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Characterization of the Major Nucleocapsid Gene *vp39* of *Spodoptera litura nucleopolyhedrovirus*

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Spodoptera litura nucleopolyhedrovirus **(SpltMNPV)** ***vp39*** **(*Spltvp39*) is one of the 37 core genes identified in all sequenced baculovirus genomes. The open reading frame of *Spltvp39* is 909 bp in length, which encodes 302 amino acids with predicted molecular weight of 33.9 kDa. Homology analysis indicated that SpltVP39 protein has 20-67% amino acid identity with those of other known baculoviruses. Amino acid sequence alignment of SpltVP39 homologs showed 6 highly conserved cysteine residues. In this study, the rabbit polyclonal SpltVP39 antiserum was prepared. *Spltvp39* transcription in SpltMNPV-infected SpLi-221 cells was first detected at 12 h post-infection (p.i.), and reached the maximal level at 96 h p.i.. The transcriptional start sites of mRNA were mapped within 3 consensus baculovirus late promoter motifs ATAAG. Time course analysis of SpltVP39 expression in SpltMNPV-infected cells showed that SpltVP39 was expressed from 24 through 96 h p.i. as a 33.9 kDa protein. Western blotting indicated that this protein appeared in the nucleocapsids of both occlusion-derived virions and budded virions. These results demonstrate that the *Spltvp39* should be classified as a baculovirus late gene similar to most other baculovirus late structural genes.**

Key words: Baculovirus, *Spodoptera litura nucleopolyhedrovirus*, *vp39*, Nucleocapsid

Baculoviruses are a very diverse group of insect-specific viruses with double-stranded, circular, and supercoiled DNA genomes that vary in size from about 80 to 180 kb[1]. During the baculovirus infection cycle, two functionally distinct virion phenotypes are produced: the budded virions (BVs) and the occlusion-derived virions (ODVs). BVs and ODVs are similar in their nucleocapsid structure and genetic material but different in the origin and composition of their envelopes[2-3]. Early in infection, newly assembled nucleocapsids egress from the nucleus and bud out from the plasma membrane, which has been modified by virus-encoded proteins to form BVs. BVs are highly infectious for most insect tissues and result in systemic infection[4]. Late in infection, nucleocapsids are retained within the nucleus and acquire their envelopes from virus-induced intranuclear membrane-derived microvesicles to form ODVs. ODVs are subsequently embedded within a proteinaceous crystalline matrix to form occlusion bodies (OBs). ODVs initiate the primary infection in a susceptible host and are required for the horizontal spread of the infection among insect hosts[4].

Baculoviruses have been reported worldwide from more than 600 host species[5], and more than 59 baculovirus genomes have been sequenced so far[6,7]. 37 genes are found to be conserved in all sequenced baculovirus genomes and are considered as baculovirus core genes, and *vp39* is one of them[6,8,9]. VP39 was found to be arranged in stacked rings around the nucleoprotein

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core[10] and was identified as the major structural protein of the baculovirus nucleocapsids[11-15]. Previous studies showed that VP39 can interact with the host cellular actin and is involved in the rearrangement and polymerization of actin[16-20]. Further studies revealed that the transportation of nucleocapsids of *Autographa californica multiple nucleopolyhedrovirus* (AcMNPV) and *Heliothis armigera single nucleopolyhedrovirus* from cytoplasm to the nucleus bases on the polymerized actin cable[17, 21-23]. Recently, it has been shown that AcMNPV VP39 is able to interact with itself and other viral proteins, such as polyhedrin, ODV-EC27, ODV-E56, IE1, 38K and ODV-E66, and with cellular kinesin-1[24-27].

