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Expression analysis of *Bombyx mori* parvo-like virus VD2-ORF1 gene encoding a minor structural protein

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Abstract: *Bombyx mori* parvo-like virus is a small, icosahedral virus containing two single-stranded linear DNA molecules (VD1, VD2). To date, little is known about this virus, how to package. VD2-ORF1 encoded a large viral structural protein with predicted molecular mass of 133 kDa, and this protein was named as P133. It is unusual for 20–22 nm icosahedral viruses to posses so large a structural protein and the function of this protein is still unknown. In this study, the transcription of P133 was examined with quantitative real-time PCR, and the results demonstrated that the mRNA of P133 could be detected from 28 h post inoculation and kept increasing until 72 h post inoculation. P133 C-terminus (P133C) and P133 N-terminus (P133N) were cloned and expressed in *E. coli* BL21; then the resulting polypeptides were used to produce antibody, respectively. Western blot analysis showed that the protein in virions recognized by anti-P133C and anti-P133N antibody had the same molecular weight, indicating that VD2-ORF1 encoded a viral structural protein without leaky scanning.

Key words: *Bombyx mori* parvo-like virus; structural protein; expression.

Abbreviations: *Bm*DNV-Z, *Bombyx mori* parvo-like virus China Zhenjiang isolate; P133, *Bm*DNV-Z structural protein with predicted molecular mass of 133 kDa; IPTG, isopropyl-β-D-thiogalactopyranoside; NCBI, National Center for Biotechnology Information; p.i., post infection; PVDF, polyvilinidendifluoride; P133C, P133 C-terminus; P133N, P133 N-terminus; qRT, quantitative real-time; RRSV, rice ragged stunt virus.

Introduction

Bombyx mori parvo-like virus infects the nuclei of columnar cells in midgut epithelium of silkworm at the early stage, then expanding to the goblet cells at the late stage, causing the flacherie (Nakagaki et al. 1980). The non-enveloped, icosahedral virions with a diameter of 20–22 nm contain two linear, single-stranded DNA molecules about 6.6 kb (VD1) and 6.0 kb (VD2), and encapsidate plus or minus DNA strands in separate virions in equal frequency. VD1 and VD2 share a 53 nucleotide-long sequence at 3' and 5' termini, but the common terminal palindromic sequences which were widely existed in Parvoviridae could not be found in VD1 and VD2, implying that it is a new type of virus with unique replication mechanisms (Bando et al. 1992, 1995). *Bm*DNV-Z also owns the self-encoding DNA polymerase motif (Hayakawa et al. 2000; Zhang et al. 2010). In the 8$^{\text{th}}$ Report of the International Committee on Taxonomy of Viruses, *Bm*DNV-2 was excluded from the family Parvoviridae because of abovementioned reasons (Ito et al. 2008).

Bombyx mori parvo-like virus contains two isolates, Japan Yamanashi isolate (*Bm*DNV-2) and China Zhenjiang isolate (*Bm*DNV-Z). The *Bm*DNV-Z genome was sequenced and the similarity between *Bm*DNV-Z and *Bm*DNV-2 was found to be 98.4%, while that between their VD2 is 97.7% (Wang et al. 2007).

*Bm*DNV-2 consists of six structural proteins with molecular weight of 120, 118, 53, 51, 49 and 46 kDa (Kawase et al. 1984; Sotoshiro et al. 1995; Hayakawa et al. 2000). The minor structural proteins of 120 KDa and 118 KDa were probably encoded by VD2-ORF1 and VD1-ORF1, respectively. The four major structural proteins were encoded by VD1-ORF2. The fusion proteins containing C-terminus of VD2-ORF1 was recognized by anti-virion antiserum, suggesting that VD2-ORF1 codes for structural proteins of *Bm*DNV-2 (Hayakawa et al. 2000). According to the amino acid sequence and peptide mapping, the minor structural protein of 120 kDa was encoded by VD2-ORF1 in *Bm*DNV-Z (Lv et al. 2011). However, the study of Sotoshiro et al. (1995) showed that anti-virion antiserum failed to recognize the minor structural protein of 120 kDa and 118 KDa encoded by VD2-ORF1 and VD1-ORF1, respectively, in the immunoblot analysis.

