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Bombyx mori nucleopolyhedrovirus ORF79 encodes a 28-kDa structural protein of the ODV envelope

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Summary. Open reading frame 79 of *Bombyx mori* nucleopolyhedrovirus (*Bm*79) is a conserved gene whose homologues have been identified in all 26 of the completely sequenced baculovirus genomes, including lepidopteran NPVs and GVs, hymenopteran NPVs, and a dipteran baculovirus. Northern blot analysis showed that the *Bm*79 transcript was about 850 nucleotides long and was initiated 12 h p.i. Temporal expression analysis revealed a 28-kDa protein, which was detected beginning 24 h p.i. using a polyclonal antibody against GST-Bm79 fusion protein. The 28-kDa protein was detected in the occlusion-derived virus envelope (ODV-E), but not in budded viruses. This observation was confirmed by observing ultrathin sections of polyhedra using immunoelectron microscopy. This demonstrated that the protein was present within the nuclei of cells. These results suggest that *Bm*79 is a functional gene that encodes a structural protein associated with the envelope of occlusion-derived virus (ODV).

Introduction

Nucleopolyhedroviruses (NPVs), which constitute a genus (*Nucleopolyhedrovirus*) of the family *Baculoviridae*, typically produce progeny virus with two virion phenotypes: 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 [19]. The two viral forms are essential for the natural propagation of the baculoviruses. They have different viral structure compositions despite containing an identical genome [7, 28]. The BV acquires its envelope early as the nucleocapsids exit from the cell surface, whereas the ODV obtains its envelope within the nucleoplasm by unknown process. It is believed that ODV envelope proteins are required to fuse with columnar cell surface proteins after occlusion bodies enter into the midgut of susceptible insects [16].

Since the sequencing of the genome of the baculovirus *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) [2], 25 other baculovirus genomes have been reported so far [33]. Based on the comparative analysis of 13 baculoviruses, 30 ORFs have been identified in common and designated as baculovirus core genes, 20 of which have known functions [13] and 10 of which remain unknown. Homologues of Bm79 have been identified in genomes of all 26 completely sequenced baculoviruses, including 16 lepidopteran NPVs and 7 GVs, 2 hymenopteran NPVs, and 1 dipteran baculovirus. Thus, *Bm*79 is one of the 30 baculovirus core genes. To date, seven of the 30 core genes have been shown to be specific for ODVs: *p*74 [20], *gp*41 [32], *odv-e*56 [5], *odv-ec*27 [6], *vp*91 [25], *pif* [17], and *odv-ec*43 [10].

In this report, the transcriptional analysis and temporal expression of the *Bm*79 gene are described, and the localization of *Bm*79 gene product has been determined. The common baculovirus gene, *Bm*79, was shown to encode a novel structural protein associated with the ODV envelope with a molecular weight of 28 kDa.

Materials and methods

Cells and viruses

BmNPV (ZJ strain) virus was propagated in BmN (BmN-4) cells, maintained at 27 °C in TC-100 insect medium supplemented with 10% (v/v) fetal bovine serum (Gibco-BRL). The titration of virus and other routine manipulations were performed according to the standard protocols [23].

Computer analysis

The functional domains and motifs of Bm79 were predicted by the software EXPASY [1]. The signal peptide was predicted by the software SignalP (http://www.cbs.dtu.dk/services/SignalP/). The data from all other baculovirus occlusion body genes compared in this paper were obtained from GenBank. Sequence alignment was performed with the software Clustal X [30] and homology shading was done using GeneDoc software [22].

Expression of Bm79 and preparation of antibody

The *Bm*79 coding region was amplified from the BmNPV genomic DNA by PCR. The *Bm*79 forward primer F 1 (5′-**ggatcc**atgttgtcaatcattttg-3′) and reverse primer R (5′-**ctcgag**ttaataaa cttggtctg-3′) were synthesized based on the genomic sequence of BmNPV T3 (GenBank accession No. NC_001962) with *Bam*HI site and *Xho*I site (shown in bold), respectively. The *Bm*79 was subcloned into the expression vector pGEX-4t-2 with a glutathione *S*-transferase (GST) at the N-terminus. Fusion protein GST-Bm79 was expressed in *E. coli* under induction conditions of 0.2 mM IPTG at 37 °C. Fusion protein GST-Bm79 was purified and extracted using SDS-PAGE. The antibody was prepared using standard techniques [12]. Purified GST-Bm79 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 within a gap of 2 weeks before exsanguinations. The polyclonal rabbit antibody against GST-Bm79 was used for immunoassay.

To prepare antibodies against ODV-E18 and GP64, the *odv-e*18 and *gp*64 coding regions were amplified from the BmNPV genomic DNA. The primers for *odv-e*18 were ODV-E18F

(5′-**ggatcc**atgttgtcaatcattttg-3′) and ODV-E18R (5′-**ctcgag**ttaataaacttggtctg-3′), and the primers for *gp*64 were GP64F (5′-**gaattc**atgctactagtaaatcagtc-3′) and GP64R (5′-**aagctt**tt aatattgtctactattacgg-3′). Subsequently, the *odv-e*18 was subcloned into pGEX-4t-2 and the *gp*64 was inserted into pET28a for expressing the fusion proteins GST-E18 and His-GP64, respectively. Purified GST-E18 and His-GP64 were used to raise the polyclonal antibodies specific for ODV-E18 and GP64, respectively.

Bm79 expressed in insect cells by Bac-to-Bac expression system

Bm79 fused with enhanced green fluorescence protein (eGFP) was expressed in the *Trichopolusia ni* cell line Tn-5B1-4. The recombinant donor vector pFastBacHTb (Invitrogen) was reconstructed to contain *egfp* and*Bm*79, designated pBacHTeGFPT-Bm79. The plasmid of pBacHTeGFPT-Bm79 was transformed into DH10 Bac *E. coli* (Invitrogen) to generate recombinant Bacmid DNA, designated rBac-EGFPBm79. For GFP-Bm79 expression, a monolayer of Tn-5B1-4 cells was transfected with rBac-EGFPBm79. Cells infected with recombinant Bacmid were harvested for SDS-PAGE and western blot analysis. Cells uninfected with virus were used as controls.

Northern blot analysis

Monolayer of BmN cells was infected with BmNPV at a multiplicity of infection (m.o.i.) of 10, virus was absorbed for 1 h in serum-free culture and then replaced with TC-100 medium with 10% FBS. Total RNA from mock- or BmNPV-infected cells was extracted using Trizol (Invitrogen) according to the manufacturer's protocol. Ten micrograms of total RNA was separated on 0.8% agarose-formaldehyde gel as described by Sambrook et al. [26]. A probe was prepared from the purified PCR product, which had been amplified previously from BmNPV genomic DNA with *Bm*79-specific primer F2 (5′-aagctttagcgtgaccacgttcac-3′) and primer R, and was labeled with DIG-dCTP. Blotting was performed using DIG High Primer DNA Labeling and Detection Starter Kit (Roche, Germany) according to the manufacturer's instructions.