Spodoptera litura nucleopolyhedrovirus (SpltMNPV), specifically infects the cotton leaf worm which is an economically important polyphagous pest in China, India, and Japan, causing considerable economic loss to many vegetable and field crops[28]. SpltMNPV is one of the important baculoviruses and has been successfully used as a commercial biological insecticide against the cotton leaf worm in China for years. Our laboratory has been working on SpltMNPV genomics. The genome of SpltMNPV is 139,342 kb and contains 141 predicted open reading frames (ORFs)[29]. Sequence analysis of SpltMNPV genome revealed that the homologue of AcMNPV ORF89 (*vp39*[30]) exists as ORF81, and the amino acid identity between SpltMNPV VP39 (SpltVP39) and AcMNPV VP39 is 37%. As a baculovirus core gene, *vp39* may have important functions in the baculovirus life cycle. We were also interested in the functions of SpltMNPV *vp39* (*Spltvp39*). In this report, for the first time, we analyzed the transcriptional profile of *Spltvp39*, prepared SpltVP39-specific polyclonal antiserum and used it to characterize the expression pattern of SpltVP39 and its structural localization in virions. The results of this study enriched the information of *vp39*.

MATERIALS AND METHODS

Computer-assisted sequence analysis

The conserved domains of SpltVP39 were predicted against NCBI's Conserved Domain Database[31], SMART[32], and HHpred analysis[33]. The amino acid sequence of SpltVP39 was searched against the nonredundant protein sequences at the NCBI database with the Position-Specific Iterated BLAST algorithm to identify SpltVP39 homologs. Multiple sequence alignments were performed and edited using ClustalX[34] and GeneDoc[35], respectively.

Cells, insect larvae and virus

The cell line SpLi-221 [*Spodoptera litura*, (*S. litura*)], donated by Dr J. Mitsuhashi (Faculty of Agriculture, Tokyo University of Agriculture and Technology, Tokyo, Japan), was cultured at 27°C in TNM-FH medium (Invitrogen Life Technologies) supplemented with 10% fetal bovine serum, penicillin (100 μg/ml) and streptomycin (30 μg/ml). Larvae of *S. litura* were reared on an artificial diet at 28°C. The SpltMNPV-G2 isolate was used. SpltMNPV OBs were produced by infecting third instar *S. litura* larvae and BV stocks were prepared by extracting hemolymph from infected insects 3 days post-infection (p.i.). Titers of BV were determined by a 50% tissue culture infective dose (TCID50) end-point dilution assay in SpLi-221 cells as described previously[36].

Plasmid construction and preparation of SpltVP39 polyclonal antibody

The following two primers, which contain *Eco*RI or *Sal*I restriction enzyme site (underlined) were designed to amplify the ORF of *Spltvp39* from SpltMNPV genomic DNA, P1, 5'-GAATTCCATGGCGCTAGTGTCCGGAGGTAAT-3', P2, 5'-GTCGACTCAAATTTCCGCAGGCGC TTGTGAA-3'. The obtained PCR products were cloned into pMD18-T vector (TaKaRa) to construct plasmid pMD-vp39. Then pMD-vp39 was digested with *Eco*RI and *Sal*I, and the corresponding fragment was cloned into the *Eco*RI and *Sal*I sites of the expression vector pET28b (+) (Qiagen). The resulting plasmid pET-vp39, in which *Spltvp39* was in frame and fused with His•Tag/thrombin/T7•Tag at the N-terminus of pET28b (+) was checked by restriction enzyme analysis and sequencing, was then transformed into *Escherichia coli* BL21 (DE3). Expressed 6×His-SpltVP39 was purified from *E. coli* BL21 (DE3) by Ni-NTA agarose under denaturing conditions as described in the handbook (QIA expressionist™). The purified 6×His-SpltVP39 protein was used as antigen to raise antiserum in New Zealand white rabbits according to the method of Sambrook *et al.*[37] using Freund's adjuvant (Gibco).

Total RNA preparation, reverse transcription (RT)-PCR and 5' rapid amplification of cDNA ends (5' RACE) analysis

SpLi-221 cells were infected with SpltMNPV at a multiplicity of infection (MOI) of 5 $TCID_{50}$. Total RNA was isolated from 1.0×10^6 mock-infected or SpltMNPV-infected SpLi-221 cells at various time points post infection using RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions, and treated with RNase-Free DNase (Promega) to eliminate DNA contamination.

