Virus Genes (2009) 38:171–177
DOI 10.1007/s11262-008-0312-3

Bombyx mori nucleopolyhedrovirus ORF51 encodes a budded virus envelope associated protein

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Received: 6 May 2008 / Accepted: 26 November 2008 / Published online: 16 December 2008
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Abstract *Bombyx mori* nucleopolyhedrovirus (BmNPV) ORF 51 (Bm51) is a gene present in many lepidopteran NPVs, but its function is unknown. In this study, Bm51 was characterized. Transcripts of Bm51 were detected from 4.5 through 72 hour post infection (h p.i.) by RT-PCR. The corresponding protein was detected from 6 to 72 h p.i. in BmNPV-infected BmN cells by western blot analysis using a polyclonal antibody against Bm51. Western blot assay of occlusion-derived virus and budded virus (BV) preparations revealed that Bm51 encodes a 23-kDa structural protein that is associated with BV and is located in the envelope fraction of budded virions. The protein was temporarily called BV-E23. In addition immunofluorescence microscopy demonstrated that the protein was present within the cytoplasm and nuclei in virus-infected cells. In conclusion, the available data suggest that Bm51 is a functional ORF of BmNPV and encodes a protein expressed in the early stage of the infection cycle that is associated with the BV envelope.

Keywords *Bombyx mori* nucleopolyhedrovirus · Bm51 · Transcript · BV-E23 · BV-associated envelope protein 23

Introduction

The *Baculoviridae* is a family of enveloped, double-stranded DNA (81.7–178.7 kb) viruses [1] that infect invertebrates, particularly insects of the order Lepidoptera. Baculoviruses typically produce two virion phenotypes of progeny virus: occlusion-derived virus (ODV) and budded virus (BV). The ODV transmits infection from insect to insect by infecting midgut columnar epithelial cells, whereas the BV is responsible for causing systemic infection within the host [2].

Baculoviruses have been extensively studied. The devastating effects on natural populations of insects have made them an obvious choice for use as biological agents to control pests [3]. They are also exploited as expression and gene therapy vectors [4–7].

Because the genome of *Bombyx mori* nucleopolyhedrovirus (BmNPV) (T3 strain) was completely sequenced [8], numerous genes from BmNPV have been characterized, including Bm79 [9], Bm67 [10, 11], Bm122 [12], Bm60 [13], DBP [14], and Bm68 [15]. However, the function of many genes in BmNPV genome remains unknown. The ORF 51 of BmNPV (Bm51) and its orthologues exist in several baculovirus genomes; however, no function has been reported so far. The transcription, expression, and localization of Bm51 were characterized in this study.

Materials and methods

Cells and viruses

BmNPV (T3 strain) virus was propagated in BmN (BmN-4) cells, which were maintained at 27°C in TC-100 media supplemented with 10% (v/v) fetal bovine serum (Gibco-BRL). The titration of virus and other routine manipulations were performed according to the standard protocol [16]. *Escherichia coli* strain BmDH10Bac, containing

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BmNPV bacmid (BmBac) DNA, was provided by Dr. Enoch Y. Park (Shizuoka University, Japan).

Computer-assisted sequence analysis and sequencing

The Bm51 gene and its deduced amino acid sequence were analyzed using Genetyx-Win (Version 5) (Software Development Co., Tokyo, Japan). The protein sequence was analyzed using the ExPASy server (www.expasy.ch) [17]. Conceptually translated Bm51 protein was compared with homologous proteins from BLAST searches of GenBank/EMBL and SWISS-PROT databases [18, 19]. Alignments of multiple sequences were carried out with ClustalW [20] and edited with Genedoc software [9]. All clones were sequenced by Shanghai Invitrogen Biotech Co. Ltd.

Transcription analysis

Monolayers of BmN cells were infected with BmNPV at a multiplicity of infection (m.o.i.) of ten, and virus was absorbed for 1 h in serum-free culture media, which was then replaced with TC-100 medium. Total RNA was extracted at 0, 1, 3, 4.5, 6, 12, 24, 48, and 72 h p.i. using the Trizol RNA extraction kit (Invitrogen) according to the manufacturer's protocol. The extracted total RNA was treated with RNase-free Dnase I (Takara) to remove contaminating genomic DNA.