Cytosine Arabinoside (Ara-C) inhibitor assay

To investigate whether the *Bm*79 was expressed as a late gene, BmNPV-infected BmN cells were treated at appropriate time points (3, 6, 12 and 24 h p.i.) with metabolic inhibitor of DNA synthesis (Ara-C, 80 ng/ml), and continuously incubated until 48 h p.i. Cells were harvested for western blot assay with anti-GST-Bm79 serum. The uninfected cells and BmNPV-infected cells were collected as controls.

Temporal expression of Bm79 in infected BmN cells

For the time course analysis, BmN cells were infected with BmNPV at a m.o.i. of 10. Cells were harvested at the designated times and washed three times with $1 \times$ PBS (0.14 M NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4). The protein concentrations of the cell extracts were determined by Bradford's method [4]. Cell lysates (20 μg) were analyzed by 10% SDS-PAGE and subsequently subjected to western blot assay.

ODV and BV preparation

Larvae of the silkworm *B. mori* were infected with BmNPV as described by Iwanaga et al. [18]. The polyhedra were purified from infected *B. mori* larvae as described by Summers and Smith [29]. The purity of BmNPV polyhedra was verified by ordinary microscopy and electron microscopy. ODVs were purified from polyhedra as described by Braunagel and Summers [7]

and Caballero et al. [9]. Briefly, polyhedra were incubated in Na_2CO_3–NaCl solution (0.1 M Na_2CO_3, 166 mM NaCl, 10 mM EDTA, pH 10.5) for 15 min at room temperature to release ODVs. The solution was centrifuged (6000 g, 10 min) to remove undissolved polyhedra. The supernatant was loaded onto 36 ml 25–56% (w/w) continuous sucrose gradient in 10 mM Tris-HCl, pH 7.5, and centrifuged at 100,000 g (Sorvall surespin 630, 23,500 rpm) for 60 min at 4 °C. The bands were collected and diluted in 0.1 × TE (10 mM Tris, pH 7.4, 1.0 mM EDTA) followed by centrifugation at 100,000 g (Sorvall T-880, 34,000 rpm) for 60 min. The pellet of ODVs was resuspended in 0.1 × TE.

BV was purified from the cell culture supernatant of infected cells as described by Braunagel and Summers [7]. A monolayer of BmN cells was infected with BmNPV at a m.o.i. of 10. After three days, the cell culture supernatant was collected and potential contaminant cells were removed by centrifugation (4000 g, 5 min) at 4 °C. The supernatant was filtered (0.45 μm filter, Millipore) to further purify the suspension. The filtrate was overlaid onto a 36 ml, 25–56% continuous sucrose gradient in 0.1 × TE and centrifuged at 150,000 g (Sorvall Surespin 630, 28,500 rpm) for 90 min at 4 °C. The BV band was collected and diluted in 0.1 × TE followed by centrifugation at 150,000 g for 90 min (Sorvall T-880, 40,000 rpm). The pellet of BVs was resuspended in 0.1 × TE.

Fractionation of ODVs into envelope and nucleocapsids

ODVs were fractionated into envelope and nucleocapsid essentially as described by Braunagel and Summers [7]. ODVs were incubated in 0.1 × TE containing 1% NP-40 and 250 units aprotinin for 30 min at room temperature with gentle shaking. The solution was layered onto 11.5 ml of 30% (v/v) glycerol in 0.1 × TE and centrifuged at 100,000 g (Sorvall T-880, 34,000 rpm) for 90 min at 4 °C. The ODV envelope proteins from the top of gradient were precipitated by acetone. The pellet of ODV nucleocapsid proteins was resuspended in 0.1 × TE. The preparations of ODV nucleocapsid and envelope were stored for western blot assay.

N-glycosylation assay

BmN cells were infected with BmNPV in the presence of 10 μg/ml tunicamycin (Sigma). Cells were harvested at 72 h p.i. and analyzed by western blot. The anti-GST-Bm79 serum was used as the primary antibody, and the antibody against BmGP64 was used as the positive control.

Western blot analysis

Western blot was performed as described by Towbin et al. [31]. Following SDS-PAGE electrophoresis, the protein samples were transferred onto a PVDF membrane (Immobilon-P, Millipore) in cold Towbin buffer (0.025 M Tris, 0.19 M Glycine, 20% methanol) with Trans-Blot Cell (Bio-Rad). After blocking with 5% skim milk in PBS-T (1 × PBS, 0.1% Tween-20), membrane was washed three times with PBS-T for 5 min and incubated with the rabbit-derived polyclonal antibody in PBS-T with 5% skim milk at 37 °C for 1 h. After washing, goat anti-rabbit IgG conjugated with HRP (1:4000) was utilized to detect the reactive band. The peroxidase activity was developed with 0.1% H_2O_2 and diaminobenzidine (DAB) as a chromogenic substrate. Molecular markers were detected by Coomassie blue staining.

Immunoelectron microscopy (IEM)

Purified polyhedra were fixed and ultrathin sections were prepared for antibody reaction as described by Flipsen et al. [11]. Sections were blocked with BL (1% BSA, 50 mM PBS, 0.02%

PEG20000, 0.1% NaN_3) for 30 min, reacted with anti-GST-Bm79 serum (1:1000 dilution) for 1 h, and washed with double-distilled water three times for 5 min. The bound primary antibody was detected using gold-conjugated protein A (10 nm) prepared as described by Slot [27]. The sections were stained with uranyl acetate and lead citrate. Samples were viewed using a JEM-1230 transmission electron microscope (JEOL).

Immunofluorescence microscopy

BmN cells were infected with BmNPV and collected at three days post infection. The harvested cells were rinsed three times with 1 × PBS and fixed in cold methanol:acetone (1:1) for 15 min, followed by three washes with 1 × PBS. To detect the Bm79 location, cells were incubated with anti-GST-Bm79 polyclonal antibody (1:400 dilution) in 1 × PBS for 2 h at room temperature. The primary antibody was removed by washing three times with 1 × PBS, and the cells were then incubated with protein G fused with enhanced green fluorescent protein for 2 h and the nucleus (DNA)-specific stain DAPI (Sigma, USA) for 1 h. Subsequently, the cells were directly observed and photographed using a Zeiss LSM 510 confocal laser scanning microscope.

Results

Sequence analysis

The Bm79 ORF contains 549 nts and is predicted to encode a 182-amino acid peptide with a predicted molecular weight of 20,900. A baculovirus consensus late transcriptional start motif TTAAG was found at 145 nt upstream of the start codon ATG (Fig. 1, shown in bold). At 70 nt and 84 nt downstream of the stop codon TAA, two typical polyadenylation signals (AATAAA) are located (Fig. 1, shown in italic). Computer analysis predicted a confident signal peptide at the N-termini (1–22 residues) and four possible *N*-glycosylation sites in the sequence (Fig. 2).