RT-PCR was performed using an RNA PCR Kit Ver. 3.0 (TaKaRa) employing 2.0 μg total RNA as the template for each time point. Synthesis of the first-strand DNA complementary to the mRNA (cDNA) was carried out using *Avian Myeloblastosis* virus reverse transcriptase according to the manufacture's instructions (Bio-Rad). The *Spltvp39*-specific primers P1 and P2 were used for PCR amplification to detect the transcription of *Spltvp39*. The obtained PCR products were analyzed in 1% agarose gels.

The 5'RACE procedure was performed using a 5' /3' RACE Kit, 2nd generation (Roche) with 2 μg of purified total RNA isolated from SpltMNPV-infected SpLi-221 cells at 48 h p.i. Briefly, first-strand cDNA synthesis was performed with a *Spltvp39*-specific primer SP1 (5'-CGACACGT TCATCACGGCCTGATAGTT-3'). The tailed cDNA was amplified by PCR using the oligo-dT anchor primer and the nested *Spltvp39*-specific primer SP2 (5'-CGGTTCCGTCGGGTATCGCTATGCTGGTC-3'). The obtained PCR products were gel-purified and cloned into pMD18-T. A total of 15 clones were selected for sequencing.

Purification and fractionation of BVs and ODVs

BVs and ODVs were purified and fractionated into envelope (E) and nucleocapsid (NC) preparations as described previous[2], with minor modifications. To purify BVs, 10 ml of hemolymph was collected from third instar *S. litura* larvae 3 days p.i. in 0.1 × TE (10 mM Tris, 1.0 mM EDTA, pH 7.4) containing 5 mM phenylthiocarbamide to inhibit prophenoloxidase activity and centrifuged twice at 3,000 × g for 10 min at room temperature, and the supernatant was then centrifuged at 100,000 × g (Beckman, SW40 rotor) for 90 min at 4°C. The pellets were re-suspended in 0.1 × TE overnight at 4°C, and then layered onto a 25% to 56% (wt/vol) continuous sucrose gradient, centrifuged at 100,000 × g (Beckman, SW40 rotor) for 90 min at 4°C. The virion band was collected, 1:4 diluted, and centrifuged at 100,000 × g (Beckman, SW40 rotor) for 90 min at 4°C. The BVs pellets were re-suspended in 0.1 × TE and stored at -20°C.

Polyhedra were isolated from infected *S. litura* larvae and were purified using a modification of the procedure described by Braunagel and Summers[2]. The concentration of the purified polyhedra was measured with a counting chamber. ODVs were purified as described previously[2, 9, 27], with modifications. Briefly, 2.0×10^9 polyhedra were incubated in polyhedra lysis buffer (0.1 M Na_2CO_3, 0.166 M NaCl, and 0.01 M EDTA, pH 10.5) at room temperature for 30 min, and centrifuged at 500 g for 5 min to remove undissolved polyhedra. Then the supernatant was overlaid onto a 25 to 56% (wt/vol) continuous sucrose gradient and centrifuged at 100,000 × g (Beckman, SW40 rotor) for 90 min at 4°C. The ODVs bands were collected, washed twice by dilution in 0.1 × TE, centrifuged at 50,000 × g (Beckman, SW40 rotor) for 60 min at 4°C, and finally re-suspended in 0.1 × TE.

BVs and ODVs were fractionated into E and NC preparations as described previously[2, 38], with minor modifications. Approximately 200 µg ODVs or BVs were incubated in a 250 µl reaction system containing 1.0% Nonidet P40 (NP-40), 10 mM Tris (pH 8.5), at room temperature for 30 min with gentle agitation. The E and NC proteins were separated by centrifugation at 150,000 × g (Beckman, SW40 rotor) for 60 min at 4°C. The E and NC proteins were in the supernatant and pellet, respectively. The NC proteins in the pellet were re-suspended in 0.1× TE. The E proteins in the supernatant were precipitated with 4 volumes of acetone, and centrifuged at 100,000 × g (Beckman, SW40 rotor) for 60 min at 4°C, and the pelleted E proteins were dissolved in 0.1× TE. ODVs, BVs, E, and NC proteins were sampled, and analyzed by Western blotting.

