Curr Microbiol (2010) 61:451–457
DOI 10.1007/s00284-010-9637-y

# Characterization of *Bombyx mori* Nucleopolyhedrovirus ORF109 that Encodes a 25-kDa Structural Protein of the Occlusion-Derived Virion

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Received: 9 February 2010 / Accepted: 19 March 2010 / Published online: 6 April 2010
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**Abstract** *Bombyx mori* nucleopolyhedrovirus (BmNPV) ORF109 (*Bm109*) is a gene present in many lepidopteran NPVs, but its function is unknown. In this study, *Bm109* was characterized. The transcript of *Bm109* was detected at 12–72 h postinfection (p.i.). Polyclonal antiserum raised to a His-BM109 fusion protein recognized BM109 in infected cell lysates from 24 to 72 h p.i., suggesting that *Bm109* is a late gene. Localization of the BM109 in BV and ODV by western analyses demonstrated that BM109 was proteins of ODV.

## Introduction

The family Baculoviridae is comprised of a diverse group of arthropod-specific DNA viruses. They are characterized by a circular double-stranded DNA genome (ranging from 80 to 180 kb) packaged within a rod-shaped capsid and enclosed by a lipid enveloped [1, 2]. They have been reported worldwide from over 600 host species, mostly from insects of the order Lepidoptera, as well as of Diptera and Hymenoptera [3].

The baculovirus life cycle typically involves the production of two virion phenotypes, budded virions (BVs) and occlusion-derived virions (ODVs). Both virions have a common nucleocapsid structure and carry identical genetic information, but are enclosed by a lipid envelope with different composition [4]. The BVs and ODVs have different functions in the Baculoviridae life cycle. BV is required for the dissemination of a viral infection throughout the tissues of an infected host. ODV is required for inter host transmission. Nucleocapsids bud through the cell membrane to become BV that can infect other cells, while ODVs remain in the nucleus and are embedded in a protein matrix, which is composed of polyhedrin during the late phase of infection [5]. *Bombyx mori* nucleopolyhedrovirus (BmNPV) contains a covalently closed circular genome of 128,413 bp, with about 136 putative ORFs [6]. Recently, a number of NPV genes have been characterized, such as *ac16* [7]; *Bm126* [8]; *Bm51* [9]; *Bm61* [10]; *Bm94* [11]. But the functions of many other genes still remain unknown, including *Bm109*. *Bm109* (nt 103,510–104,172) is a homologue of *Ac132* of AcMNPV [6], encoding a putative protein of 221 amino acids (predicted molecular mass of 25.2 kDa). Sequence analysis performed with Inter ProScan program could not reveal any function for BM109.

In this study, we studied the transcription of BM109, characterized its structural localization and the protein expression pattern.

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## Materials and Methods

### Cells, Virus, Bacterial Strains, and Antibiotics

The BmNPV (Zhenjiang strain) virus was propagated in BmN (BmN-4) cells. The BmN cell line was cultured at 27°C in TC-100 insect medium (Gibco, Tulsa, USA) supplemented with 10% (v/v) fetal bovine serum (Gibco) using standard techniques.

### Bioinformatic Analysis

The sequence analysis for *Bm109* gene and its encoding protein were performed by BLASTP and FASTA programs using updated GenBank/EMBL, SWISS-PROT databases. Sequence alignment was performed with the software ClustalX, and homology shading was done using GeneDoc software. The functional domains of *Bm109* were predicted by the softwares from EXPASY (Swiss Institute of Bioinformatics, Lausanne, Switzerland).

### Expression of *Bm109* and Preparation of Antibody

*Bm109* coding sequence was amplified with primers 5′-A*GGATCC*ATGCTATCCTGGTTATGG-3′ (containing the BamHI site) and 5′-CCG*CTCGAG*TTACAATACTT CTTGTAT-3′ (containing the XhoI site) from the BmNPV genomic DNA by PCR. The *Bm109* was subcloned into the pET30a (+) expression vector (Novagen, Madison, USA) in frame with the N-terminal 6×His tag. The recombinant plasmid, pET-*Bm109*, was verified by PCR, restriction analysis and DNA sequencing. The recombinant plasmid was transformed into *E. coli* BL21 cells and the fusion protein was expressed under induction conditions of 1 mM IPTG at 37°C for 8 h. The 6×His-tagged recombinant BM109 protein was purified by a $Ni^{2+}$-NTA column (Novagen) and used to raise polyclonal antibodies in rabbits.