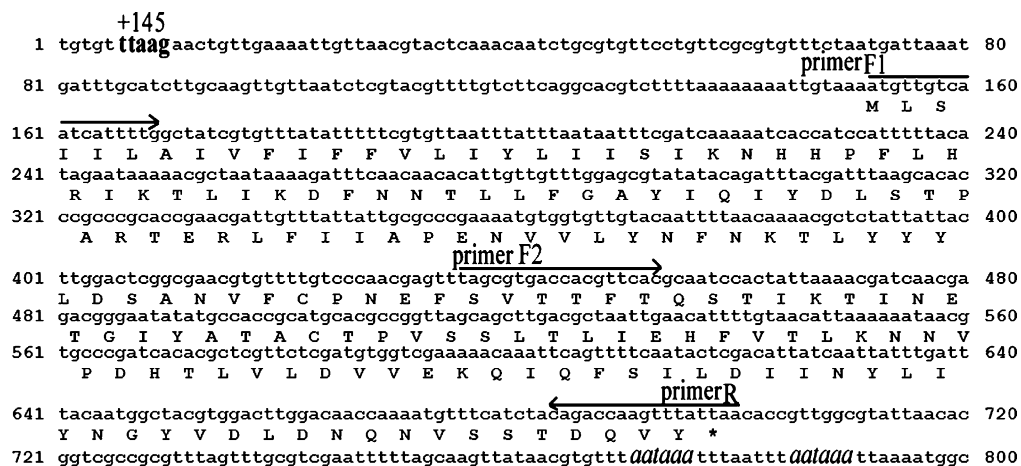

Fig. 1. Nucleotide sequence and deduced amino acid sequence of *Bm*79. A baculovirus consensus late transcriptional start motif TTAAG is shown in bold. The primers used in this study are indicated by arrows. Two typical polyadenylation signals (AATAAA) are shown in italics

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                                                                            Signal peptide
                           *        20         *        40         *        60         *        80
BmNPV       :---------------------------------------------------MLSIILAIVFIFFVLIYLIISIKNHH---FFL : 29
AcMNPV      :---------------------------------------------------MLSIMLAIVFVLFVLIYLIISIKNHH---FFL : 29
NeseNPV     :--------------------------------------------------MLFSIIALILILIITVFLCYYYTSQIN---FYV : 30
NeleNPV     :--------------------------------------------------MLIAMLTLGVLLLLLITTGIYFAIEFN---FYE : 30
RoMNPV      :---------------------------------------------------MLSIMLTILFVLFVLIYLIVAIKNHH---FFL : 29
CfDefNPV    :---------------------------------------------------MLSIMVVIAFLVALFMYVAVALRDHH---FFL : 29
CfNPV       :---------------------------------------------------MLSIMVVIVFVVALLMYVALSLRNHH---FFL : 29
EppoNPV     :---------------------------------------------------MLSIMVLIVFLVTLFMYVALSLKNHH---FFL : 29
OpMNPV      :---------------------------------------------------MLFMMAAIALAVLLFMYVAVALRDHH---FFL : 29
HearNPV     :------------------------------------------------MFINIASTLIGAVAIVCLLIFFIVIALIN---FYR : 32
HearNPV-G4  :------------------------------------------------MFINIASTLIGAVAIVCLLIFFIVIALIN---FYR : 32
HzSNPV      :------------------------------------------------MFSNIASTLIGAVAIVCLLIFFIVIALIN---FYR : 32
MacoNPV-A   :------------------------------------------------MLSKISSVLIAAIAILCLLIFIVALLYIN---FYR : 32
MacoNPV-B   :------------------------------------------------MLSKISSVLIAAIAILCLIVFIVALLYIN---FYR : 32
SeMNPV      :---------------------------------------------------MTSTTVIGAVAILCLLIFLIALLYIN---FYR : 29
LdMNPV      :------------------------------------------------MFSKIWLTLIAAAAIVCLMVFIATIAFIN---FYR : 32
AdhoNPV     :MTKISFSKFLLDSTTFSSPVNEFFNSKLSTVSRIVVTLIVEVSRFCLKIFSNMSLTLIAAIAIIIMSVLVLFANLIN---FLN : 80
SpliNPV     :----------------------------------------------------MVSTLIWLGVMVCLIAFVYALSLIN---FYR : 28
CrleGV      :-------------------------------------------------MSSTDMA-VLLIVIALIFLIVNT--LDN---FLI : 28
CpGV        :-------------------------------------------------MSSTDMITVFLLGIALLILIVTT--PAN---FLI : 29
PhopGV      :-------------------------------------------------MSFIDMLTAILLIIALVIALFSN--LFN---FLI : 29
AdorGV      :-------------------------------------------------MSSTDMITMTSLGVGLVVTILLLFWYMN---FLI : 31
XecnGV      :------------------------------------------------------ML-TVLIVVCLCILLLGVY-LYS---FLT : 24
AgseGV      :--------------------------------------------------MFMDIVVVPLLFLAIFALLVIT-LLFN---FVE : 29
PfxyGV      :--------------------------------------------------MDKDILIQIIVCVSIFILIIVIY-YFN---IFN : 29
CuniNPV     :------------------------------------------------MFEALPIVVLILCVLLIAFALGQTVQTVRGGGIVR : 35