SDS-PAGE and Western blotting

Protein samples were mixed with an equal volumes of 2 × protein sample buffer (0.25M Tris-Cl [pH 6.8], 4% sodium dodecyl sulfate [SDS], 20% glycerol, 10% 2-mercaptoethanol, and 0.02% bromophenol blue) and incubated at 95°C for 5 min, and then were analyzed by SDS–12% PAGE (sodium dodecyl sulfate-polyacrylamide gel

electrophoresis) according to the method of Sambrook *et al*.[37]. Proteins from the gel were transferred onto a PVDF membrane (MILLIPORE), and probed with one of the following primary antibodies: one was rabbit polyclonal SpltVP39 antiserum (1: 500 dilution) raised as described above, and the other was pre-immune serum (1: 200 dilution). Alkaline phosphatase-conjugated goat anti-rabbit antibody (1: 2,000) (Zymed Laboratories, Inc.) was used as a secondary antibody. Immunoreactive proteins were visualized using a BCIP (5-bromo-4-chloro-3-indolylphosphate)-Nitro Blue Tetrazolium kit (Roche Diagnostic Corporation).

RESULTS

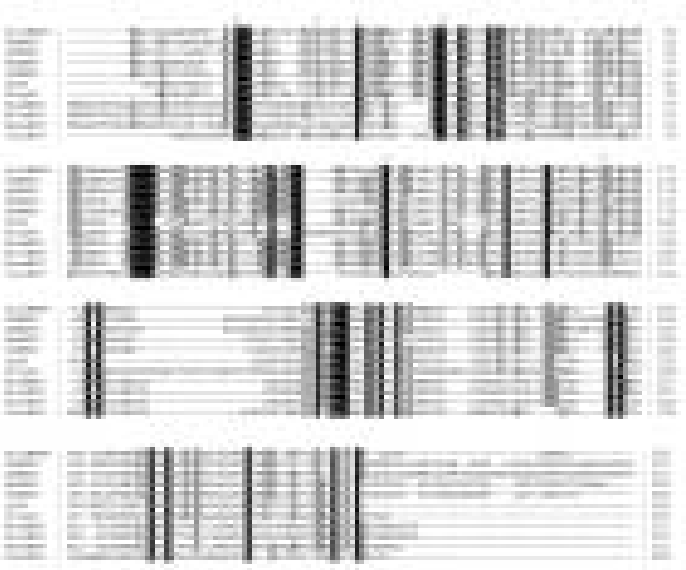
Sequence analysis of *Spltvp39*

The *Spltvp39* is located between the nucleotides (nt) 77,450 and 78,358 of the SpltMNPV genome[29], potentially encoding 302 amino acids with a predicted molecular weight of 33.9 kDa. Computer analysis revealed that the 5' upstream sequence of *Spltvp39* contains three baculovirus consensus late promoter motifs ATAAG at nucleotide residues -27 to -31, -75 to -79 and -284 to -288, with respect to the translational start codon ATG. The canonical polyadenylation signal AATAAA was found 103 nt downstream of the translational stop codon TGA.

Baculovirus *vp39* is a core gene that is conserved among all of the baculoviruses, the genomes of which have been sequenced. Homology analysis indicated that SpltVP39 shares 20-67% amino acid identity with those of other known baculoviruses. Amino acid sequence alignment of the selection of SpltVP39 homologs from each baculovirus genus showed 6 highly conserved cysteine residues (Fig. 1).

Expression of SpltVP39 in *E. coli*

The *Spltvp39* was confirmed to be correctly inserted into the pET28b (+) by restriction enzyme analysis and sequencing, and then transformed into *E. coli* BL21 (DE3). The BL21 (DE3) cells were induced with IPTG (Isopropylthio-β-D-galactoside) (1 mmol/L) for 5 h and subjected