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In parvoviruses, leaky scanning is usually applied to express different proteins (Kerr et al. 2006). In *Bm*DNV-2, VD1-ORF2 encodes the four major structural proteins (Hayakawa et al. 2000). It is unclear whether VD2-ORF1 uses this expression strategy. In this paper, quantitative real-time (qRT) PCR was performed to investigate the transcription of VD2-ORF1 in the midgut of virus-infected larvae at different time point. The 5'-terminus (933 bp) and 3'-terminus (1,020 bp) segments of VD2-ORF1 were expressed using pET-28a, and then anti-P133 C-terminus (P133C) and anti-P133 N-terminus (P133N) antibodies were prepared. Western blot analysis was carried out using anti-P133C and anti-P133N antibodies to detect the presence of *Bm*DNV-Z structural protein with predicted molecular mass of 133 kDa (P133) in virions and in midgut of *Bombyx mori* larvae infected with *Bm*DNV-Z.

Material and methods

qRT PCR

The total RNA was extracted from infected midgut tissue of susceptible silkworm strain Huaba 35 at different time points, using Trizol reagent (Invitrogen, Carlsbad, Canada) according to the manufacturer's protocol, and then treated with 0.2 U/µL of DNase I (TaKaRa) at 37℃ for 2 h. The first-strand cDNA was synthesized from 2 µg of total RNA in a final volume of 25 µL using SYBR RT-PCR Kit A (TaKaRa).

qRT PCR information was obtained using an integrated system for thermal cycling, real-time fluorescence detection and subsequent analysis (STRATAGENE Mx 3000P). The specific primers used to examine VD2-ORF1 gene were as follows: forward, 5'-TCATTGGCAACTGGA ACTG-3'; reverse, 5'-ATAAGATGCGATAGGAGCA-3'. The primers for the constant expressed gene, *Bm*actinA3, were: forward, 5'-GGATGTCCACGTCGCACTTCA-3'; reverse, 5'-GCGCGGCTACTCGTTCACTACC-3', designed based on the National Center for Biotechnology Information (NCBI) GenBank (Benson et al. 2010) sequence (Accession No.: X04507). Each final reaction contained 10 µL of SYBR Premix Ex Taq, 1 µM primer and 1 µL of cDNA in a total volume of 20 µL. Thermal cycling conditions were: denaturation at 95℃ for 5 min, followed by 40 cycles of 10 s at 95℃, 20 s at 56℃ and 20 s at 72℃. After PCR, the specificity of amplification was confirmed by automated melting curve analysis and agarose gel electrophoresis of the products. The transcription level was determined with the ΔCt and the transcription level of the target gene which was normalized with *Bm*actinA3 in the same samples as previously described (Chen et al. 2007).

Prokaryotic expression of P133N and P133C and preparation of antibodies

The primers 5'-CGGGATCCATGAATTTAAAGAGGTTA TACGT-3' (the *Bam*HI site underlined) and 5'-CCCTCGA GACGAGGTGCTAATTCTACTTG-3' (the *Xho*I site underlined) were designed to amplify the 933 bp fragment encoding the N-terminus of P133 with viral genome as the template, while the primers 5'-CGGGATCCATGCCTTATGG AGTTGTAGA-3' (the *Bam*HI site underlined) and 5'-CCCTCGAGAGTACAGATTACTGTTCTAATAGTATT-3' (the *Xho*I site underlined) were designed to amplify the 1,020 bp fragment encoding the C-terminus of P133. These PCR products were cloned into the plasmid vector pET-28a to express the peptides as fusion proteins with 6×His-tag at their terminus, and the resulting plasmids were designated as pET-28a-201n and pET-28a-201c, respectively.

BL21 cells harbouring recombinant plasmids, pET-28a-201n and pET-28a-201c, were grown at 37℃ to an OD_{600} of about 0.6. The cells were induced with isopropyl-β-D-thiogalactopyranoside (IPTG) at final concentrations ranging from 0.05 to 1.6 mM for 5 h at 37℃ and then harvested by centrifugation at 8,000 rpm for 5 min at 4℃. The fusion protein presented in the cells was separated in 10% SDS-PAGE and stained with Coomassie brilliant blue. The specific bands corresponding to the two fusion peptides were excised manually from the polyacrylamide gel with a sterile scalpel and digested with trypsin (sequencing grade; Promega, Madison, WI) and then analyzed by a matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) mass spectrometer (Bruker Daltonics, Germany). Peptide mass fingerprinting was performed by comparing the masses of identified peptides to NCBI protein database using the MASCOT search engine (http://www.matrixscience.com/). Western blot analyzed the fusion peptides using anti-6×His (1:5000) and horseradish peroxidase conjugated with goat antirabbit IgG. The immunoreactive proteins were visualized by diaminobenzidine staining.