                   *       100         *       120         *       140         *       160
BmNPV       :HRIKTLIKDFNNTLLFGAYIQIYDLSTP-ARTERLFIIAPENVVLYNFNKTLYYYLDS--ANVFCP-NEFSVTTFTQSTIKTI :108
AcMNPV      :HRIETLIQDFNNTLLFGAYVQIYDLSTP-ARTERLFIIAPENVVLYNFNKTLYYYLDS--ANVFCP-NEFSVTTFTQSTIKTI :108
NeseNPV     :YLKETVVKSHENSLFYGTQIEVYDDNNVSG-ISRLLLIKPTNIIIYNLHDTVYAYLQN-SVGSTCSQQESFVIAINRNNISDI :111
NeleNPV     :RLRNTVANAHKNSLFYGTEIDVYDDKNVTG-ISRLLLIKPTNIIVYNRDNTVYAYLQN-SVGSTCGKNEYQVIAINRDNISDV :111
RoMNPV      :HRIETLIQDFNNTLLFGAYVQIYDLSTP-ARTERLFIIAPENVVLYNFNKTLYYYLDS--ANVFCP-NEFSVTTFTQSTIKTI :108
CfDefNPV    :NRIQTFVRDFDNTLLYGTHVQIYDLSTP-ARTERLFIIAPRNVVLYNFDKTLYYYLDS--ANVFCP-NEFTVTRFTSATIQTV :108
CfNPV       :NRIHALLRDFDNTLLYGTHVRIYDLSTP-ARTERLFIIVPENVVLYNFDKTLYYYLDS--GNVFCP-NEYTVAKFTSATIRTV :108
EppoNPV     :QRIEIFLRDFDNTLLYGTHVQIYDLSTP-ARTERLFIIKPENVVLYNFDKTLYYYLDS--ANVFCP-NEYSVTRFTRATIQTV :108
OpMNPV      :NRVCALLRDFDNTLLYGTHVRIYDLSTP-ARTERLFIIAPENVVLYNFDKTLYYYLDS--ANVFCP-NEYTVAKFTGATIRTV :108
HearNPV     :NNVKKLIEDHKRTLQFGAYIDVEDLSTSSAHVERLFLIRPENVVLYNFDGALWYYLES--GSVLCP-REFAIVRFTFNDIKTV :112
HearNPV-G4  :NNVKKLIEDHKRTLQFGAYIDVEDLNTSSAHVERLFLIRPENVVLYNFDGALWYYLES--GSVLCP-REFAIVRFTFNDIKTV :112
HzSNPV      :NNVKKLIEDHKRTLQFGAYIDVEDLSTSSAHVERLFLIRPENVVLYNFDGALWYYLES--GSVLCP-REFAIVRFTFNDIKTV :112
MacoNPV-A   :NDAKKLVHDHAHTLQFGAYIEVYDLSTP-ARVERLFVIKPENVILYNLDGALFYYLES--SSVFCP-REFSLVRFSQDEIRSV :111
MacoNPV-B   :NDAKKLVHDHAHTLQFGAYIEIYDLSTP-ARVERLFVIKPENVILYNLDGALFYYLES--SSVFCP-REFSLIRFSRDEIRSV :111
SeMNPV      :TQVLKLIHDHQNTLQFGAYVDVYDLSVPPDRVERLFLIRPENVLLYNLDGTLFYYLES--SSVFCP-REFSLVRFNKNDIEAI :109
LdMNPV      :DNIDKIVRDHADTLQFGAYIDIYDLTVPPDRAERLFIVRPENVVLYNTNGALFYYLES--SSALCP-REFALVRFSKAEIEAV :112
AdhoNPV     :NEIDKIINDHTNTLQFGTYIEIYDASFK-NNVERLFVIKPENVMIYNTHGQLFYYLES--SSVICP-REFSLLRISAADIWSI :159
SpliNPV     :NQLQKLIDDHADTTHFGVFVDVEDLSINENNIERLLIVRPENVIVYNVGDVVYYYLES--GSVLCP-REFAVVRFTSDNIASI :108
CrleGV      :MIGTRLIESTR--VKVGPFIHVLERDGD----R-LFVIEPEQTLLYNTAGLIYYYFEGGASRRFCPVGECAIVRIGLTDIGLI :104
CpGV        :MIGARLVDSTR--VNVGAYIHVLERDGD----R-LFVVEPERALLFNTAGVLYYYFEGGASRRFCPAGECAIVRIGTADIRLI :105
PhopGV      :RIGRQLVDTAH--VNVGAYINVEQRDGD----R-LFIVEPDKVVMYNTAGVLYYYFEGGASRRMCPDGECAVIKFTTNDIQSF :105
AdorGV      :ILGNQLIRYGH--VYLGFVVNVLERDND----K-LIVIEPDDTVLINTAGILYYYFEGGASQRMCPSNEYAVVRMARRDIVLI :107
XecnGV      :NFANKLLVTTR--VCVYPSIRVMERDGD----R-LFVLEPEQIIIYNTAGVLYYYLEGGASGRLCPKNEFAIVRFDRNDINLI :100
AgseGV      :DFAAEMVEATV--VNVLPLINVEEKGND----R-LFVIEPEKSVIYNTHGALYYYFEGGVSGRLCPHNEYAVVRITAADIELI :105
PfxyGV      :LYRLKIKRSAY--LDIQPHIKLMSRDNE----R-LFLINPERVVIYNPGDVIYYYFEGGVSQRVCPDKEYAVVRISGADIKMI :105
CuniNPV     :HAADTLVCAFRKTFTYGIEVEVYDLDAESS-ANRMFIVRPENTLIYNNAGNVYVYLTVA-RGVRCAFNQDVVVQFNRDTIFNS :116

                 *       180         *       200         *       220         *       240
BmNPV       :NETGIYATACTPVSSLTLIEHFVTLKNNVPDHTIVLDVVEKQIQFSILDIINYLIYNGYVDIDNQNVSSTDQVY--------- :182
AcMNPV      :NETGIYATACTPVSSLTLIEHFATLKNNVPDHTIVLDVVDQQIQFSILDIINYLIYNGYVDLLAE------------------ :173
NeseNPV     :EN-GVENIVCSTVSEYNLLNYFVGASEPAFKINPSYDTEL----FTIIDVINYGLSTGVLNFNP------------------- :170
NeleNPV     :SD-GIEKIVCTTISDYNLLEYFI-AETPVLTLMPTYDTEL----FTIVDVINYCIN-GVLNFN-------------------- :167
RoMNPV      :NETGIYATACTPVSSLTLIEHFATLKNNVPDHTIVLDVVDQQIQFSILDIINYLIYNGYVDLLAE------------------ :173
CfDefNPV    :NDTGVYSTACTSVGSLTLIEHFAGLKNNTPDHMIVLDAAE-QIQFTIMDIINYLIYNGYVDIGVALK---------------- :174
CfNPV       :NDTGVYSTACTSVGSLTLIDHFVGLKNNAPDHIIALDADE-QIQFTIMDIINFLIFNGFVNLSNAQTSLLTTKNKHIF----- :185
EppoNPV     :NDTGVYSTACTSIGSLTLIEHFTGLKNNSPDHTIVLDAAE-QIQFTIMDIINYMIYNGFVDL--------------------- :169
OpMNPV      :NDTGVYSTACTVVGSLTLIEHFVGLKNNSPDHTIVLDVAE-QIQFTIMDVINYLIYNGYVDIAAG------------------ :172
HearNPV     :NESGLENIVCTNVNALTLIEHFMTLKNGLADERIIINLQ--NINFSIIDVINLLIHKGYVYLE-------------------- :173
HearNPV-G4  :NESGLENIVCTNVNALTLIEHFMTLKNGLADERIIINLQ--NINFSIIDVINLLIHKGYVYLE-------------------- :173
HzSNPV      :NESGLENIVCTNVNALTLIEHFMTLKNGLADERIIINLQ--NINFSIIDVINLLIHKGYVYLE-------------------- :173
MacoNPV-A   :NESGLYNTVCTNVNSLVVLEHFLTLKNNVSDERILLTVD--EIHYSILDIINLLIYTGYVLVT-------------------- :172
MacoNPV-B   :NESGLYNTVCTNVNSLVVLEHFLTLKNNVSDERILLTVD--EIHYSILDIINLLIYTGYVLVT-------------------- :172
SeMNPV      :NDSGIYNTVCTNVNSLVILEHFLTLKNNVSDMRVLLTVD--EIQYTIIDIINYLIHLGLVQIK-------------------- :170
LdMNPV      :NESGAESTACTNVNSLTVLEHFVALKNNEPDERVALSLD--EIDYSILDIINALIAGGLAQVY-------------------- :173
AdhoNPV     :NESGTETTLCTNVNSLALLEHFIVLKNNIADERILLFVD--DIHYSILDLINLIISLGYVYIE-------------------- :220
SpliNPV     :NENGVEDTICTGVNSLSLIEHFMTLKQGVPDVRAIMSVETGEINYSIMDIINYMIHNGLVKM--------------------- :170
CrleGV      :NENGIYNVSCTNTSSWDLYEHFKNDSF-EWKLPTFYEK------TSIIDIINELIRYGYVRIGYQ------------------ :162
CpGV        :NETGTYNITCTGTGSLGLYEHFKSDSV-EWQMSVYNDS------NSIIDIINYLIRSGSAQIT-------------------- :161
PhopGV      :NETGEYNISCTRINALTLIEHFS-ESP-SIRLPMPEDQ------FSALDVINKIIQFGYAKINFN------------------ :162
AdorGV      :NENNSYNIACTPTSALALKRHFESAILSKLSFPIPWDAS-----YTVIDVINMLIDNGVIGIYS------------------- :166
XecnGV      :NEDGSENVSCTTASSLAMYQHFDSIKIRETTIPIR-DLE-----YNVLQVINFIIRRGFAKIR-------------------- :157
AgseGV      :NETGVYDIYCSPVGSRTIYSHFTSDSF-RYQAIIP-KMT-----YTLLDVINYLIETGFALVY-------------------- :161
PfxyGV      :NQSGVYNVACTGISSLSLLDNFS-ETVSQQNIPIA-DAR-----FTLLDILNFLIANGYASVA-------------------- :161
CuniNPV     :-IFGEFRQMCTSLRALDLIEFFRTQQP-EPRLRFAVELDG-TREYTVIDMVNFIHERGLIEYGEGTDVRLRGRRKDSDQVHRW :196
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The alignment of aa sequences showed that nine amino acids were identical in the 26 baculoviruses (Fig. 2, indicated with stars). Comparison analysis showed that Bm79 had the highest identity (93%) with ORF96 of AcMNPV in NPV group I, 61% with SpliNPV ORF87 in Group II, and 46% with PlxyGV ORF71. In addition, Bm79 even had 42% identity with the dipteran baculovirus, CuniNPV ORF90, 46% with NeseNPV ORF60, and 47% with NeleNPV ORF57 of hymenopteran NPVs.

