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Characterization of a Late Gene, ORF60 from *Bombyx mori* Nucleopolyhedrovirus

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Open reading frame 60 of *Bombyx mori* nucleopolyhedrovirus (*Bm*60) is located between 56,673 and 57,479 bp in the BmNPV genome which encodes 268 amino acid residues with predicted molecular weight of 31.0 kDa. Bm60 and its homologues have been identified in 11 completely sequenced lepidopteran NPVs. The transcript of Bm60 was detected by RT-PCR at 18-72 h post-infection (p.i.), while the corresponding protein could be detected at 24-72 h p.i. in BmNPV-infected BmN cells by Western blot analysis using a polyclonal antibody against Bm60. The expression of Bm60 was inhibited in the presence of Ara-c, an inhibitor of viral DNA synthesis. These results together indicated that Bm60 was a late gene. The size of Bm60 product was found to be a 31 kDa in BmNPV-infected BmN cells, consistent with predicted molecular weight. Immuno-fluoresence analysis showed that the Bm60 product was first detected in the cytoplasm at 24 h p.i and also located in nucleus during later infection. In conclusion, the available data suggest that *Bm*60 is a functional ORF of BmNPV and encodes a 31kDa protein expressed in the later stage of infection cycle.

Keywords: BmNPV, Bm60, Expression, RT-PCR, Subcellular location

Introduction

Baculoviruses have a circular double-stranded DNA genome of 80-180 kb. The Baculoviridae is a diverse family of pathogens that is infectious for arthropods, particularly insects of the order Lepidoptera and is used as a natural control agent of insect pests (Moscardi *et al*., 1999).They are taxonomically subdivided into two genera, the nucleopolyhedrovirus (NPVs) and the granuloviruses (GVs) according to the morphology of occlusion bodies (OBs) (Durantel *et al*., 1998). The NPVs have large OBs containing numerous virions, whereas the GVs have single virions occluded within small granular OBs. The Lepidoptera NPVs can be further divided into groups I and groups II on the basis of phylogenetic analysis (Bulach *et al*., 1999; Herniou *et al*., 2001).

The *Bombyx mori* NPV (BmNPV) is a major pathogen of the mulberry silk worm and cause disastrous effect on output of *Bombyx mori* silk. It is also second in popularity only to AcMNPV as a baculovirus expression system. A BmNPV-based expression system is an economic alternative for large-scale synthesis of commercially important biomolecules, since the silkworm larvae, which are easy to rear on a synthesis or natural diet, can be used instead of the cultured cell lines (Meada, 1989). For development of such application, it is important to understand the molecular basis of baculovirus infection.

As an extensively high-level expression of recombinant genes in insect caterpillars, the complete sequence and genetic organization of the BmNPV have been elucidated. The DNA genome is 128,413bp and potentially encodes 136 genes (Gomi *et al*., 1999). So far, the functions of several genes in BmNPV have been characterized, such as orf8 (Imai *et al*., 2004), orf6 (Iwanaga *et al*., 2002), orf42 (Acharya *et al*., 2002 a), lef-8 and lef-9 (Acharya *et al*., 2002b) etc. But the functions of many other genes still remain elusive, including orf60.

BmNPV orf60 (Bm60) is located between 56,673 and 57,479 bp in the BmNPV genome, encodes a 268 amino acids residues with predicted 31.0 kDa molecular weight, and is transcribed in the opposite orientation of polyhedrin gene. Though Bm60 homologues are identified in several lepoidoptera NPVs, it is uncertain whether Bm60 and its homologues are

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functional genes. In this study, we studied the transcript, expression, subcellular location of Bm60.

Materials and Methods

Virus and cell lines. The *Bombyx mori* cell line, BmN, was mantained in TC-100 medium (Gibco, Carlsbad) with 10% fetal bovine serum (Gibco). The BmNPV (Zhenjiang strain) was used for infections as wild type and propagated in BmN cells.