For cDNA synthesis and PCR, total RNA was extracted at each time point and transcribed by AMV reverse transcriptase (Takara) and an oligo-p(dT)18 primer for first strand cDNA synthesis. The coding region of the Bm51 gene was amplified by PCR with two primers: P1, 5′-atgtac aataaatttctg-3′(nt 45935-45953); P2, 5′-ttacatattatatttagca ag-3′(nt 46381-46402). The product of RT-PCR of beta-actin with two primers (ActinF, 5′-AATGGCTCCGG TATGTGC-3′; ActinR, 5′-TTGCTCTGTGCCTCGTCT-3′) was set as the positive control.

Production of anti-Bm51 antiserum

The Bm51 coding region was amplified from the BmNPV genomic DNA by PCR using an upstream primer (5′-GGATCCATGTACAATAAATTTCTG-3′) with a *Bam*HI site (underlined) and a downstream primer (5′-CTCG AGTTACATATTATATTTAGCAAG-3′) with an *Xho*I site (underlined). The amplified fragment was inserted into the pTA2 (Toyoba Co. LTD, Japan) vector and further sequenced. The correct Bm51 coding region was then inserted into the expression vector pGEX-4t-2 (Pharmacia, USA) with a glutathione S-transferase sequence (GST) at the N-terminus. The recombinant plasmid was transformed into *Escherichia coli* BL21 cells for expression under the induction conditions of 3.2 mM IPTG at 37°C for 8 h. The fusion protein GST-Bm51 was separated by 12% SDS-PAGE gel and then retrieved from the gel. The polyclonal antibody against GST-Bm51 was prepared using standard techniques [9]. In brief purified protein (about 2 mg) in complete Freund's adjuvant was injected subcutaneously to immunize New Zealand white rabbits (Sigma, USA), which was followed by two booster injections in incomplete Freund's adjuvant 3 weeks later before exsanguinations. The prepared polyclonal rabbit antibody against GST-Bm51 was used for immunoassays.

Temporal expression of Bm51 in infected BmN cells

Monolayers of BmN cells, infected with BmNPV T3 strain BVs (m.o.i. of 10) at the designated times (mock 1, 3, 4.5, 6, 12, 24, 48, and 72 h p. i.), were washed three times with cold PBS (0.14 M NaCl, 2.7 mMKCl, 10.1 mM Na2HPO4, 1.8 mM KH2PO4, pH 7.4). The protein concentration of the cell extracts was determined by Bradford's method [21]. Cell lysates (20 mg) were analyzed by SDS-PAGE (12% gel) and subsequently subjected to Western blot assay.

ODV, BV, BV-E, and BV-NC preparation

Fifth-instar larvae of *B. mori* were injected with 5 µl ($TCID_{50}$/ml = 10^9) of BmNPV T3 budded viruses. The infected larvae were fed at 25°C for 5 days, and the polyhedra were purified and verified by ordinary microscopy and electron microscopy [22]. ODV was purified from polyhedra as described by Braunagel and Summers [23] and Caballero et al. [24].

BV was purified from the cell culture supernatant of infected cells as described by Braunagel and Summers [23]. In brief BmN cells were infected with BmNPV at an m.o.i. of 10. After 3 days, the cell culture supernatant was centrifuged (4,000*g*, 5 min, 4°C) to remove cell debris. The supernatant was then filtered (0.45 µm filter, Millipore) to further purify the suspension, and the filtrate was pelleted, resuspended, and then overlaid onto a 36-ml, 25–56% continuous sucrose gradient in 0.1 × TE and centrifuged at 150,000*g* for 90 min at 4°C. The BV band was collected and diluted in 0.1 × TE, which was followed by centrifugation at 150,000*g* for 90 min. The pellet of BV was resuspended in 0.1 × TE. Separation of BV envelope (BV-E) and nucleocapsid fractions (BV-NC) after treatment with NP-40 was done as described previously [25].