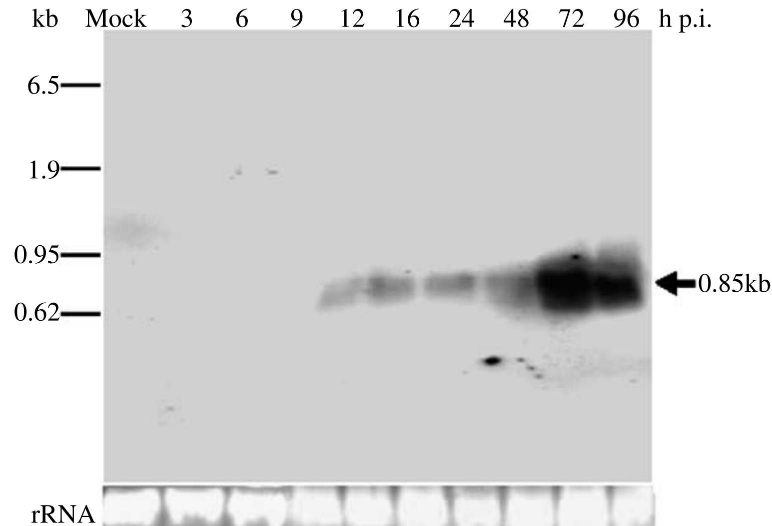

Fig. 3. Northern blot analysis of *Bm*79 transcription in mock- and BmNPV-infected BmN cells. Ten micrograms of total RNA was separated on a 0.8% agarose-formaldehyde gel, transferred onto a nylon membrane, and hybridized to a DIG-labeled DNA probe. The sizes of RNA markers are shown on the left and the size of detected RNA is indicated on the right. The portion of the gel containing rRNA stained with ethidium bromide are shown (lower panel)

Fig. 2. Amino acid sequence alignment of baculovirus Bm79 homologues. The predicted signal peptide is marked and four possible sites of *N*-glycosylation are outlined in the BmNPV amino acid sequence. The sources of sequences are: BmNPV (GenBank, NC_001962), AcMNPV (GenBank, NC_001623), CfDefNPV (GenBank, NC_005137), CfMNPV (GenBank, NC_004778), HearNPV (GenBank, NC_003094), AdhoNPV (GenBank, NC_004690), NeseNPV (GenBank, NC_005905), RoMNPV (GenBank, NC_004323), MacoNPV-B (GenBank, NC_004117), MacoNPV-A (GenBank, NC_003529), HzSNPV (GenBank, NC_003349), SpliNPV (GenBank, NC_003102), EppoNPV (GenBank, NC_003083), HearNPV-G4 (GenBank, NC_002654), LdMNPV (GenBank, NC_001973), OpMNPV (GenBank, NC_001875), SeMNPV (GenBank, NC_002169), AgseGV (GenBank, NC_005839), CrleGV (GenBank, NC_005068), AdorGV (GenBank, NC_005038), PhopGV (GenBank, NC_004062), CpGV (GenBank, NC_002816), PlxyGV (GenBank, NC_002593), XecnGV (GenBank, NC_002593), CuniNPV (GenBank, NC_003084) and NeleNPV (GenBank, NC_005906). GeneDoc software was used for homology shading. Identical amino acids are shown with stars and two shading levels are set: black for 100% similarity groups and grey for 80% similarity groups

Transcriptional analysis of Bm79

The temporal regulation of the *Bm*79 transcript was examined by Northern blot analysis using total RNA isolated from BmNPV-infected BmN cells. The result revealed one transcript of about 0.85 kb (Fig. 3). This transcript was detectable as early as 12 h p.i., reached a peak at 72 h p.i., and remained very steady until 96 h p.i.

Temporal expression of Bm79 in infected cells

For the purpose of detecting Bm79 protein expression, a GST-Bm79 fusion construct was generated and the fusion protein was used to raise polyclonal antiserum in rabbits. To verify that the anti-GST-Bm79 serum detected Bm79, we expressed the *Bm*79 gene as a fusion protein with a 6Xhis epitope and GFP in insect cells using the Bac-to-Bac system. Western blot analysis with anti-GST-Bm79 serum and anti-6His mouse monoclonal antibody was performed (Fig. 4). The two sera detected bands of the same size. Thus, the antibody prepared against GST-Bm79 recognized the Bm79 protein.

To determine the time course of Bm79 protein expression, infected cell extracts were sampled at designated time points and analyzed by western blot using anti-GST-Bm79 serum. The results revealed that a protein with an apparent molecular weight of 28,000 showed a strong reaction (Fig. 5). This band was detectable as early as 24 h p.i., increased to high levels at 48 h p.i. and lasted until

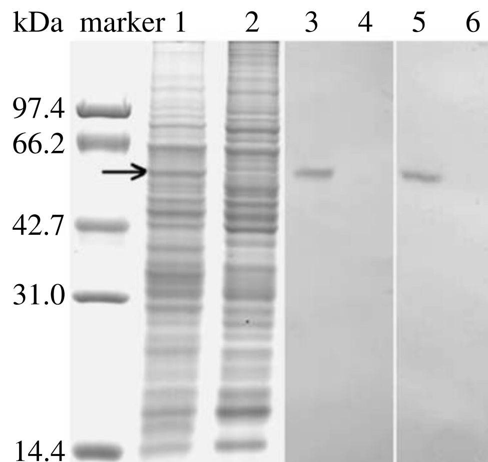

Fig. 4. Western blot analysis of the Bm79 protein expressed in *Trichopolusia ni* cells. The Bm79 fused with eGFP was expressed in the cell line Tn-5B1-4 using the Bac-to-Bac system. The cell extracts were separated by SDS-PAGE and transferred onto PVDF membrane for western blot assay. *1*, lysates of cells infected with recombinant Bacmid rBac-EGFPBm79. Fusion protein is indicated by an arrow. *2*, uninfected cell lysates. *3*, western blot analysis of *Bm*79 expressed in insect cells, mouse His-tag monoclonal antibody was used as primary antibody and goat-anti-mouse serum conjugated with HRP was used as secondary antibody. *5*, western blot analysis of *Bm*79 expressed in the insect cells, anti-GST-Bm79 serum was used as primary antibody. *4* and *6*, uninfected cells as control