The 6×His-tagged recombinant proteins were purified on an Ni^{2+}-NTA column (Novagen) and used to raise polyclonal antibodies in rabbits. The antibody was prepared using standard techniques (Harlow et al. 1988). Purified proteins (about 2 mg) was injected subcutaneously to immunize New Zealand white rabbits in complete Freund's adjuvant, followed by two booster injections in incomplete Freund's adjuvant within a gap of 2 weeks before exsanguinations. The polyclonal rabbit antibody against P133C and P133N were used for immunoassay.

SDS-PAGE

The virion was purified as described previously (Wang et al. 2007) from the faeces of 4th instar larvae of *B. mori* strain Huaba infected with *Bm*DNV-Z. Twenty µL of the purified virion sample were mixed with 4 µL of 5×SDS-PAGE loading buffer solution containing 250 mM Tris-HCl (pH 6.8), 10% SDS, 0.5% bromphenol blue, 50% glycerin and 5% β-mercaptoethanol, and boiled for 10 min. Electrophoresis was performed at 60 V for 30 min and then at 100 V for 90 min at room temperature. The protein molecular weight standard was from TaKaRa (Dalian, China). The gel was stained with Flamingo fluorescent gel stain (BIO-RAD) according to the instruction manual.

Western blot assay

The Western blot assay was performed according to the method described by Towbin et al. (1979). Viral structural polypeptides from purified *Bm*DNV-Z virions were resolved on a 10% SDS-PAGE gel, and then were transferred to a polyvilinidendifluoride (PVDF) membrane (Millipore). Viral structural polypeptides on the PVDF membranes were reacted with rabbit anti-P133C and anti-P133N antibodies, respectively, which were then incubated with the goat anti-rabbit IgG antibody conjugated with peroxidase. The membranes reacted with anti-P133N and anti-P133C were visualized by chemoluminescence.

Time course of P133 expression in midgut of day 2 larvae of the 5th instar infected with 5 µL of infected midgut lysate was analyzed by Western blotting. The midgut was