Sub-cellular localization analysis of Bm51 proteins in infected cells

Monolayers of BmN cells infected with BmNPV (m.o.i. of 10) at 48 h p.i. were washed three times with cold PBS, and

then cells were fixed in a 1:1 methanol to acetone solution for 15 min, followed by three washes in cold PBS. Cells were incubated with GST-Bm51 polyclonal antiserum (diluted with PBS, 1:100) for 1 h at room temperature. Cells were washed three times with cold PBS and then incubated with protein G fused to enhanced green fluorescent protein (EGFP), and DAPI (Sigma, USA) for 1 h. Cells were directly observed and photographed using a Leica TCS-SP5(Germany) confocal laser scanning microscope.

Results

Sequence analysis of Bm51

The Bm51 ORF contains 468 nucleotides and encodes a 155 amino acid peptide with a predicted molecular mass of 18.5 kDa (http://www.expasy.ch/tools/pi_tool.html). A baculovirus early consensus transcriptional start motif (CAGT) is 26 nt upstream of the start codon (Fig. 1, shown in box), and a TATA box is 31 nt upstream of the CAGT motif (shown in box), suggesting that Bm51 might be an early transcriptional gene. In additional, a typical polyadenylation signal (AATAAA) is 454 nt downstream of the translation stop codon TAA (Fig. 1, shown in box), and no signal peptide sequence, transmembrane region, nuclear localization signal, or membrane retention signal is predicted in the deduced amino acid sequence using EXPASY tools.

Blast search results show that Bm51 homologues exist in four completely sequenced Group I NPV genomes (AcMNPV, BmNPV, RoMNPV, and PlxyMNPV) and eight Group II NPV genomes (TnSNPV, ChChNPV, HearNPV, HearNPV-G4, HzSNPV, MarvMNPV, EcobNPV, and LdMNPV). Its homologue is also found in *Thysanoplusia orichalcea* NPV, whose genome is partially sequenced. Sequence comparison of the amino acid (aa) sequences shows that Bm51 has its highest identity (93%) with AcMNPV ORF63 and its lowest identity (22%) with LdMNPV ORF117 Fig. 2. No homologue was found in GV that have sequenced genomes, or baculoviruses isolated from non-lepidopteran invertebrates. Thus, the Bm51 homologue is specific to some lepidopteran NPVs.

Transcriptional analysis of Bm51

To determine the initial time of Bm51 transcription, RT-PCR analysis was performed using total RNA isolated from BmNPV-infected host cells. A band with an expected size of 468 bp was amplified at 4.5 h p.i., which remained detectable up to 72 h p.i. (Fig. 3). The result suggests that Bm51 might be an early gene, which is consistent with the presence of the early gene motif in the putative promoter region.

Temporal expression of Bm51 in infected BmN cells

To study the expression of Bm51, a time course of BmNPV-infected BmN cells was analyzed by western blot using anti-GST-Bm51antiserum. A specific immunoreactive band was first observed at 6 h p.i. and remained detectable up to 72 h p.i. (Fig. 4). These data are consistent with the results of transcriptional analysis; however, the protein was detectable 1.5 h later than the gene transcript (Fig. 4), which is probably due to the low amount of