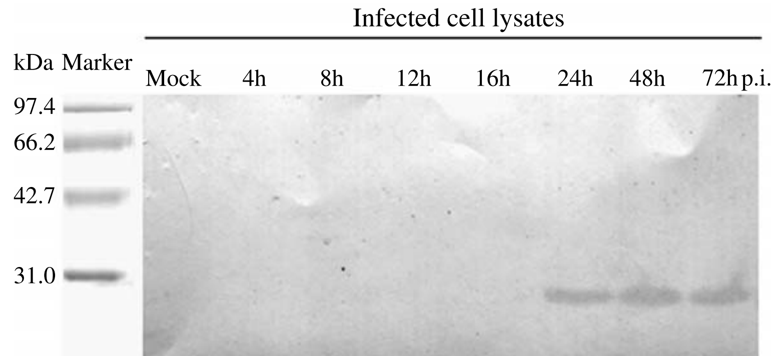

Fig. 5. Western blot analysis of the *Bm*79 gene product in BmN cells. The cells were collected at mock, 4, 8, 12, 16, 24, 48, 72 p.i., and 20 μg cell lysates at each interval was subjected to western blot analysis using anti-GST-Bm79 serum. The binding was developed with diaminobenzidine (DAB) as a chromogenic substrate. Protein markers are indicated on the left

72 h p.i. However, the molecular weight of the detected protein was larger than the predicted putative *Bm*79 gene product (20.9 kDa). This might be due to post-translational modifications or other processing functional form.

Ara-C inhibitor treatment analysis

To examine whether the *Bm*79 gene was a late gene, BmNPV-infected cells were treated with Ara-C at appropriate time points, and harvested for western blot assay at 48 h p.i. The result showed that the immuno-reactive band of 28 kDa could be detected only when Ara-C was added to the BmNPV-infected cells at 24 h p.i. and it disappeared when Ara-C was treated at 3, 6, or 12 h p.i. (Fig. 6). In contrast, Bm79 was present in the sample of BmNPV-infected cells, and no

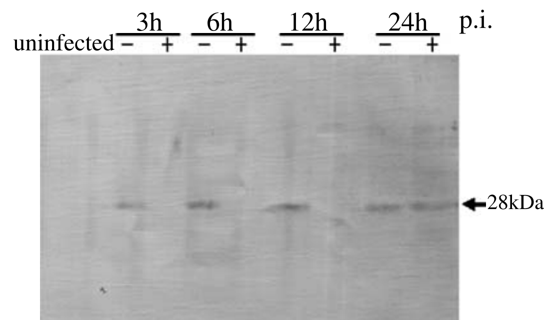

Fig. 6. Western blot analysis of the expression of Bm79 in BmNPV-infected BmN cells treated with Ara-C. The Ara-C was treated when the BmN cells were infected at appropriate time points (3, 6, 12, 24 h p.i.), and cells were continuously incubated until 48 h p.i. Harvested cells were subjected to western blot assay using anti-GST-Bm79 serum. Uninfected cells and BmNPV-infected cells were used as controls. The sample treated with Ara-C is indicated by plus (+) and the untreated sample by minus (−)

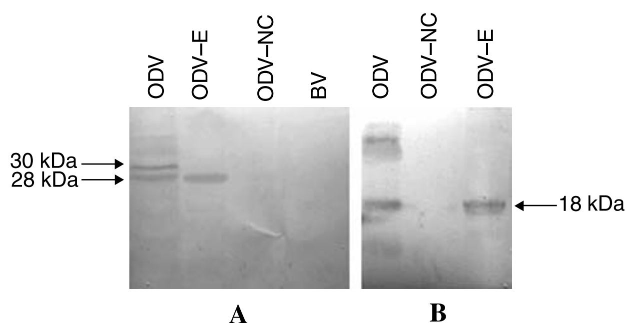

Fig. 7. Western blot analysis of ODVs, ODV-E, ODV-NC and BVs of BmNPV. **A** Preparation of ODVs, ODV envelope (ODV-E), ODV nucleocapsid (ODV-NC) and purified BVs were subjected to western blot analysis using anti-GST-Bm79 serum. **B** Preparation of ODVs, ODV-NC, and ODV-E were subjected to western blot analysis using anti-ODV-E18 serum. The sizes of reactive bands are indicated by arrows

band was detected in uninfected cells (Fig. 6). Along with the results of sequence analysis, Northern blot and western blot, this observation confirmed that *Bm*79 is a late gene.

Immunodetection of the Bm79 protein in ODV

To determine if the Bm79 protein was a structural component of BmNPV, western blot analysis of purified BVs and ODVs was performed. The ODV fraction showed two reactive bands of 28- and 30-kDa (Fig. 7A, lane ODV), and the size of the 28-kDa protein was in agreement with that detected in lysates from infected cells. In contrast, no band was detected in the BV fraction (Fig. 7A, lane BV). Thus, the Bm79 protein appeared to be a structural protein specific for ODV. As a control, the ODV-NC and ODV-E fractions were used in a western blot with an antibody raised against the ODV-E18 protein [6] (Fig. 7B). The result showed a positive band of 18 kDa that was detected only in the ODV-E fraction (Fig. 7B, lane ODV-E), and no band was detected in the ODV-NC fraction (Fig. 7B, lane ODV-NC). Thus, the separation of ODV-NC and ODV-E was considered to be pure. When an antibody against Bm79 was used, the 28-kDa protein was detected in the ODV-E fraction (Fig. 7A, lane ODV-E) instead of in the ODV-NC fraction (Fig. 7A, lane ODV-NC).

N-glycosylation analysis of Bm79

Because the Bm79 was larger than the theoretical size, and four possible *N*-glycosylation sites were predicted by computer analysis, the BmNPV-infected cells were treated with an inhibitor of *N*-glycosylation, tunicamycin. The western blot result showed that the size of Bm79 was not altered by the presence of tunicamycin (Fig. 8). The positive control, the BmGP64 protein, a *N*-glycosylated protein of BV envelope [21], was altered in size due to tunicamycin treatment (Fig. 8). Thus, Bm79 was thought to be a non *N*-glycosylated protein.