Computer-assisted sequence analysis. The protein sequence was analyzed using softwares of the ExPASy (www.expasy.ch) for the prediction of motifs, domains, transmembrane regions and signal peptides (Appel *et al*.,1994). Homologues were explored by using BLASTP searching tool in the updated GenBank/EMBL and SWISS-PROT databases (Pearson *et al*., 1990; Altschul *et al*., 1997). The sequence alignment was carried out with ClustalW (http://www.ebi.ac.uk/clustalw) and edited with Genedoc software (Ver.2.04) (Free Software Foundation, Inc.).

RT-PCR. BmN cells were infected with BmNPV at a multiplicity of infection (m.o.i) of 10. Total RNA was extracted at 0, 3, 6, 12, 18, 24, 48 and 72 h p.i. using Trizol (Invitrogen) according to manufacturer's protocol. RNA was dissolved in diethylprocarbonate (DEPC)-treated water and quantified by optical density measurement at 260 nm.

For cDNA synthesis and PCR experiment, the total RNA was first treated with DNase to eliminate any potential genomic DNA contamination. First-strand cDNA was synthesized from DNase-treated total RNA by using oligo(dT) primer and Superscript II reverse transcriptase (Invitrogen) following the manufacturer's protocol. The first-strand cDNA was amplified by PCR using an upstream primer P1 (5'-AGGATCC ATGaaaataaatttgtgc-3') with a *Bam*HI site (underlined) and a downstream primer P2 (5'-ACTCGAG tcatgtttctttttgaa-3') with a *Xho*I site (underlined). The PCR product was electrophoresed in 1.0% agarose gel.

Expression of the Bm60 gene in *E.coli.* and generation of anti-Bm60 antiserum. The Bm60 coding region amplified by RT- PCR was ligated into pGEM-T easy vector (Promega).The insert was retrieved by digestion with *Bam*HI and *Xho*I, and was inserted into the expression vector pGEX-4T_2 (Amersham Pharmacia). Bm60 was fused in in-frame with the C-terminal glutathione S-transferase (GST) tag in pGEX-4T_2 plasmid. The DNA sequence encoded GST and Bm60 was further confirmed by sequencing to ensure that ORF60 was translated correctly.

The recombinant plasmid, designated as pGEX-GST-Bm60, was transformed into *Escherichia coli* BL21 cells and fusion protein expression was induced by 0.1 mM isopropyl β-D-thiogalactoside (IPTG) at 28°C in the presence of ampicillin. The recombinant protein was purified using a GST binding column (Amersham Pharmacia) and used to raise polyclonal antibodies in rabbits.

New Zealand white rabbits received four injections of 200 μg of purified GST-Bm60 fusion protein, the first immunization was carried out hypodermically in 50%complete Freund's adjuvant (Sigma) followed by the second injection intramuscularly in 50% incomplete Freund's adjuvant (Sigma) with 3 weeks interval. Two weeks after the second injection the rabbits were injected third time using purified fusion protein. One week later the last injection was performed to boost immunization with purified fusion protein. Serum was collected one week after the last injection. Western blot analyses using *E.coli*.BL21 cell extracts, *E.coli*.BL21 cell extracts expressing GST-Bm60 and column-purified GST-Bm60 were used to test the specificity of antisera.

Western blot analysis. BmN cells were infected with BmNPV at an m.o.i. of 10 $TCID_{50}$ units per cell. Cells were harvested at designated time points (0, 3, 6, 12, 24, 48, 72 h p.i.), pelleted at 3,000 g, resuspended in PBS and lysed in SDS-PAGE loading buffer by boiling for 10 min. Protein samples were separated by 12% SDS-PAGE and transferred onto an Immobilon-P nitrocellulose membrane (Millipore) by semi-dry electrophoresis transfer (Ausubel *et al*., 1994). The membranes were immersed in 3% skimmed milk powder in PBST at 4°C overnight followed by incubation with the anti-Bm60 polyclonal antiserum diluted 1 : 5000 for 1 h at room temperature. Subsequently, the membrane was incubated with a goat anti-rabbit IgG conjugated to HRP diluted 1 : 5000 for 1 h at room temperature. The signal was detected with a DAB substrate solution.