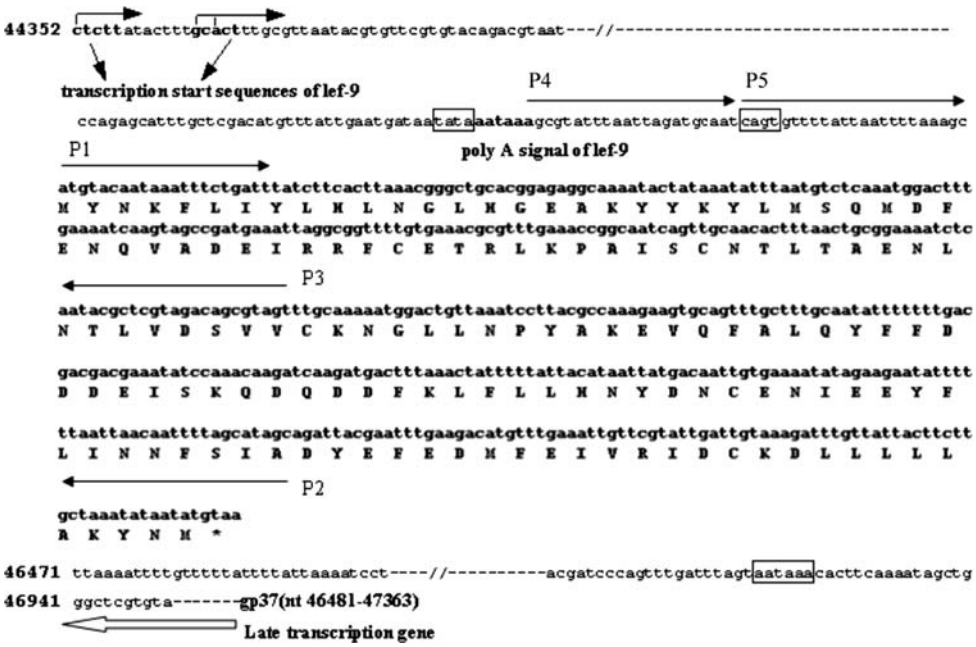

Fig. 1 Schematic diagram of the genomic region with Bm51 and the transcripts of adjacent genes. The location of the early initiation motif (CAGT), TATA box, and a typical polyadenylation signals (AATAAA) are denoted in boxes. Primers are indicated with arrows. The transcription start sequences of Bm50 (lef-9) "CTCTT" and "GCACT", as well as the consensus polyadenylation signal (AATAAA), are indicated in bold. The transcription of gp37 is marked with a hollow arrow. The positions of the PCR primers used for RT-PCR analysis are also indicated

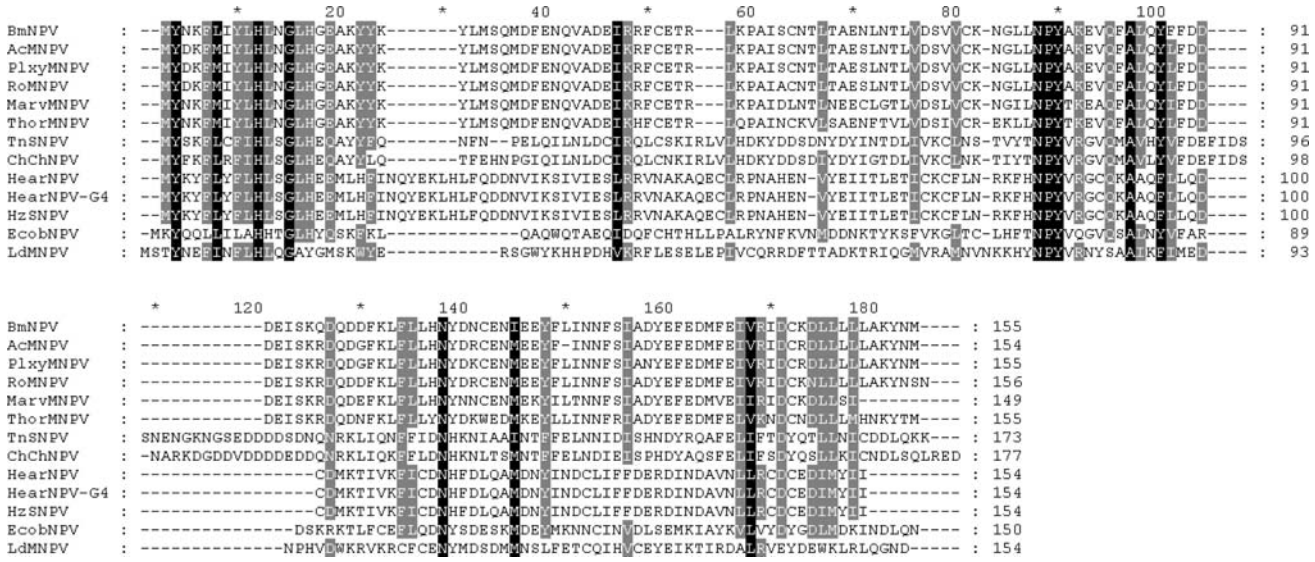

Fig. 2 Amino acid sequence alignment of baculovirus Bm51 homologues. The alignment was edited with GeneDoc software. Black shading: 100% similarity. Grey shading: 80% similarity. The sources of the sequences are: BmNPV (GenBank, NC_001962), AcMNPV (GenBank, NC_001623), PlxyMNPV (GenBank, NC_008349), RoMNPV (GenBank, NC_004323), MarvMNPV (GenBank, NC_008725), ThorMNPV (GenBank, AF169480), TnSNPV (GenBank, NC_007383), EcobNPV (GenBank, NC_008586), ChChNPV (GenBank, NC_007151), HearNPV (GenBank, NC_003094), HearNPV-G4 (GenBank, NC_002654), HzSNPV (GenBank, NC_003349), LdMNPV (GenBank, NC_001973)