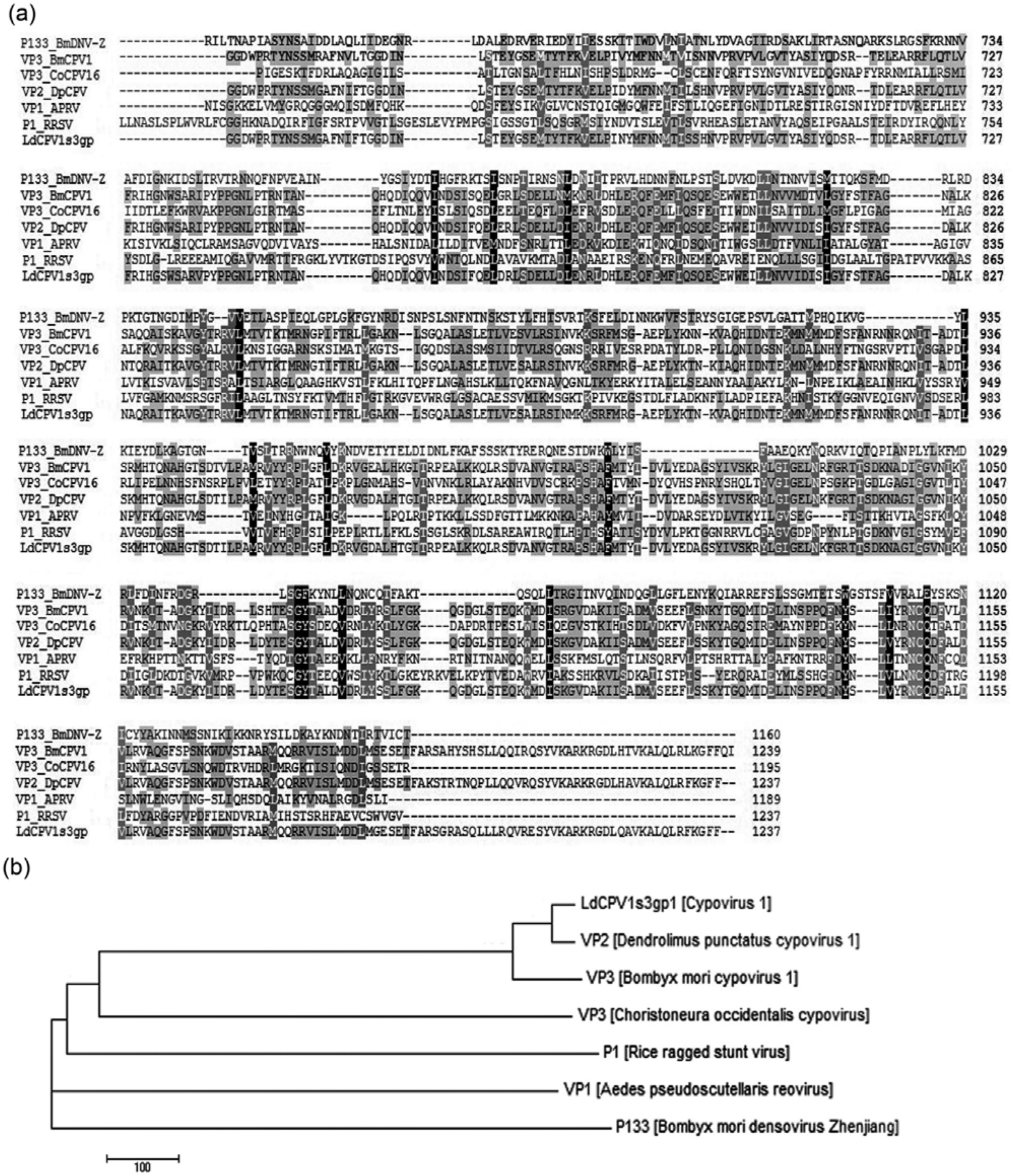

Fig. 1. (a) Multiple sequence alignments of C-terminus of P133 and other structural proteins in Reoviridae. Identity of 100%, 80% and 60% was marked by black, dark gray and light gray, respectively. *Bombyx mori* parvo-like virus (P133_BmDNV-Z, P133; GenBank: ACD68164.1), *Bombyx mori* cypovirus1 (VP3_BmCPV1, VP3; AAK20303.1), *Choristoneura occidentalis* cypovirus 16 (VP3_CoCPV16, VP3; ACA53381.1), *Dendrolimus punctatus* cypovirus1 (VP2_DpCPV, VP2; AAN86620.1), *Aedes pseudocutellaris* reovirus (VP1_APRV, VP1; AAN86620.1), rice ragged stunt virus (P1_RRSV, P1; NP_620514.1), *Lymantria dispar* cypovirus1 (LdCPV1s3gp, LdCPV; NP_149148.1). (b) Tree built with full-length sequences of P133 and other structural proteins deriving from Reoviridae.

then excised 2, 12, 24, 36, 48, 60 and 72 h after virus infection and the total proteins were extracted by RIPA lysis buffer (Beyotime) according to the manufacturer's protocol. A total of 240 μg proteins were separated by 10% SDS-PAGE and subsequently transferred onto PVDF membrane for Western blotting. Following antibodies were used: anti-P133N (1:1000), anti-P133C (1:1000), anti-VP (1:1000) and HRP-conjugated secondary antibodies (1:5000).

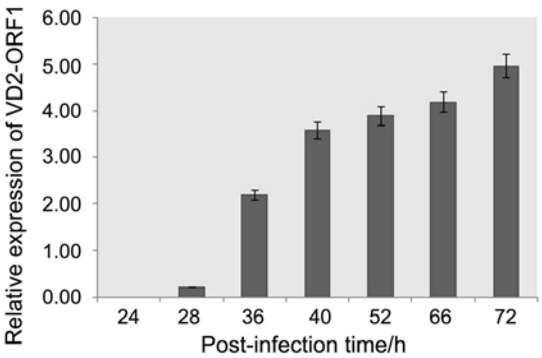

Fig. 2. RT qPCR for relative expression level of VD2–ORF1. Each analysis was repeated at least three times for each set of RNA samples. Each point represents the mean value ± SD. The relative amounts of VD2–ORF1 were normalized using the BmactinA3 as a standard.

Results

Comparison of the amino acid sequences of P133 homologues

In order to discover the additional function of P133, amino acid sequence of P133 was submitted to GenBank at NCBI for BLASTp and conserved domain searches (Altschul et al. 1990). No conserved domain could be found, but the result of BLASTp showed that six homologues of P133 were found in the family Reoviridae. These homologues were all viral structural protein. Sequence alignment demonstrated that the C-terminus among these proteins was conserved (Fig. 1a).