Purified 6×His-BM109 protein (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 with a gap of 2 weeks before exsanguinations. The polyclonal rabbit antibody against 6×His-BM109 was used for the immunoassay.

### Mass Spectrometry Analysis

Protein spots were manually excised from SDS-PAGE gels. Gel slices were washed with 100 ml of 50% acetonitrile, 50 mM ammonium hydrocarbonate, pH 8.0. Gel slices were then dehydrated with acetonitrile and vacuum dried. After rehydration in 10 ml of 50 mM ammonium hydrocarbonate pH 8.0 containing 0.5 mg of porcine trypsin (Promega, France), samples were incubated with the same buffer for overnight (16–18 h) at 37°C. Peptide fragments from digested proteins were then crystallized with α-cyano-4-hydroxycinnamic acid as a matrix and subjected to MALDI-TOF (Bruker Daltonics, Germany) for peptide mass fingerprinting. The mass of identified peptides was obtained by searching NCBI protein database with the Mascot software (Matrix Science; http://www.matrixscience.com/search_form_select.html).

### Transcription of *Bm109* in Infected BmN Cells and Quantitative Real-Time PCR Analysis

Total RNA was extracted from BmNPV-infected BmN cells at a multiplicity of infection (MOI) of 5 with Trizol (Invitrogen, Carlsbad, USA) at 0, 6, 12, 24, 48, and 72 h postinfection (h p.i.). RNA from each time point was treated with RNase-Free DNase Set (Takara, Dalian, China), and RT-PCR was performed using the RNA PCR Kit Ver.3.0 (Takara) with 2 μg total RNA as the template per time point. First-strand cDNA was synthesized using an oligo(dT) primer and AMV reverse transcriptase (Takara), according to the manufacturer's guidelines. The PCRs were performed using 40 cycles of of 94°C for 20 s, 58°C for 30 s, and 72°C for 15 s by the gene-specific primers 5′-GATGTGGGCAACAGGTTTG-3′ and 5′-CACCGTCA TAGAGCACTTCCA-3′. A quantitative real-time PCR (QRT-PCR) was performed with SYBR Premix ExTaq (Takara) using the iCycler iQ Muticolor Real-Time PCR Detection System (Bio-Rad) by the gene-specific primers 5′-TGCCAGAATCACAAGCGA-3′ and 5′-GGTACTTCC CAACTGTCACTA-3′.

### Expression of *Bm109* in Infected BmN Cells

For the time course analysis, BmN cells were infected with BmNPV at a MOI of 10. Cells were harvested at the designated times (0, 6, 12, 24, 48, and 72 h p.i.), pelleted at 4,000×*g*, resuspended in 1 × PBS, and lysed in 2% SDS by boiling for 10 min. The protein concentrations of the cell extracts were determined by Bradford's method [12]. Cell lysates (20 μg) were analyzed by 12% SDS-PAGE and subsequently subjected to western blot assay.

### BVs and ODVs Purification

Hemolymph-derived BVs were purified from BmNPV-infected larvae as described previously [13, 14]. Polyhedra and ODV were extracted from the cells infected with BmNPV at 96 h p.i. and purified as previously described [15]. For analysis of structural proteins, BV and ODV fractions were analyzed by western blot. BmNPV-infected BmN cells were used as positive control.