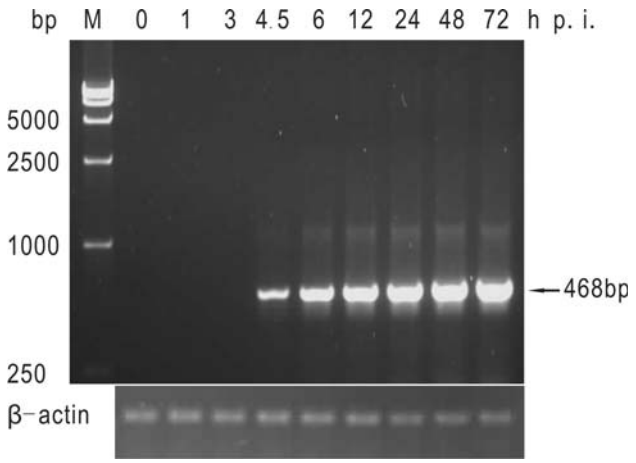

Fig. 3 RT-PCR analysis of Bm51 transcription in BmNPV-infected BmN cells. Total RNA was isolated from BmNPV-infected cells at 0, 1, 3, 4.5, 6, 12, 24, 48, and 72 h p.i. Nested PCR was conducted with the Bm51-specific primers P1 and P2. Size of the transcript is indicated in bp on the right side of the panel. The β-actin RT-PCR product was used as the control. The products were separated on a 1.0% agarose gel

protein produced shortly after transcription. No immunoreactive band was detected in the mock-infected control. The detection of the Bm51 protein at 6 h p.i. indicated that it was synthesized early in infection. The size of the immunoreactive protein was about 23 kDa, larger than the predicted molecular weight of 18.5 kDa, suggesting that there may be post-translational modification events.

Immunodetection of the Bm51 protein in BVs

To investigate whether Bm51 was a structural component of BmNPV, western blot analyses of purified BV, BV envelope (BV-E), BV nucleocapsid (BV-NC), and ODV were carried out. The BV fraction and BV envelope (BV-E) showed a reactive band (Fig. 5), and the size was in agreement with that detected in lysates from infected cells. In contrast, no band was detected in the ODV fraction (Fig. 5). The efficacy of the fractionation was examined by immunoassay with antibodies against VP80 (a BV-NC and ODV-NC component) [26], GP64 (a component of BV-E) [27], and Bm56 (a component of ODV-NC) [28]. The positive bands at 80 kDa were detected as expected in the BV-NC fraction, and BV or ODV preparations with antibody against VP80. The bands of 64 kDa were detected as expected in the BV-E and BV sample using anti-GP64 antiserum, and the band of 42 kDa was only detected in ODV using anti-Bm56 antiserum (Fig. 5). Hence, the separation of BV-N, BV-E, and ODV was considered to be pure. Thus, the above results suggest that the Bm51 gene encodes a structural protein associated with the BV-E.

Subcellular location in infected BmN cells

The intracellular localization of the Bm51 protein was determined by immunofluorescence using anti-GST-Bm51 polyclonal antiserum and cells infected with BmNPV Fig. 6. The results revealed that Bm51 was clearly visible