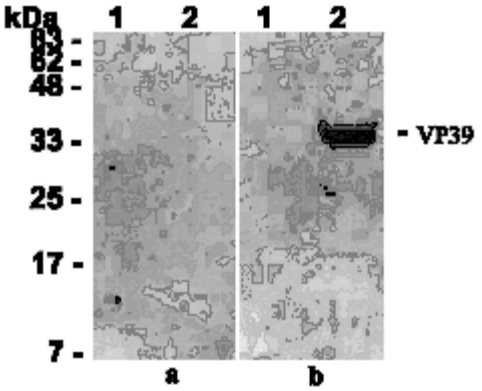


Eleven representative sequences were selected and aligned using Clustal X 1.83 and were edited with GeneDoc software. The VP39 protein sequences used are as follows: SpltMNPV (NP_258349.1), *Autographa californica* MNPV (AcMNPV, NP_054119.1), *Orgyia pseudotsugata* MNPV (OpMNPV, NP_046246.1), *Heliothis armigera single* NPV (HaSNPV, YP_002332627.1), *Spodoptera exigua* MNPV (SeMNPV, NP_037835.1), *Cydia pomonella granulovirus* (CpGV, GenBank accession number NP_148880.1), *Plutella xylostella* GV (PlxyGV, NP_068298.1), *Neodiprion abietis* NPV (NeabNPV, YP_667940.1), *Neodiprion lecontei* NPV (NeleNPV, YP_025289.1), *Neodiprion sertifer* NPV (NeseNPV, YP_025196.1) and *Culex nigripalpus* NPV (CuniNPV, NP_203328.1). Amino acids with black shading denote 100% conservation in all sequences. Those with dark gray and light gray shading represent 80% and 60% conservation in all sequences, respectively. The asterisks indicate the location of the conservative cysteine amino acids

Fig. 1. Amino acid sequence alignment of SpltVP39 and its selected baculovirus homologs

to SDS-PAGE, followed by Western blotting. The results indicated that SpltVP39 could be highly expressed as an approximate 33.9 kDa protein in the cells of BL21 (DE3) (data not shown).

Expressed 6×His-SpltVP39 was purified and used as antigen to raise antiserum in New Zealand white rabbits. Western blotting was performed to assess the specificity of the antiserum. A specific immunoreactive band of approximately 33.9 kDa, which is in accordance with the predicted molecular mass of SpltVP39, was detected in the BL21 (DE3) (Fig. 2b); yet the pre-immune serum did not show any positive reaction (Fig. 2a), indicating that the antiserum prepared in this study is specific and can be used in the further study.



The recombinant BL21 (DE3) cells were mock-induced (1) or induced (2) with IPTG for 5 h and were harvested. Western blotting was performed to assess the anti-VP39 serum prepared in this study (b), and the pre-immune serum was used as a control (a). The pre-stained protein standards (NEB) are indicated on the left and the corresponding band to SpltVP39 is indicated on the right

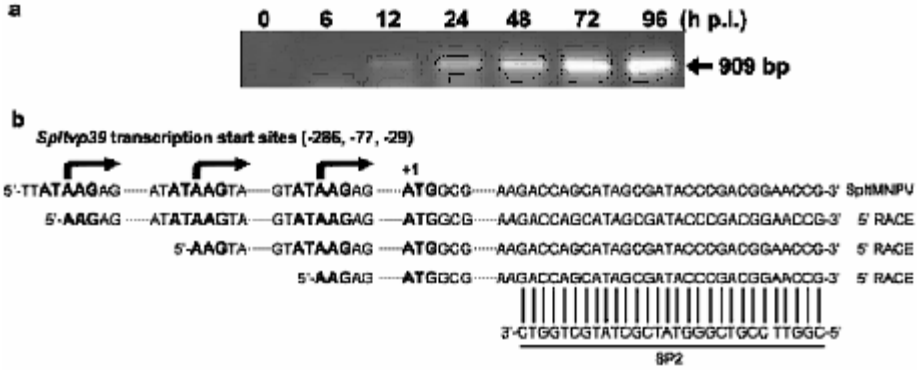
Fig. 2. Assessment of anti-VP39 serum prepared in this study

RT-PCR analysis of *Spltvp39* transcripts

To investigate the transcription profile of *Spltvp39*, RT-PCR was performed using the total RNA extracted from SpltMNPV-infected SpLi-221 cells at different time points. The primers (P1 and P2) within *Spltvp39* ORF were used to amplify the full-length 909 bp of *Spltvp39*. The 909 bp *Spltvp39*-specific transcripts were first detected in SpltMNPV-infected SpLi-221 cells at 12 h p.i., the transcripts level increased in abundance at 72 h p.i. and remained at steady-level up to 96 h p.i. (Fig. 3a). The RT-PCR products obtained were cloned into pMD18-T and sequenced. As expected, the obtained sequences matched the *Spltvp39* sequence. No signals were obtained when reverse transcriptase was not added prior to the PCR step (data not shown), indicating no possible contamination of the SpltMNPV DNA.