Cytosine Arabinoside (Ara-c) inhibitor assay. To investigate whether the Bm60 was expressed as a late gene, BmN cells infected with BmNPV was treated at 6 h p.i. using the metabolic inhibitor, Ara-C, with a final concentration of 80ng/ml in medium. The Ara-C treated samples were collected at 12, 18, 24, 48 and 72 h p.i. and processed for western blot analysis with anti-Bm60 antiserum. The uninfected cells and BmNPV-infected cells were collected as controls.

Subcellular location. Monolayers of BmN cells were infected with BmNPV (M.O.I. of 10). The cells were collected in designated time points. The cells were washed three times in PBS and fixed with 4% paraformaldehyde in PBS for 10 mins at room temperature. The cells were washed three times and permeabilized with 0.2% Triton X-100 in PBS for 10 min at room temperature, and again washed three times with PBS. The cells were incubated with the anti-Bm60 serum diluted 1 : 5000 for 1h at room temperature. The anti-Bm60 serum was removed by washing three times with PBS. Then cells were allowed to react with proteinG fused with enhanced green fluorescent protein (EGFP) (ProteinG-EGFP) diluted 1 : 8000 for 1 h at room temperature. After washing three times with PBS, the cells were examined under a Zeiss LSM510 confocal laser-scanning microscope for fluorescence. The BmNPV-infected cells reacted only with ProteinG-EGFP as negative controls.

Results

Sequence analysis. To identify any similarities to potential biologically significant domains or motifs from the existing protein families, the deduced Bm60 protein sequence was compared with proteins in the PROSITE database. Four Protein kinase C phosphorylation sites(aa 29-31, aa 47-49, aa

199-201 and aa 224-226), three N-glycosylation sites (aa 45-48, aa 62-65, aa 197-200), seven Casein kinase II phosphorylation site (aa 52-55, aa 58-61, aa 70-73, aa 105- 108, aa 106-109, aa 224-227, aa 235-238), two Tyrosine sulfation sites (aa 116-130, aa 240-254) one Tyrosine kinase phosphorylation site (159-165) and one cAMP- and cGMP-dependent protein kinase phosphorylation sites (aa 265-268) were found in the Bm60 amino acid sequences. In addition, no other significant domains or motifs was searched by protein searching tool PROSITE.

The puptative transcription start sites, such as baculovirus consensus early CA(G/T)T and late TAAG promoter motifs were analyzed in the regions upstream of Bm60. The result suggested that a late transcription motif TAAG was found 14 nt upstream of the translation start codon ATG of Bm60, six early transcription motifs CA(G/T)T were observed 189nt, 158nt,141nt,133nt,127nt and 81nt upstream of the ATG codon of Bm60.Two polyadenylation signal sequences AATAAA were located 207nt and 275nt of translation stop codon TAA.

Search in protein databases, GenBank and SWISS-PROT, revealed that the putative Bm60 protein was homologous to the predicted products from 10 NPV ORFs, including: *Autographa californica* MNPV (ORF74) (Ayres, *et al*., 1994), *Helicoverpa zea*SNPV(ORF70) (Chen,*et al*., 2002), *Helicoverpa armigera* NPV (ORF68) (zhang *et al*., 2005), *Spodoptera.litura* NPV (ORF71) (pang *et al*., 2001), *Lymantria dispar* NPV (ORF135) (Kuzio *et a*l., 1999), *Rachiplusia ou* NPV (ORF71) (Harrison *et al*., 2003), *Choristoneura fumiferana* defective NPV(ORF69) (GenBank accession No.AY327402), *Choristoneura fumiferana* NPV (ORF69) (GenBank accession No. NC 004778), *Orgyia pseudotsugata* NPV (ORF77) (Ahrens *et al*., 1997) and *Epiphyas postvittana* NPV (ORF66) (Hyink *et al*., 2002). No homologues were explored in other lepoidoptera NPVs sequenced, like *Adoxophyes honmai* NPV, *Spodoptera exigua* NPV and *Mamestra configurata* NPV. Sequence alignments of Bm60 and its homologues from other NPVs revealed that only 1 completely conserved amino acid residue, which likely play important role in function of Bm60 and its homologues (Fig. 1).