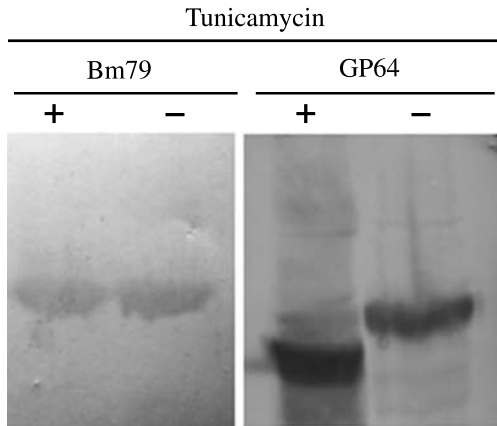

Fig. 8. *N*-glycosylation analysis of Bm79. BmN cells were infected with BmNPV in the presence of tunicamycin. Cells were harvested at 72 h p.i. for western blot analysis. Anti-GST-Bm79 and anti-BmGP64 sera were used as primary antibodies. The samples treated with tunicamycin are indicated by plus (+) symbols and untreated samples are indicated by minus (−) symbols

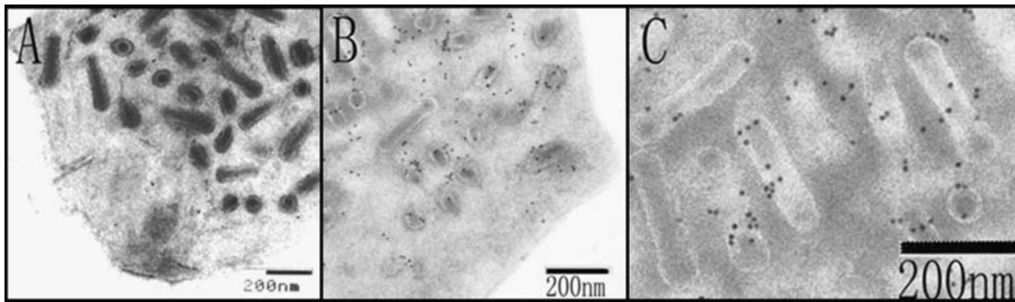

Fig. 9. Immunoelectron microscopy of BmNPV polyhedra. Polyhedra were fixed and ultrathin sections were reacted with anti-GST-Bm79 serum and 10-nm gold conjugated protein-A was used to detect antibody binding. **A** Ultrathin sections of polyhedra were treated with pre-immune serum. **B** and **C** Ultrathin sections of polyhedra were treated with anti-GST-Bm79 serum

Immunogold electron microscopy

To confirm the localization of Bm79, immunogold electron microscopy (IEM) was performed using the polyclonal antibody against GST-Bm79. The result revealed that most of the gold particles concentrated around nucleocapsids within polyhedra (Fig. 9B and C), whereas labeling rarely occurred in other parts (Fig. 9B). In contrast, the background cross-reactivity was very limited when pre-immune serum was used (Fig. 9A). Thus, the result of IEM confirmed that the Bm79 was a constituent of the BmNPV ODV.

Immunofluorescence microscopy

The sub-cellular localization of the Bm79 proteins was investigated by immunofluorescence using a confocal laser scanning microscope. At 72 h p.i., the nuclei of infected BmN cells were enlarged, and cells were collected for fluorescence examination. A significant amount of fluorescence was closely concentrated within the nucleoplasm and no fluorescence was detected in the cytoplasm (Fig. 10). In

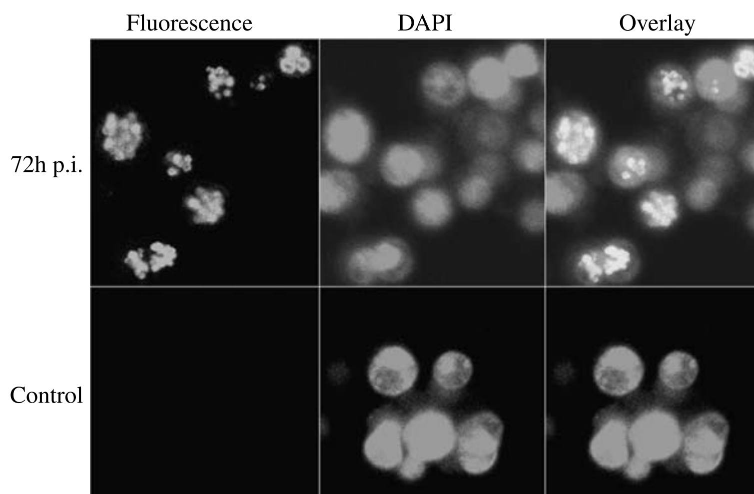

Fig. 10. Intracellular localization of Bm79 in BmNPV-infected BmN cells. The cells were collected at 72 h p.i., washed with 1 × PBS and reacted with anti-GST-Bm79 serum, fluorescence was developed by incubating with protein G fused with enhanced green fluorescent protein. As a control, preimmune serum was used as the primary antibody. The nuclei were stained with DAPI. The samples were viewed using a confocal laser fluorescence microscope

the control batch, no fluorescence was detected when preimmune serum was used (Fig. 10). Thus, the Bm79 protein displayed an intranuclear localization where ODV acquires its envelope protein. Therefore, this observation indirectly supports the evidence that Bm79 is specific for the ODV envelope (ODV-E) protein.

Discussion

In this study, a transcriptional analysis of the *Bm*79 gene of BmNPV as well as the localization of the gene product in sub-cellular compartments and within virions was performed. Northern blot analysis detected the initiation of *Bm*79 transcripts at 12 h p.i. (Fig. 3, lane 12 h p.i.) and expression of Bm79 could be detected as early as 24 h p.i. (Fig. 5, lane 24 h p.i.). However, we observed that the size of the immuno-reactive protein (28 kDa) was larger than the predicted molecular weight of 20.9 kDa. Tunicamycin treatment (Fig. 8) showed that the increased molecular weight was not due to the *N*-glycosylation, suggesting that other type of post-translational modification might have occurred. A similar phenomenon was also observed in several other structural proteins, such as ODV-EC56 [5], ODV-EC43 [10], ODV-E18 [6] etc. Furthermore, western blot was performed to determine if Bm79 was a structural protein. The immuno-reactive band was present only in ODVs (Fig. 7A, lane ODV) and not in BVs (Fig. 7A, lane BV), and Bm79 was further located in ODV-E (Fig. 7A, lane ODV-E). These results indicate that Bm79 is a specific ODV protein, and this was further confirmed by IEM using ultrathin sections of polyhedra reacted with anti-GST-Bm79 serum (Fig. 9B and C). Surprisingly, a 30-kDa protein immunoreacted strongly in ODVs,

but was absent in ODV-NC and ODV-E (Fig. 7A). This 30-kDa protein might be a cross-reactive band and degraded during the process of ODV fractionation.

Computer analysis predicted that Bm79, consistent with PIF [17], ODV-E25, and ODV-E66 [15], had a potential hydrophobic membrane-spanning domain at the N-terminus (Fig. 2). The *pif* mutant virus revealed an absence of per os infectivity [17]. Additionally, it has been reported that the ODV-E25 and ODV-E66 are associated with nuclear membranes and only the N-terminal sequences are sufficient to direct reporter proteins to the nuclear envelope, intranuclear microvesicles, and the envelope of occlusion derived virus [8, 15]. The Bm79 possibly had a similar function to ODV-E25 and ODV-E66. Previous report supported that proliferation of the nuclear envelope or inner nuclear membrane produced the intranuclear membranes essential for ODV nucleocapsid envelopment [3]. Four ODV envelope proteins, P25 [24], ODV-E66 [14], ODV-E56 [5], and ODV-E18 [6], have been shown to associate with microvesicles induced in infected cell nuclei. Moreover, the ODV-EC27 complex has been shown to function as a multifunctional cyclin to benefit virus infection [3]. It has not been determined if other structural proteins are also involved in functions of cell cycle regulation. Studies on proteins that either interact with Bm79 or are associated with viral-induced intranuclear microvesicles will be investigated in our further studies.