To evaluate the evolutionary relationship of the virus in the family Reoviridae and *Bm*DNV-Z, a phylogenetic tree was constructed by the neighbour-joining method based on these structural proteins and it revealed that *Bm*DNV-Z was more closely related to *Aedes pseudoscutellaris* reovirus (Fig. 1b).

Transcription of VD2-ORF1 in vivo

qRT PCR was used to determine the VD2-ORF1 mRNA expression in the midgut infected with *Bm*DNV-Z at different time points. The transcription level of the target gene was normalized with *Bm*actinA3 in the same samples. The results showed that mRNA of VD2-ORF1 was presented from 28 h post infection (h p.i.), and kept increasing till 72 h p.i. (Fig. 2).

Prokaryotic expression of P133C and P133N

Expression of fusion protein 6×His-P133N in *E. coli* resulted in the production of a 36 kDa protein, while expression of fusion protein 6×His-P133C resulted in a 39 kDa protein. Ten-% SDS-PAGE analysis indicated that expressions of the two fusion proteins could be induced by different concentrations of IPTG (Fig. 3A-1 and 3B-1). Western blot analysis using the anti-His antibody further confirmed that the expected 36 kDa and 39 kDa fusion proteins were induced (Fig. 3A-1 and 3B-1). The fusion proteins were excised for mass spectrometer analysis. The results indicated that the 36 kDa and 39 kDa fusion proteins surely were the N-terminus and C-terminus of P133, respectively (Fig. 3A-2 and 3B-2).

Examination of P133 in the virions and in midgut of virus-infected larvae

To determine whether P133 was present in virions, viral structural polypeptides were resolved by SDS-PAGE and Western blot analyses were performed using the anti-P133N and anti-P133C antibody. The same spe-

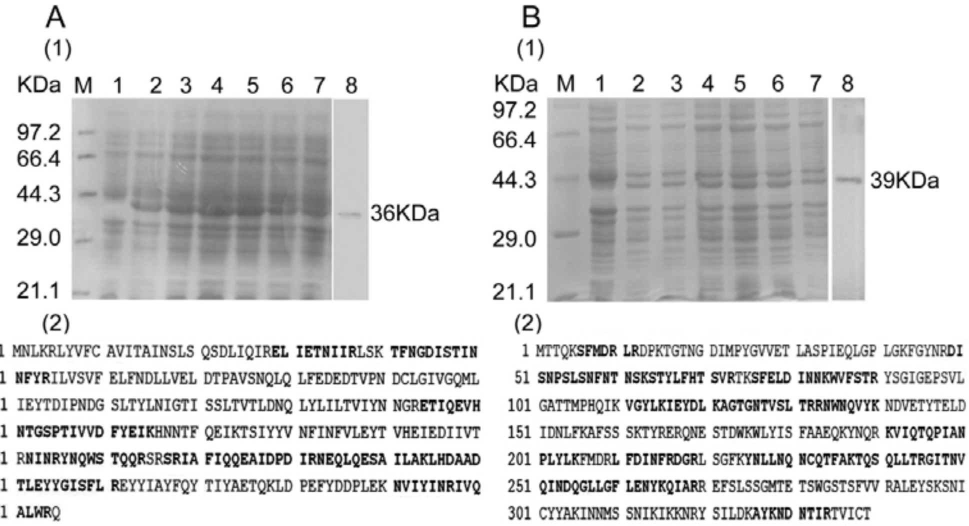

Fig. 3. Expressing and identification of P133N and P133C. (A) Prokaryotic expressing of P133N. (1) Lane M: protein marker; lane 1: *E. coli* BL21 with pET-28a induced without IPTG; lanes 2–7: *E. coli* BL21 with pET-28a-201n induced by IPTG at different final concentration (0.05–1.6 mM); lane 8: the fusion protein was examined with the antibody against 6×His tag. (2) Amino acid sequence of P133N. Matched peptide sequences are shown with bold characters. (B) Expressing and identification of P133C. (1) Lane M: protein marker; line 1: *E. coli* BL21 with pET-28a induced without IPTG; lanes 2–7: *E. coli* BL21 with pET-28a-201c induced with IPTG at different final concentration (0.05–1.6 mM); lane 8: the fusion protein was examined with the antibody against 6×His tag. (2) Amino acid sequence of P133C. Matched peptide sequences are shown with bold characters.