RT-PCR. Temporal regulation of Bm60 transcript was examined by RT-PCR analysis using total RNA isolated from BmNPV-infected host cells at designated time intervals (0, 3, 6, 12, 18, 24, 48 and 72 h p.i.). The result revealed a band with an expected size of 818 bp (Fig. 2). The Bm60 transcript was

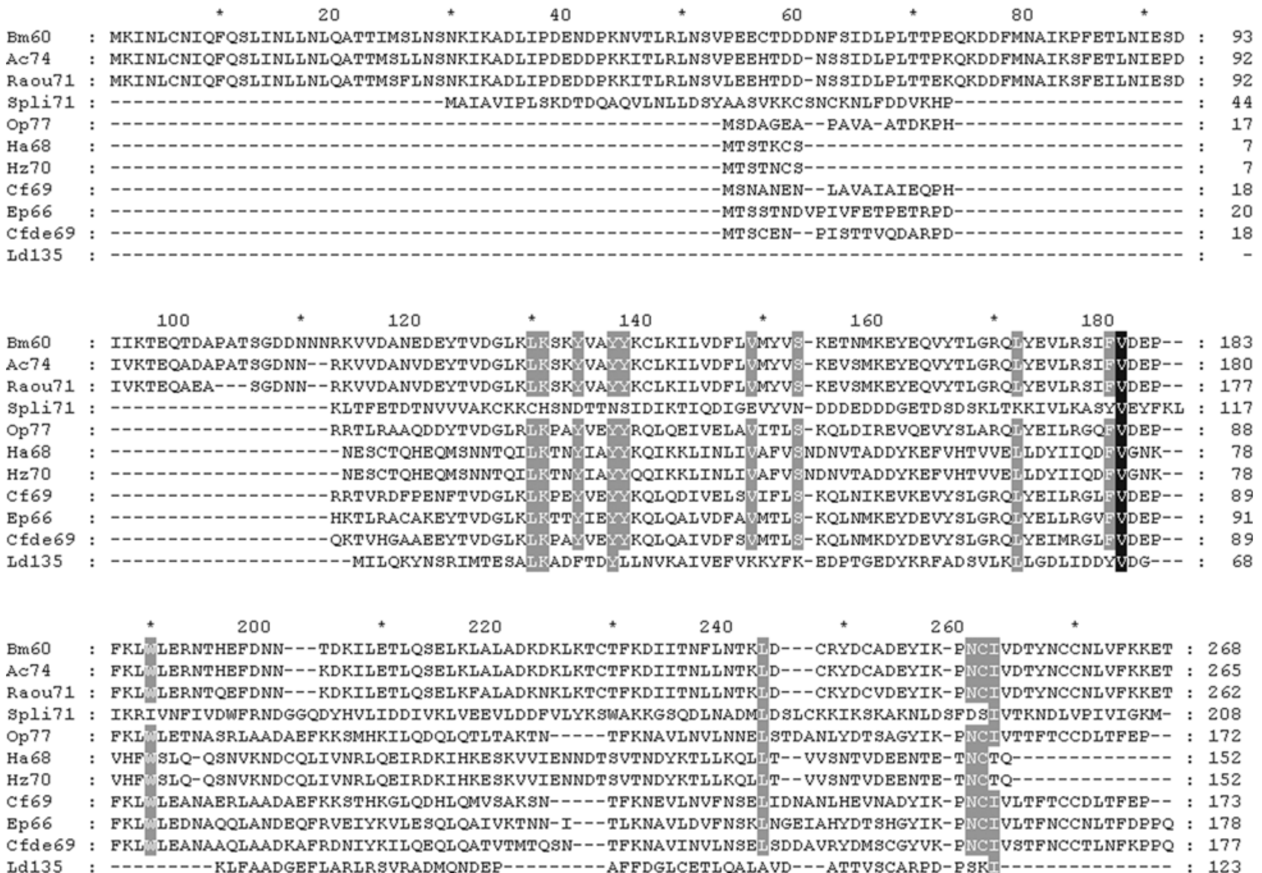

Fig. 1. Alignment of amino acid sequences of Bm60 and its homologues. The alignment was edited with GeneDoc software (Ver.2.04) Two shading were set: Black shading for 100% identity and Grey shading for 80% identity. The sources of sequences are indicated in paper.

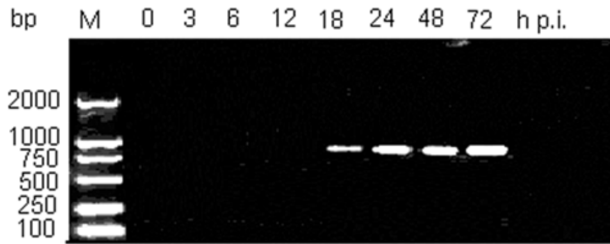

Fig. 2. RT-PCR analysis of the BmNPV ORF60 transcript. The analysis performed on total RNA extracted from BmNPV-infected BmN cells at 0, 3, 6, 12 ,18, 24, 48 and 72 h p. i.(lane 1-7). times p.i. are indicated above the lanes. Size marker are indicated in bp (2000, 1000, 750, 500, 250, 100).

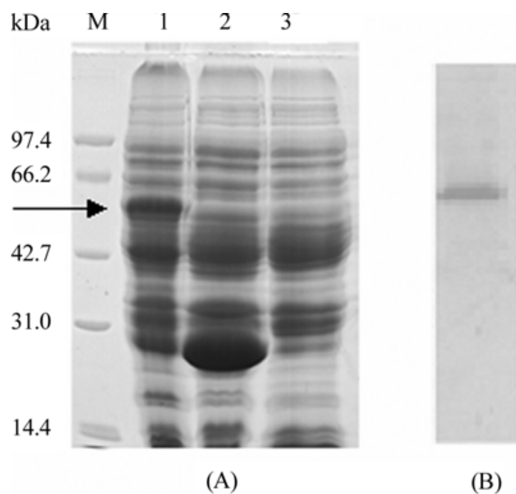