## Results

### Sequence Analysis of *Bm109*

The coding region of *Bm109* is 663 bp in length, which could encode a 221-aa peptide with a predicted molecular weight of 25.2 kDa. It is transcribed in the same direction as the *polyhedrin* gene. A putative late transcription motif,

```
gttgacaacaaataagcatgtccgacaaaacgccgacaaaaaaagcggcggccatgcc
                          M  S  D  K  T  P  T  K  K  S  G  G  H  A
atgacgttacgagagcgcggcgtaacaaaacccccaaaaaagtctgaaaagttgcagcag
 M  T  L  R  E  R  G  V  T  K  P  P  K  K  S  E  K  L  Q  Q
tacaagaaagctatcgctgccgagcaaacgctgcgcaccacagcagatgtttcttctttg
 Y  K  K  A  I  A  A  E  Q  T  L  R  T  T  A  D  V  S  S  L
cagaaccacggggagagtgccgtttttcaagagttggaaagattagagaatgtggttgta
 Q  N  H  G  E  S  A  V  F  Q  E  L  E  R  L  E  N  V  V  V
gtattagaaaatgaacaaaaacgattgtatcccatattaaatacgcctcttgataatttt
 V  L  E  N  E  Q  K  R  L  Y  P  I  L  N  T  P  L  D  N  F
attgttgcattcgtgaatccgacatatcccatggcctattttgtcaataccgattacaaa
 I  V  A  F  V  N  P  T  Y  P  M  A  Y  F  V  N  T  D  Y  K
ttaaaactagaatgtgccagaatcacaagcgatttactttacaaaaacaaaaacgaagtc
 L  K  L  E  C  A  R  I  T  S  D  L  L  Y  K  N  K  N  E  V
gctatcaacaggcctaagatatcgtcttttaaattgcaattgaacgacgtaattttagac
 A  I  N  R  P  K  I  S  S  F  K  L  Q  L  N  D  V  I  L  D
actatagaaactattgaatacgatttacaaaataaagttctcacaattactgcacccgtt
 T  I  E  T  I  E  Y  D  L  Q  N  K  V  L  T  I  T  A  P  V
caagatcaagaactaagaaaatccatcatttattttaatattttaaatagtagtgacagt
 Q  D  Q  E  L  R  K  S  I  I  Y  F  N  I  L  N  S  S  D  S
tgggaagtaccaaagtacatgaaaaatttgtttgatgaaatgcaattggaacccccgcc
 W  E  V  P  K  Y  M  K  N  L  F  D  E  M  Q  L  E  P  P  A
attttaccattaggtctttagatttggtaaagttagcacgtcgacatcatgtttgcgtcg
 I  L  P  L  G  L  -
ttgacctcagagcaaaagttgttattaaaaaaa
```

**Fig. 1** Nucleotide sequence and deduced amino acid sequence of Bm109. The baculovirus consensus late transcriptional start motif ATAAG is shown in *box*, a typical polyadenylation signals (AATAAA) is shown in *ellipse*

ATAAG, was found at 6 nts, upstream from the start codon, ATG, suggesting that *Bm109* might be a late transcriptional gene. A polyadenylation signal sequence (ATTAAA) was located at 63 nts downstream of the translation stop codon, TAG (Fig. 1).

Sequence alignment for *Bm109* with its homologues from 11 NPVs is shown in Fig. 2. Comparison analysis showed that *Bm109* had the highest identity (93%) with *ORF132* of AcMNPV. The homologues from the other 10 NPVs shared 30–89% identity with BmMNPV *Bm109*.

### Transcriptional Analysis of *Bm109* and QRT-PCR

To determine the temporal expression profile of *Bm109* transcripts at early, late, and very late phases of virus infection, RT-PCR was performed using total RNA isolated from BmNPV-infected BmN cells at different time points. We used BmNPV *i.e-1* gene and *p10* gene as the controls for the early gene and very late gene, respectively. As expected, the 237-bp *Bm109* fragment was amplified from 12 to 72 h p.i., indicating that the *Bm109* is a late gene. By contrast, the 193-bp *p10* fragment was detectable from 24 to 96 h p.i. The 315-bp *i.e-1* fragment was detectable from 6 to 72 h p.i. (Fig. 3a). To further analyse the temporal expression of *Bm109* transcript, a QRT-PCR was also performed. QRT-PCR analysis showed that the *Bm109* transcript was detected from 24 to 72 h p.i. and reached a maximal level at 72 h p.i. (Fig. 3b).