Mapping of the transcription start sites of *Spltvp39*

To map the transcription start sites of *Spltvp39*, 5'RACE was performed with total RNA isolated from SpltMNPV-infected SpLi-221 cells at 48 h p.i. (Fig. 3b). Three PCR products, which were about 500bp, 300bp and 250 bp in length respectively, were detected as *Spltvp39* transcripts.



(a) RT-PCR analysis of *Spltvp39* transcripts performed on total RNA extracted from SpltMNPV-infected SpLi-221 cells at the designated time points. The size of the PCR products is indicated on the right. (b) 5'RACE analysis of the *Spltvp39* transcription start sites (arrows). Three sequences below the SpltMNPV genome were obtained from 5'RACE analysis. Three baculovirus consensus late promoter motifs ATAAG and the translation start codon ATG are indicated in bold. The first nucleotide of the translation start codon is indicated as +1. The SP2 primer used for 5'RACE analysis is underlined

Fig. 3. Transcriptional analysis of *Spltvp39* in SpltMNPV-infected SpLi-221 cells

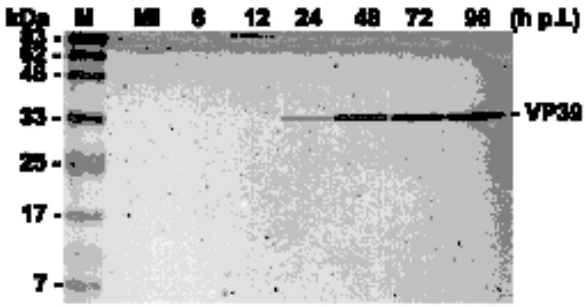
Fifteen clones derived from the products were sequenced and the results revealed that transcriptions of *Spltvp39* initiated from the second A of the three canonical baculovirus late promoter motifs ATAAG, mapping 29 nt, 77 nt and 286 nt upstream of the ATG translation initiation codon. Taken together with the results of the RT-PCR, it can be concluded that *vp39* of SpltMNPV is transcribed actively in the late stage of infection.

Time course analysis of SpltVP39 expression in SpltMNPV-infected cells

In order to study the expression of the *Spltvp39*, time course in SpltMNPV-infected SpLi-221 cells were analyzed by Western blotting using SpltVP39 antiserum. A specific immunoreactive band of approximately 33.9 kDa was first clearly observed at 24 h p.i., stained maximally by 72 h p.i. and then remained constant (Fig. 4). The results of expression profile of SpltVP39 further confirmed that SpltVP39 is expressed as a late gene.

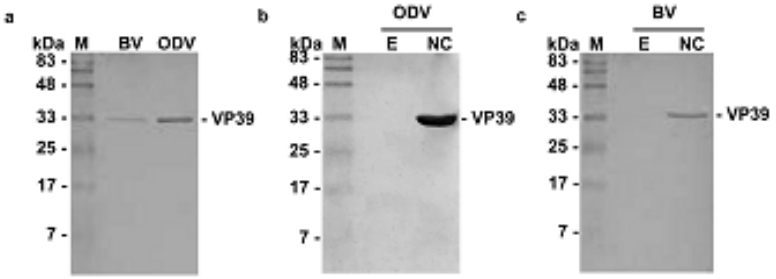
Structural localization of SpltVP39 in virions

To investigate whether SpltVP39 is a structural component of virions or not, BVs and ODVs from SpltMNPV-infected *S. litura* larvae were purified and fractionated into E and NC fractions and SpltVP39 was detected by Western blotting with SpltVP39 antiserum. The results indicated that SpltVP39 was detected in ODVs and BVs (Fig. 5a), while the pre-immune serum did not show any positive reaction (data not shown). The detected molecular weight of SpltVP39 was approximately 33.9 kDa. Meanwhile, SpltVP39 was also clearly detected in NC fractions of ODVs and BVs. In contrast, no detectable bands were present in E fractions of ODVs and BVs (Fig. 5b, 5c). These results suggested that SpltVP39 is the component of the nucleocapsids of ODVs and BVs.