Fig. 3. Expression of Bm60 in *E. coli*. BL21 cells. BL21 cells transformed with pGEX-GST-Bm60. fusion plasmid were induced by IPTG and the cell extracts were separated by SDS-PAGE. (A) SDS-PAGE analysis. M. protein molecular weight markers. lane 1. proteins from BL21 cells transformed with pGEX-GST-Bm60 (the expressed protein is indicated with an arrow). lane 2. proteins from BL21 transformed with pGEX-4T-2 plasmid. lane3. BL21 cell proteins. (B) Western blot analysis of expressed protein using anti-GST antiserum.

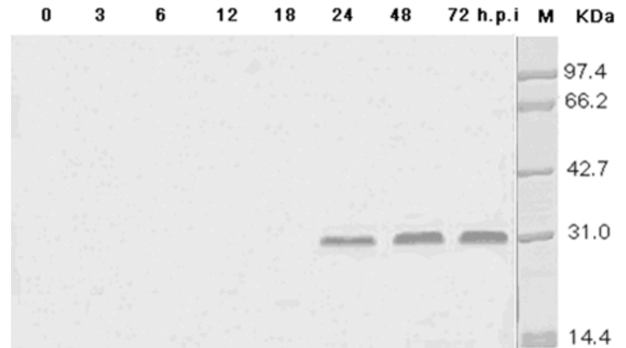

Fig. 4. Temporal expression analysis of *Bm60* product in BmNPV-infected BmN cells by Western blot. The BmNPV-infected BmN cells were collected at designated time (shown at the top). The total protein were separated on 12% SDS-PAGE gel and transferred to a PVDF membrane. western blot analysis was carried out using anti- Bm60 antiserum and detected with a diaminobenzidine (DAB) substrate. Size standards are indicated in kDa and immunoreactive proteins are indicated by arrows.