### Expression of *Bm109* and Immunodetection of BM109 Protein in Infected Cells

We expressed the protein encoded by Bm109 using pET30a expression vector that introduces an extra 5-kDa N-terminal tag (His plus S tag) [16]. Thus, the apparent molecular size of the expressed fusion protein is about 30 kDa. Expression of 6×His-BM109 fusion gene in *E. coli* resulted in the production of a 30-kDa protein. Western blot analysis using specific anti-His antiserum confirmed that the 30-kDa protein fused with 6×His tag (data not shown). The purified fusion protein was used to immunize rabbits to produce the specific antiserum against BM109. A time course analysis of BM109 in BmNPV-infected BmN cells was performed by western blot using anti-BM109 antiserum. The result revealed that a specific immunoreactive band with approximate molecular mass of 25 kDa was first detected form 24 to 72 h p.i. (Fig. 4). No immunoreactive band was detected in the mock-infected control. The size of immunoreactive protein was in agreement with the prediction (25 kDa). Thus, our data suggested that the post-translational modification events may be limited, despite the presence of many putative post-translational modification sites.

### Mass Spectrometry Analysis

The Mascot (Matrix Science, London, UK) search was performed with carbamidomethyl as the fixed modification

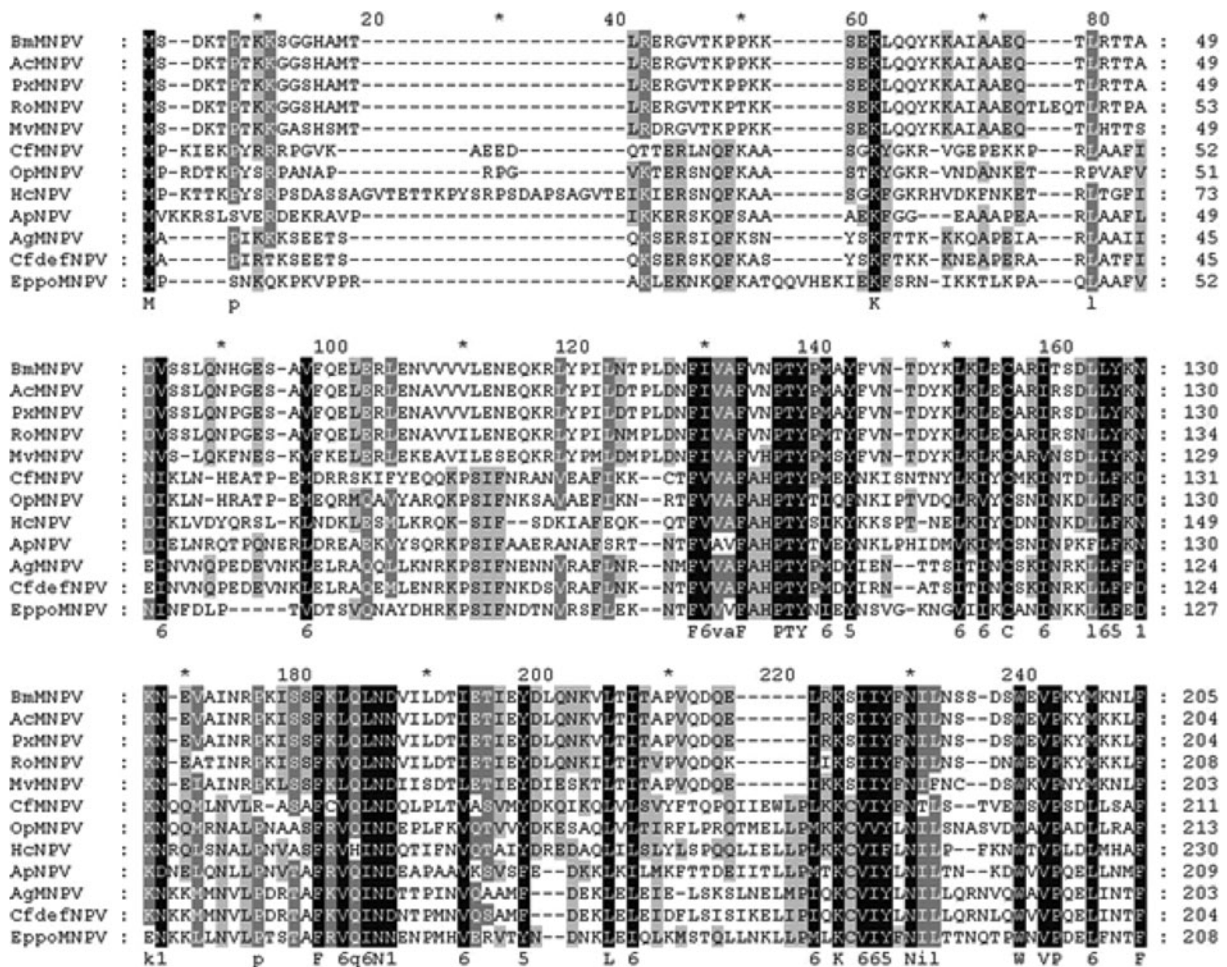


**Fig. 2** Amino acid sequence alignment of baculovirus Bm109 homologues. The sources of sequences are: BmNPV (GenBank, NP_047529), AcMNPV (GenBank, NP_054162), PxMNPV (GenBank, YP_758596), RoMNPV (GenBank, NP_703119), MvMNPV (GenBank, YP_950831), CfNPV (GenBank, NP_848436), OpNPV (GenBank, NP_046286), HcNPV (GenBank, YP_473212), ApNPV (GenBank, YP_610996), AgNPV (GenBank, YP_803522), CfdefNPV (GenBank, NP_932736), EppoNPV (GenBank, NP_203286)

of cysteine and variable N-terminal Gln-pyroGlu. The protein was confirmed to be homologous to AcMNPV ORF132 by a Mascot score of 74, with 6 peptides matched and 30% amino acid coverage (Fig. 5). The result indicated that the protein was ORF109 (*Bm109*).

## Localization of the BM109 Protein in Cell, BV, and ODV

To investigate whether the BM109 protein was a structural protein, western blot analysis of purified BVs, ODVs, and BmNPV-infected BmN cells were carried out for immunodetection. The data (Fig. 6) showed that BM109 could be detected in ODV but not BV, total proteins of infected BmN cells as control. Preparations of BmNPV BV were subjected to western blot analysis using anti-Bm51 [9]. These results confirmed that BM109 was associated with ODV virions and may function as a structural protein.

## Discussion

In this report, we described some preliminary characteristic of *Bm109*, a gene that has thus far not been characterized. Homologues of *Bm109* have been identified in genomes of all group I NPVs and exclusive to all group I NPVs [17]. Here, we presented the transcription, expression, and structural localization analysis of the *Bm109*.

The transcription analysis of *Bm109* by RT-PCR showed that the *Bm109* started transcription at 12 h p.i., and remained until at least 72 h p.i. This result suggested that *Bm109* might be a late gene. Baculovirus infection progresses in three phases, early, late, and very late, in cultured insect cells. The separation between early and late transcription is always the onset of DNA replication. The early genes are transcribed prior to DNA replication, whereas late and very late genes are activated during or after replication [18]. The early genes usually encode

