results demonstrated that the XSV has only one mRNA, which has a potential ORF with a product of 17 kDa (13, 16). However, there are two AUGs (separated by 33 bases) in the same frame, which are both in a favorable context with regard to Kozak's rule (13). Our result demonstrated that CP17 and CP16 are independently translated from the same mRNA of the XSV. The two protein products are at almost equal molarity, similar to that in the viral particle (4, 13), suggesting that the respective translation of these two proteins is equally efficient and well-regulated in shrimp and insect cells.

It is well known that the N- and C-termini of nucleocapsid proteins play important roles in RNA genome packaging and viral particle assembly in most viruses (6, 7). CP17 and CP16 are both structural proteins of viral particles. The expressed CP17 and CP16 in insect cells, as well as the CP17 or CP16 alone in *E. coli* were found to assemble into VLPs which displayed a similar structure and size under electron microscopy as the native XSV particles. However, if we deleted 10 aa at the C-terminus of CP17, the expressed protein did not assemble into VPLs. possible explanations for this result are: 1) the expressed protein couldn't correctly fold and thus affected the assembly of VLP; 2) the C-terminus, which is rich in hydrophobic amino acids (ALVT YWDSEQ), plays a key role for VLP assembly.

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