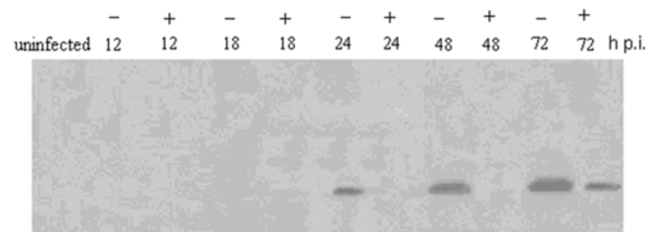

Fig. 5. Western blot analysis of the expression of Bm60 in BmNPV-infected BmN cells treated with Ara-C. The BmN-infected cells were treated with Ara-C at the time point of 6 hrs p.i.. The treated cells then were collected at different time points (12, 18, 24, 48, 72 hrs p.i.) and processed for western blot using anti-Bm60 antiserum followed by incubated with a goat anti-rabbit IgG conjugated to HRP. The signal was detected with DAB substrate. The uninfected cells and BmNPV-infected cells were collected as controls. The cells treated with Ara-C are indicated with plus (+) and untreated cells are indicated with minus (-).

detected at 18 h p.i. and remained detectable until 72 h p.i. (Fig. 2).

Expression of Bm60 gene and immunodetection of Bm60 protein in infected cells. In order to express the Bm60, The vector pGEX-GST-Bm60 was introduced into *E. coli* BL21 (DE3). The GST-Bm60 protein was overexpressed in BL21, the expressed production was a 57-kDa protein in size (Fig. 3A). Western blot analysis using the anti-GST antiserum further confirmed that the 57-kDa polypeptide was the fusion protein (Fig. 3B). The fusion protein was purified and used to immunize rabbits for producing the anti-Bm60 antiserum.

To see whether a protein was encoded by Bm60 ORF, Protein extracts of BmNPV-infected BmN cells isolated at different time (0, 3, 6, 12, 24, 48 and 72 h p.i.) were separated by 12% SDS-PAGE. Western blot was performed with anti-Bm60 antiserum. The result revealed that a specific immuno-reactive band with approximately 31 kDa, which first detected at 24 h.p.i. and could be detected until 72 h.p.i. (Fig. 4). No immunoreactive band was detected in mock-infected control. The 31 kDa immunoreactive band was consistent with predicted 31 kDa molecular weight, suggesting that no major post translational modification of the Bm60 protein occurred.

Ara-C inhibitor analysis. In order to examine whether Bm60 was a late gene, BmN cells infected with BmNPV were incubated with Ara-C, a metabolic inhibitor of DNA synthesis. Western blot result revealed that the immuno-reactive band of 31 kDa was disappeared at 24 and 48 h p.i. and only detected in 72 h p.i. in Ara-C treated samples, suggesting that the

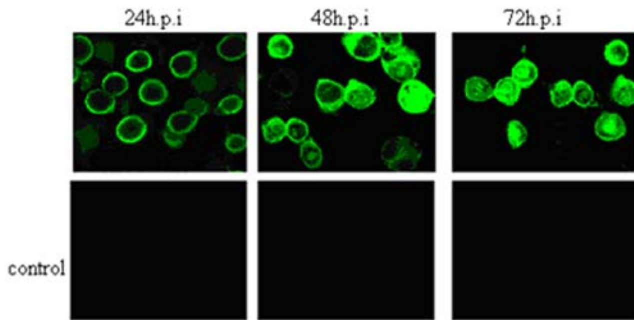

Fig. 6. Subcellular localization of Bm60 in in BmNPV-infected BmN cells. The cells were collected at 24, 48 and 72 h p.i., washed with 1 × PBS and reacted with anti –Bm60 serum followed by incubated with protein G fused with enhanced green fluorescent protein. preimmune serum was used as control . Cells were photographed under a confocal laser fluorescence microscope.