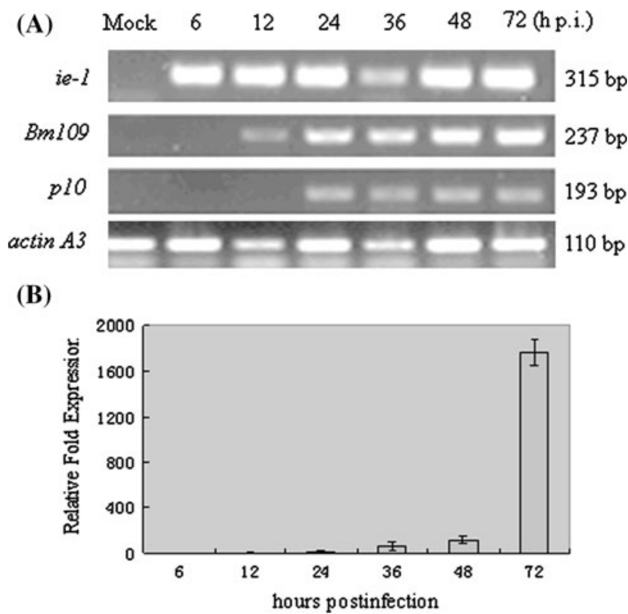


**Fig. 3** **a** RT-PCR analysis of *Bm109* transcription. Total RNA was extracted from BmNPV-infected cells at different times post infection (p.i.). PCR products of different genes are indicated on the *left*. Times p.i. are indicated above the *lanes*. The sizes (in bp) of different PCR products are indicated on the *right*. *i.e-1* and *p10* are positive controls for early and late genes. The actin A3 RT-PCR product was used as the control**. b** QRT-PCR analysis of *Bm109* transcription. Total RNA was extracted from BmNPV-infected cells at different times post infection (p.i.). The values showed the results of three independent replication assays with *error bars* indicating standard deviation

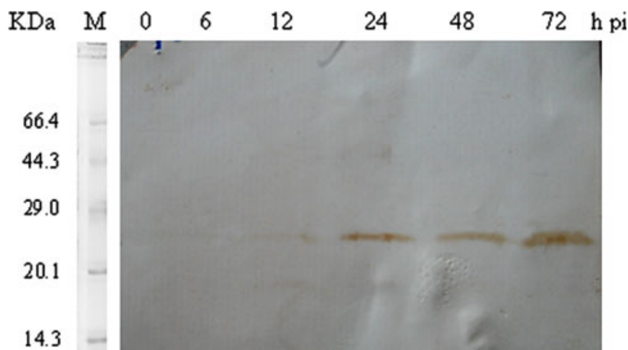


**Fig. 4** Western blot analysis of the BM109 protein in BmN cells. The cells were collected at 0, 6, 12, 24, 48, and 72 h p.i., and processed for western blot using anti-BM109 antiserum followed by incubated with a goat antirabbit IgG conjugated to HRP. The signal was detected with diaminobenzidine (DAB) as a chromogenic substrate

proteins with regulatory functions, such as transcription, replication, and modification of host processes. Late genes include baculovirus structural proteins, whereas very late genes are those involved in the processes of occlusion and cell lysis [18]. Late proteins are frequently involved in the regulation of late and very late gene expression [19, 20]. Baculovirus late genes were transcribed using a

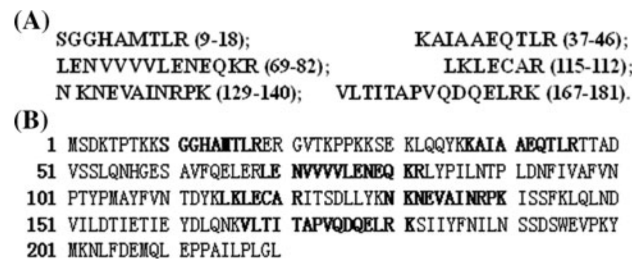


**Fig. 5** Identification of BM109 protein by MALDI-TOF analysis. **a** Peptide sequences identified by mass spectrometry. **b** Amino acid sequences of BM109 protein. Matched peptide sequences are shown as *bold* character