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References

1. Appel RD, Bairoch A, Hochstrasser DF (1994) A new generation of information retrieval tools for biologists: the example of EXPASY WWW server. Trends Biochem Sci 19: 258–260
2. Ayres MD, Howard SC, Kuzio J, Ferber ML, Possee RD (1994) The complete DNA sequence of *Autographa californica* nuclear polyhedrosis virus. Virology 202: 586–605
3. Belyavskyi M, Braunagel SC, Summer MD (1998) The structural protein ODV-EC27 of *Autographa californica* nucleopolyhedrovirus is a multifunctional viral cyclin. Proc Natl Acad Sci USA 95: 11205–11210
4. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254
5. Braunagel SC, Elton DM, Ma H, Summers MD (1996) Identification and analysis of an *Autographa californica* nuclear polyhedrosis virus structural protein of the occlusion-derived virus envelope: ODV-E56. Virology 217(1): 97–110
6. Braunagel SC, He H, Ramamurthy P, Summers MD (1996) Transcription, translation, and cellular localization of three *Autographa californica* nuclear polyhedrosis virus structure proteins: ODV-E18, ODV-E35, and ODV-EC27. Virology 222(1): 100–114

7. Braunagel SC, Summers MD (1994) *Autographa californica* nuclear polyhedrosis virus, PDV, and ECV viral envelopes and nucleocapsids: structural proteins, antigens, lipid and fatty acid profiles. Virology 202: 315–328
8. Braunagel SC, Williamson ST, Saksena S, Zhong Z, Russell WK, Russell DH, Summers MD (2004) Trafficking of ODV-E66 is mediated via s sorting motif and other viral proteins: Facilitated trafficking to the inner nuclear membrane. Proc Natl Acad Sci USA 101: 8372–8377
9. Caballero P, Zuidema D, Santiago Alvarez C, Vlak JM (1992) Biochemical and biological characterization of four isolates of *Spodoptera exigua* nuclear polyhedrosis virus. Biocontrol Sci Technol 2: 145–157
10. Fang M, Wang H, Wang H, Li Y, Chen X, Vlak JM, Hu Z (2003) Open reading frame 94 of *Helicoverpa armigera* single nucleocapsid nucleopolyhedrovirus encodes a novel conserved occlusion-derived virion protein, ODV-EC43. J Gen Virol 84: 3021–3027
11. Flipsen JTM, Van Lent JWM, Goldbach RW, Vlak JM (1993) Expression of polyhedron and p10 in the midgut of AcNPV infected *Spodoptera exigua* larvae: An immunoelectron microscopic investigation. J Invert Pathol 61: 17–23
12. Harlow E, Lane D (1988) Antibodies: A Laboratory Manual, 1st edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
13. Herniou EA, Olszewski JA, Cory JS, O'Reilly DR (2003) The genome sequence and evolution of baculoviruses. Annu Rev Entomol 48: 211–234
14. Hong T, Braunagel SC, Summers MD (1994) Transcription, translation and cellular localization of PDV-E66: A structural protein of the PDV envelope of *Autographa californica* nuclear polyhedrosis virus. Virology 204: 210–222
15. Hong T, Summers MD, Braunagel SC (1997) N-terminal sequences from *Autographa californica* nuclear polyhedrosis virus envelope proteins ODV-E66 and ODV-E25 are sufficient to direct reporter proteins to the nuclear envelope, intranuclear microvesicles and the envelope of occlusion derived virus. Proc Natl Acad Sci USA 94: 4050–4055
16. Horton HM, Burand JP (1993) Saturable attachment sites for polyhedron-derived baculovirus on insect cells and evidence for entry via direct membrane fusion. J Virol 67: 1860–1868
17. Iryna K, Seraffn G, Liliane C, Guy C, Miguel LF (2002) Characterization of *pif*, a gene required for the *per os* infectivity of *Spodoptera littoralis* nucleopolyhedrovirus. J Gen Virol 83: 3013–3022
18. Iwanaga M, Kang WK, Kobayashi M, Maeda S (2000) Baculovirus infection blocks the progression of fat body degradation during metamorphosis in *Bombyx mori*. Arch Virol 145: 1763–1773
19. Keddie BA, Aponte GW, Volkman LE (1989) The pathway of infection of *Autographa californica* nuclear polyhedrosis virus in an insect host. Science 243: 1728–1730
20. Kuzio J, Jaques R, Faulkner P (1989) Identification of *p74*, a gene essential for virulence of baculovirus occlusion bodies. Virology 173: 759–763
21. Masmudur Rahman Md, Gopinathan Karumathil P (2003) Characterization of the gene encoding the envelope fusion glycoprotein GP64 from *Bombyx mori* nucleopolyhedrovirus. Virus Res 94: 45–57
22. Nicholas KB, Nicholas HB Jr, Deerfield DW (1997) GeneDoc: analysis and visualization of genetic variation. EMBnet News 4: 14
23. O'Reilly DR, Miller LK, Luckow VE (1992) Baculovirus Expression Vectors: A Laboratory Manual. Freeman, New York
24. Russell RL, Rohrmann GF (1993) A 25-kDa protein is associated with the envelopes of occluded baculovirus virions. Virology 195: 532–540

25. Russell RL, Rohrmann GF (1997) Characterization of P91, a protein associated with virions of an *Orgyia pseudotsugata* baculovirus. Virology 233: 210–223
26. Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: A Laboratory Manual, second edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
27. Slot JW, Geuze HJ (1985) A new method of preparing gold probes of multiple-labelling cytochemistry. Eur J Cell Biol 38(1): 87–93
28. Smith GE, Summers MD (1981) Application of a novel radio-immunoassay to identify baculovirus structural proteins that share interspecies antigenic determinants. J Virol 39: 125–137
29. Summers MD, Smith GE (1975) *Trichoplusia ni* granulosis virus granulin: a phenol-soluble, phosphorylated protein. J Virol 16: 1108–1116
30. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 24: 4876–4882
31. Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamidae gels to nitrocellulose sheets: Procedure and some applications. Proc Natl Acad Sci USA 76: 4350–4354
32. Whitford M, Faulkner P (1992) Nucleotide sequence and transcriptional analysis of a gene encoding gp41, a structural glycoprotein of the baculovirus *Autographa californica* nuclear polyhedrosis virus. J Virol 66: 4763–4768
33. Zhang CX, Ma XC, Guo ZJ (2005) Comparison of the complete genome sequences between C1 and G4 isolates of the *Helicoverpa armigera* single nucleocapsid nucleopolyhedrovirus. Virology 333: 190–199

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