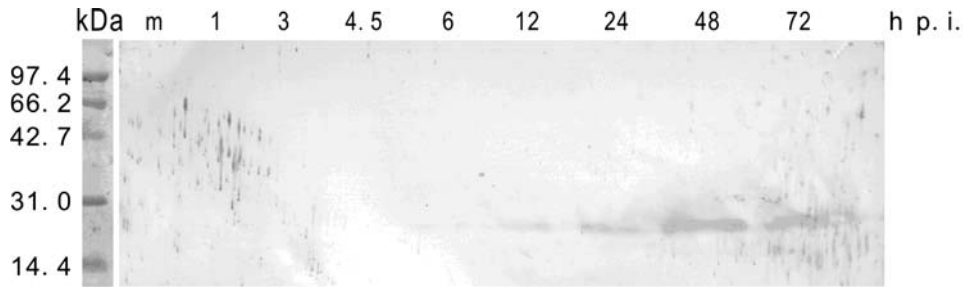

Fig. 4 Western blot analysis of Bm51 in BmNPV-infected BmN cells. The cells were collected for the mock infection control and at 1, 3, 4.5, 6, 12, 24, 48, and 72 h p.i., and 20 μg cell lysate at each interval was subjected to western blot analysis using anti-GST-Bm51 serum. The binding was developed with diaminobenzidine (DAB) as a chromogenic substrate. Protein markers are indicated on the left and mock-infected cell lysate is indicated (m)

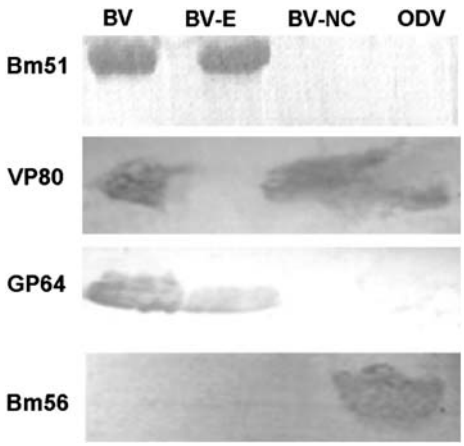

Fig. 5 Structural localization of Bm51. Preparations of BmNPV BV, BV envelope (BV-E), BV nucleocapsid (BV-NC), and purified ODV were subjected to western blot analysis using anti-Bm51, anti-VP80, anti-GP64, or serum anti-Bm56. The primary antibodies used in the experiments are indicated to the left of each panel

in the cytoplasm and nucleus of infected cells. In the control, no obvious fluorescence signal was observed in virus-infected cells exposed to pre-immune serum and the EGFP-conjugated protein G (Fig. 6).

Discussion

In this study, the preliminary characteristics of Bm51 were studied. The search for homologues of Bm51 revealed that homologous genes were present in 12 completely sequenced members of lepidopteran NPVs, suggesting that Bm51 and its homologues are specific to some lepidopteran NPVs and might play an important role in the virus life cycle for infection of these lepidopteran hosts.

NPV genes have been classified into at least three groups: immediate-early, early, and late genes, and the expression of viral genes upon infection is temporally regulated [29, 30]. RT-PCR results showed transcripts of Bm51 were detectable at 4.5 h p.i. Transcripts of the *Bm51* neighboring gene, *lef*-9, whose transcript signals were detected at 12 h p.i., did not overlap with *Bm51* [31]. The transcription of the another *Bm51* adjacent gene *gp*37 initiated 24 h p.i. [32], which was quite different from the early transcription initiation of Bm51. To determine the possible 5′ end of the Bm51 transcript, total RNA was extracted from BmN cells infected with BmNPV after 24 h p.i. RT-PCR showed that a band of about 210 bp as expected could be amplified when using the pair primers P5 (5′-cagtgttttattaattttaaagc-3′) (Fig. 1) and P3 (5′-ctacgctgtctacgagcg-3′), while no band could be detected by using the pair primers P3 and P4(5′-gcgtatttaattagatgcaat-3′) (Fig. 1). When control genomic DNA was used as a template, normal products could by amplified by using either of the about two pair primers. The result suggested that the transcriptional initiation site for Bm51 is near the early transcriptional start motif (CAGT). Our results indicate that *Bm51* is a gene expressed early in the viral infection cycle and the early transcriptional start motif (CAGT) may be used.

Western blot analysis showed that the size of Bm51 protein was about 23 kDa, which is larger than the predicted molecular weight of 18.5 kDa, suggesting that there may be post-translational modification events. Though an N-glycosylation site (aa 124–127) and three casein kinase II phosphorylation sites (aa 26–29, 62–65, and 95–98) were predicted; if the protein is actually N-glycosylated, -phosphorylated, -lapidated, or otherwise modified, remains to be determined.