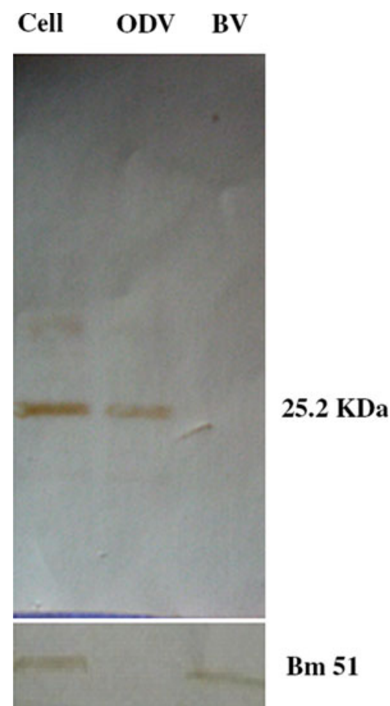


**Fig. 6** Localization of BM109 protein in BmNPV BVs, ODVs, and BmN cells. BV, ODV, cell lysates samples were separated by SDS-PAGE and analyzed by western blotting

conventional RNA polymerase II promoter [21]. This promoter [22] was determined to contain a core sequence motif of ATAAG, GTAAG, or TTAAG, but not CTAAG. By analyzing the sequence of *Bm109*, we found the motif ATAAG that was at 6 nts upstream of the start codon ATG. Our data further confirmed that *Bm109* was a late gene that could only be detected from 24 to 72 h p.i. by BM109 polyclonal antiserum.

We also carried out sub-cellular localization experiment to check the biosynthesis and transport of BM109. Theoretically, the ODV structural proteins will be synthesized in cytoplasm and then transported into nucleus for envelope assembly and polyhedrin formation [23]. We did detect the

expression of BM109 in the cytoplasm (data not shown). However, to our surprise that BM109 could not be detected in nucleus even after 72 h of BmNPV infection. One possibility is this protein was only incorporated into the ODV at the very late stage, which may require more time than 72 h p.i. There were some proteins localized in the cytoplasm in early stage and localized in the nucleus in late stage in infection, such as *Bm60* [24], *Ha83* [25], *vp39* [26]. There are indeed a few structural proteins that localized in cytoplasm from 24 to 72 h p.i., such as *Bm67* [13], *Ha128* [27]. *Bm67* was required for the production of infectious budded viruses and for assembly of envelope and nucleocapsids [28]. However, the product of BM67 was a non-structural protein that is different from BM109. Therefore, further experiments will have to be performed to investigate the localization of BM109 protein expressed in BmNPV-infected cells.

Western blot was used to determine whether BM109 is a structural protein. The immunoreactive band was present only in ODVs and not in BVs. The result was consistent with the proteomic analysis [4]. ODVs and BVs are genetically identical but differ in their envelope composition [15]. Many component proteins contain an N-terminal hydrophobic signal sequence in combination with several adjacent positively charged amino acids, targeting these proteins to intranuclear microvesicles that are the likely precursors from which the envelopes of occluded virus are derived [29]. In addition, there are some proteins that were likely to be present in ODV nucleocapsids, for example, ODV-E66 [30]; ODV-E25 [30]; ODV-EC43 [4]; ODV-E18 [4], and *Per os* infectivity factors (Ac138 (*P74-pif*), Ac22 (*pif-2*), Ac115 (*pif-3*), Ac119 (*pif-1*), Ac145, and Ac150). The analysis of baculovirus core gene *ac142* by McCarthy and Theilmann [31] has shown that in the absence of a complete *ac142*, nucleocapsids cannot acquire nuclear viral envelopes to form ODV. Without an envelope, *ac142* nucleocapsids are not occluded within the polyhedra and the polyhedra produced are empty of virions. Thus, it is also possible that the BM109 may be involved in the production of the nuclear viral envelopes to form ODV.

In summary, our data implicated that BM109 is a structural protein for the ODV of BmNPV, and may play an important role in the replication of lepidopteran NPVs. Our work provides good materials and platforms for subsequent detailed investigation of the cellular roles of BM109 in the BmNPV-infected cells.

**Acknowledgments** We thank Dr. Haifeng Shi for his support and assistance. This work was supported by the National Program of High-Tech Research and Development (863 High-Tech Program, No. 2008AA10Z145), the grants from Jiangsu Sci-Tech Support Project-Agriculture (No. BE2008379), National Basic Research Program of China (No. 2005CB121006).

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