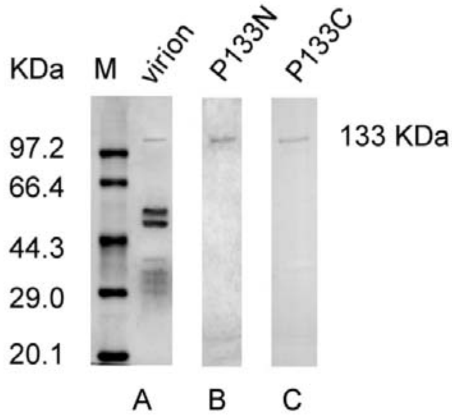

Fig. 4. Identification of P133 in *Bm*DNV-Z virions by western blot. (A) Lane M: protein marker; Lane virion: polypeptides were separated by 10% SDS-PAGE from *Bm*DNV-Z, stained by Flamingo fluorescent gel stain. (B) and (C) Western blot analyses with anti-P133N and anti-P133C antibody, respectively. The band was visualized by chemoluminescence.

cific protein band about 133 kDa was recognized by anti-P133N and anti-P133C antibody (Fig. 4), suggesting that VD2-ORF1 coded for a structural protein of *Bm*DNV-Z. No other proteins could be detected, indicating that leaky scanning expression strategy did not happened during VD2-ORF1 expression, which was commonly used in parvovirus.

To investigate the expression of P133 in midgut of larvae infected with viruses, the midgut was excised from virus-infected larvae at different time points and the total proteins were extracted for Western blot analysis using anti-P133C and anti-P133N antibodies. Time course analysis of P133 expression showed that P133 could not be detected by anti-P133N or anti-P133C antibody in the midgut of virus-infected larvae at any time point, while the major structural protein VP could be recognized by anti-VP antibody in the same samples (data not shown).

Discussion

*Bm*DNV-Z is a viral agent which causes the flacherie disease and is disadvantageous to sericulture. *Bm*DNV-Z VD2-ORF1 encodes a structural protein P133. In this study, the expression of P133 was characterized.

P133 is a minor structural protein of *Bm*DNV-Z based on its low molar ratios within virion. We did not succeed in detecting P133 in midgut of larvae infected with *Bm*DNV-Z at different times, but the major structural protein VP could be detected from 48 h p.i. in the same samples (data not shown). Moreover, the transcripts of P133 were able to be examined from 28 h p.i. to 72 h p.i. with increasing abundance (Fig. 2). It was possible that the expression level of P133 was too low to be detected in midgut of virus-infected larvae. Minor components of virions sometimes performed important functions for the viral replication other than for maintaining the virion structure (Hayakawa et al. 2000). Two minor components VP1 and VP3 of rotavirus have the RNA-dependent transcriptase and RNA-capping activities (Sandino et al. 1986). The phospholipase A_2 property was identified in the unique portion of the VP1 protein, a minor component of the BmDNV-1 capsid (Li et al. 2001).

Amino acid sequence alignment illustrated that the C-terminus was conserved between P133 and some viral structural proteins in family Reoviridae. Viruses in family Reoviridae are double-stranded RNA viruses. Usually, double-stranded RNA viruses contain all of the necessary enzymatic machinery to synthesize complete mRNAs within the core without the need for disassembly (Lawton et al. 2000). Rotavirus, member of the family Reoviridae, consists of three layers (shells) of proteins, contained in the double-stranded RNA genome, RNA polymerase VP1, guanylyltransferase VP3, RNA-binding protein VP2 (Patton et al. 1999) and VP6, which is essential for transcriptase activity (Sandino et al. 1986).

Leaky scanning expression was the common way used to produce multiple structural proteins in the family Parvoviridae (Kerr et al. 2006). For example, from the single VP mRNA of *Galleria mellonella* densovirus, *Casphalia extranea* densovirus and *Bm*DNV-1, four to five in-frame methionines stretching over a length of about 1,100 nucleotides in the 5'-terminus of the transcripts initiate the translation of four to five isoforms of the capsid protein (Li et al. 2001; Fediere et al. 2002; Tijssen et al. 2003). As shown in Figure 4, the same specific protein band was recognized by anti-P133N and anti-P133C antibody and no other proteins could be detected in virions, suggesting that there was no leaky scanning expression strategy in expression of P133.

In conclusion, this study suggested that P133 was certainly a component of *Bm*DNV-Z, and the expression of VD2-ORF1 did not employ the leaky scanning strategy. Although the exact role of P133 is unclear, we are hopeful that our study will lead to a better understanding of the structure of virion and function of the structural proteins by providing a foundation for further research of this topic.

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