BV is broadly specialized to infect many internal tissues of the host including tracheoblasts, hemocytes, and fat bodies. BV entry occurs by receptor-mediated, absorptive endocytosis [33]. In this study, structural location showed the Bm51 protein was associated with the BV but not with the ODV, suggesting that the Bm51 protein may either play a role in the assembly of BV or be related to BV infection of cells. Previous studies revealed that the BV-specific protein GP64 plays important roles in attachment and budding from cells [27, 34–36]. However, GP64 is only present in group I

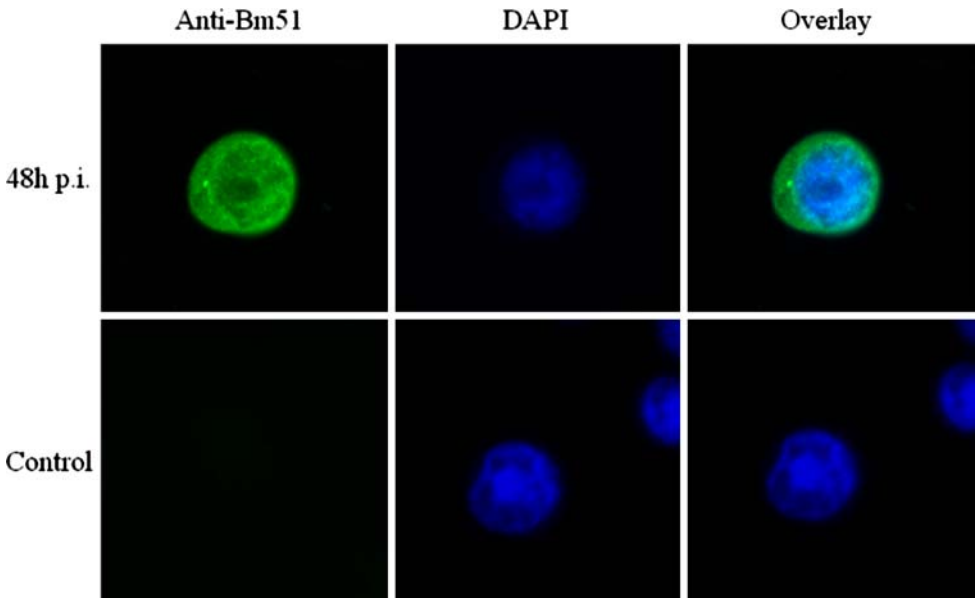

Fig. 6 Intracellular localization of Bm51 in BmNPV-infected BmN cells. The cells were collected at 48 h p.i., washed with 1 × PBS and reacted with anti-GST-Bm51 serum. Fluorescence was developed by incubation with protein G fused to EGFP. As a control, pre-immune serum was used as the primary antibody. The nuclei were stained with DAPI (blue). The samples were observed under a confocal laser scanning fluorescence microscope

NPV. Bm68 was also found to be a structural protein associated with only BV, and fractionation of virions revealed that Bm68 was present in the envelope fraction, but not the capsid fraction of BV. Immunohistochemical analysis showed that the Bm68 protein was localized mainly in the nucleus of infected cells [15], which is similar to Bm51. Subcellular localization of Bm51 showed that it was present both in the cytoplasm and in the nucleus of infected cells, suggesting that transport of Bm51 from the cytoplasm into the nucleus may happen during the infection. Similar transport was found for many other baculovirus proteins, such as ODV-E66 [37] and P74 [38], with their transportation from cytoplasm to nucleus mediated by FP25 K [39]. Several other BV-specific proteins were also identified, such as Ha33 (BV-e31) [40], Ha39 [41], Ld130 [42], and Ac23 [43].

In summary, Bm51 is transcribed delayed early post infection. The virion structural location showed that Bm51 encodes a structural protein of the BV envelope.

Acknowledgments This project was supported by the National Program of High-tech Research and Development (863 High-Tech Program, No. 2006AA10A119) and the National Natural Science Foundation of China (Project No. 30570074).

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