SpLi-221 cells were mock-infected (mi) or infected with SpltMNPV at an MOI of 5. The cells collected at the corresponding time points indicated above the lanes were analyzed by immunoblotting with SpltVP39 antiserum. The pre-stained protein standards are indicated on the left and the corresponding band to SpltVP39 is indicated on the right

Fig. 4. Time expression profile of SpltVP39



ODVs were purified from SpltMNPV-infected third instar *S. litura* larvae 7 days p.i. and BVs were prepared by extracting hemolymph from SpltMNPV-infected third instar *S. litura* larvae 3 days p.i.. ODVs and BVs were fractionated into envelope (E) and nucleocapsid (NC) fractions, respectively. The SpltVP39 was detected using SpltVP39 antiserum. The pre-stained protein standards (NEB) are indicated on the left and the corresponding band to SpltVP39 is indicated on the right. (a) Western blotting of SpltVP39 in purified BVs and ODVs. (b) Western blotting of SpltVP39 in E and NC fractions of ODVs. (c) Western blotting of SpltVP39 in E and NC fractions of BVs

Fig. 5. Structural localization of SpltVP39 in BVs and ODVs

DISCUSSION

Baculovirus *vp39* is a core gene that is highly conserved in all sequenced baculovirus genomes. In SpltMNPV, the characterization of *vp39* is still unknown.

In this report, the temporal transcription, translation and structural localization of SpltVP39 were studied. Amino acid sequence alignment of SpltVP39 homologs from each baculovirus genus showed that they contain 6 highly conserved cysteine residues (Fig. 1). These cysteine residues probably form disulfide bonds which may be critical for virions integrity.

Transcriptional analysis of *Spltvp39* by RT-PCR showed that the *Spltvp39* transcript was first detected at 12 h p.i. and increased until 96 h p.i. (Fig. 3a). In AcMNPV-infected Sf21 cells, *vp39* was transcribed maximally at 12 and 24 h p.i.[14]. Mapping of *Spltvp39* transcripts by 5'RACE indicated that, like other baculovirus *vp39*[11-14], *Spltvp39* initiated transcription from the second A of the three baculovirus late promoter motifs ATAAG (Fig. 3b). Multiple initiation sites may allow the capsid gene to compete more effectively for RNA polymerase, thus allowing the message RNA to be transcribed at high levels.

Western blotting showed that SpltVP39 was detected from 24 h p.i. to 96 h p.i., which appeared not to correspond to the transcription data. However, we could not exclude the possibility that SpltVP39 was expressed at 12 h p.i. with low expression level. A similar condition occurs for AcMNPV ODV-E56[39]. In *Orgyia pseudotsugata* MNPV infeced *Lymantria dispar* cells, VP39 was first detected by Western blotting at 24 h p.i.[12]. *Spltvp39* was predicted to encode a protein with molecular weight of 33.9 kDa, which was close to the actual size of the protein determined by SDS-PAGE and Western blotting. These results suggested that the SpltVP39 protein is not extensively modified post-translationally. Structural localization of SpltVP39 in virions indicated that SpltVP39 is a nucleocapsid protein of BVs and ODVs, which is consistent with the previous works[11-15].

In summary, transcriptional analysis and Western blotting demonstrated that the *Spltvp39* should be a baculovirus late nucleocapsid gene, which is consistent with other baculovirus *vp39*, indicating that *Spltvp39* is highly conserved in baculoviruses. These results obtained in this study laid a foundation for further researching the functions of *Spltvp39*.

Baculovirus VP39, as a major nucleocapsid protein, is thought to be an important protein involved in the transport and assembly of nucleocapsids[16, 17, 25]. Nevertheless, the exact role of VP39 in virus replication is still unknown. Additionally, VP39 can interact with many viral and host proteins[25-27]. Thus, it may play critical roles in viral entry into and egress from the infected cells. These possibilities are currently being investigated in our laboratory.

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