expression of Bm60 could be inhibited by Ara-C (Fig. 5). As control, Bm60 was present in the samples of BmNPV-infected cells and no signal was detected in uninfected cells (Fig. 5).

Subcelluar location of Bm60 proteins in infected cells. To visualize the distribution of Bm60 during BmNPV infection, the subcellular location of Bm60 protein was detected by immunofluorescence using anti-Bm60 antiserum and cells infected with BmNPV.

Since Bm60 protein was first detected at 24 h p.i., the time points of 24, 48 and 72 h p.i. were chosen for observation. The result suggested that Bm60 was localized primarily in the cytoplasm and hardly present in the nucleus at 24 h p.i., and was detected in the nucleus from 48 till 72 h p.i. except that cytoplasm (Fig. 6). As control experiment, the BmNPV-infected cells reacted only with ProteinG-EGFP at all time points did not show detectable fluorescence signal (Fig. 6).

Discussion

In this study, we described some preliminary characteristic of Bm60, a gene that has thus far not been characterized. Based on the phylogenetic analysis, 63 genes were conserved among all sequenced lepidopteran NPVs (Herniou *et al*., 2001). These genes were considered as core genes for all lepidopteran NPVs. the search for homologues of Bm60 revealed that homologues were present in 10 completely sequenced members of lepidopteran NPVs, not in all completely sequenced lepidopteran NPVs, suggesting Bm60 and its homologues were specific genes for 11 completely sequenced lepidopteran NPVs and might play important role in function related to these hosts.

Western blots of total infected cell proteins probed with a Bm60-specific antiserum detected a single protein migrating at 31 kD, compatible with the predicted size. The treatment of BmNPV-infected BmN cells with tunicamycin showed no alteration in 31 kD size by western blot using anti-Bm60 serum (data not shown). These results indicated that no major post-translational modifications occurred in the Bm60 protein, despite the presence of several potential post modification motifs in Bm60 amino acid sequence. In this study, the transcription analysis of Bm60 by RT-PCR showed that the Bm60 started to transcribe at 18 h p.i. and remained until at least 72 h p.i.. This result suggested that Bm60 might be a late gene and probably used only one late transcription start motif, TAAG, found 14nt upstream of the translation start codon ATG of Bm60, despite six early transcription motifs present in the Bm60 sequence. Western blot analysis further confirmed this result for Bm60 protein was detected from 24 to 72 h p.i., late in infection cycle. The inhibitor analysis of Ara-C confirmed directly that Bm60 was a late expressed gene. Bm60 protein, compared with its mRNA, was first detected later, at 24 h p.i.. This could be due to the fact that the western blot was not sensitive enough to detect low levels of protein at early time or/and the affinity of the antiserum to protein is low.

The subcellular location revealed that Bm60 localized primarily in the cytoplasm at 24 h p.i. and both in the cytoplasm and nucleus at 48 and 72 h p.i.. Computer analysis of Bm60 did not predict the existence of a membrane spanning domain in this protein. The similar protein location were found in other baculovirus proteins, such as Ha122 (Long *et al*., 2003) and Ha83 (Wang *et al*., 2006), this transporting from cytoplasm to nucleus was mediated by FP25K (Braunagel *et al*., 1999). Therefore, Bm60 protein possibly form a protein complex with one of proteins involved into transporting process and was interacted with proteins that were transported to the nucleus.

Although some basic characteristics were described in this paper, much information about this gene keeps unknown. To further understand the functions of Bm60, analyses of knock-out mutants of Bm60 are necessary.

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