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Characterization of the Open Reading Frame 35 of *Bombyx mori* Nucleopolyhedrovirus

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Open reading frame 35 (*bm35*) of the *Bombyx mori* nucleopolyhedrovirus (BmNPV) is a special gene whose homologues are only found in some group-I nucleopolyhedroviruses, suggesting that *bm35* plays a specific role in the viral life cycle. This paper described the characterization of BmNPV *bm35*. Computer-assisted sequence analysis shows that a putative RING finger motif is observed in the protein, Bm35 encoded by *bm35*. The coding sequence of *bm35* was amplified and subcloned into the vector pET30a(+) and the $(His)_6$-tagged fusion protein His-Bm35 was expressed in the *Escherichia coli* BL21 (DE3) LysS cells. The *bm35* transcript and Bm35 protein were detected in BmNPV-infected BmN cells at 12~48 h post infection (p.i.) by RT-PCR and Western blot analysis using the polyclonal antibody generated by immunizing a rabbit with purified $(His)_6$-tagged Bm35, suggesting that *bm35* is synthesized in the late stage of BmNPV infection cycle. Bm35 was not a structural component associated with budded virus (BV) and occlusion derived virus (ODV). These data indicated that *bm35* is a functional gene in the BmNPV life cycle.

Key words: BmNPV, *bm35*, Expression, Structural component

Introduction

Baculoviridae is a family of DNA viruses that have a large, circular, supercoiled and double-stranded DNA genome within a rod-shaped nucleocapsid. Previously, this family has been assigned to contain two genera, *Nucleopolyhedrovirus* (NPV) and *Granulovirus* (GV) (Theilmann *et al*., 2005), but a recent proposed reclassification has expanded the family to four genera: *Alphabaculovirus, Betabaculovirus, Deltabaculovirus* and *Gammabaculovirus* (Jehle *et al*., 2006).

The important silkworm pathogen, *Bombyx mori* nucleopolyhedrovirus (BmNPV), is a member of genus *Alphabaculovirus*. Since the complete genome of BmNPV (T3 strain) has been sequenced (Gomi *et al*., 1999), many genes have been well-characterized, such as *orf60* (Du *et al*., 2006), *orf42* (Acharya and Gopinathan, 2002), *orf79* (Xu *et al*., 2006), *lef-7* (Wang *et al*., 2006), chitinase (Daimon *et al*., 2007), *vfgf* (Katsuma *et al*., 2006), *orf67* (Chen *et al*., 2007). BmNPV *bm35* is located at 31903~32298 bp in the genome of BmNPV T3 strain. It is 396-bp long and predicted to encode a 131 amino acid peptide with a deduced molecular weight of 15.0 kDa. Homologues of *bm35* are found only in the genomes of some group-I NPVs, but are not present in genomes of hymenopteran or dipteran NPVs or GVs, implying that *bm35* and its homologues may play a specific role in individual virus life cycle. In this study, we analyzed the *bm35* gene by examining transcription of the gene and expression of its protein product in BmNPV infected BmN cells.

Materials and Methods

Cells, larvae, virus, bacterial strain

The *B. mori* cell line, BmN, derived from ovary of silkworm (Grace, 1967), was cultured at 27ºC in TC-100 insect medium (Sigma-Aldrich, USA) supplemented with 10% (v/v) fetal bovine serum (Gibco-BRL, USA) using standard techniques (King and Possee, 1992). BmNPV (T3 strain) virus was propagated in BmN cells. The *B. mori* larvae (F1 hybrid JingSong × HaoYue) were reared on fresh mulberry leaves at 25ºC with a 12:12 h light/dark

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photoperiod. The wild-type virus BmNPV T3 strain, whose titer of BV was determined by end-point dilution (King and Possee, 1992), was used to infect 5th instar larvae. *Escherichia coli* BL21 (DE3) LysS cells were maintained in our lab.

Computer-assisted sequence analysis

The protein sequence was analyzed using ExPASy server (http://www.expasy.org) for the prediction of motifs, domains, transmembrane regions and signal peptides. Homologues were explored by using BLASTP searching tool in the updated GenBank/EMBL and SWISS-PROT databases. Sequence alignment was performed with the software Clustal X (http://www.ebi.ac.uk/Tools/clustalw2/index.html) and edited with GeneDoc software.

Cloning, expression of *bm*35 in *E. coli* and preparation of an antiserum

The complete coding sequence of *bm35* was amplified by PCR from the BmNPV genomic DNA using primers 35-F: 5'-AGGATCC ATG CAC GAC GGT CGC GTT-3' (*Bam*HI site underlined) and 35-R: 5'-GCTCGAG TTA CAA AGT TTT GTA TTT-3' (*Xho*I site underlined), and cloned into *Bam*HI and *Xho*I sites of vector pET30a(+) (Novagen, USA), to produce plasmid pET30-bm35. After being verified by sequencing with T7 promoter primer, the pET30-bm35 was transformed into *E. coli* BL21 (DE3) LysS cells. The $(His)_6$-tagged protein, His-Bm35 was induced by addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG; Sigma-Aldrich, USA), and analyzed by 12% SDS-PAGE and Western blot with anti-$(His)_6$ monoclonal antibody (Qiagen, Germany). His-Bm35 was purified using Ni^{2+}-NTA agarose beads (Qiagen, Germany) under native conditions following the manufacturer's instructions, and used to generate polyclonal antiserum according to the procedure described previously (Wang *et al*., 2005).

Transcriptional analysis

A monolayer (about 1.0×10^6, 80~90% confluence) of BmN cells were infected with BVs of BmNPV at 5.0 $TCID_{50}$/cell. Total RNA was extracted at 0, 6, 12, 18, 24 and 48 h p.i. by adding Trizol reagent (Invitrogen Life Technologies, USA). The RNA was dissolved in 50 μl of double-distilled water, quantified by A_{260} measurement, and treated with RNase-free DNase to eliminate any potential BmNPV genomic DNA contamination. First-strand cDNA was synthesized with AMV reverse transcriptase XL and oligo(dT) primer (TaKaRa Bio, Japan). The obtained cDNA mixtures were amplified by PCR using primers 35-F and 35-R. RNA from mock-infected cells was used as a negative control.

Purification of BV and ODV particles

At 72 h p.i., infected BmN cells were centrifuged at 3000 rpm for 5 min at 4°C. The supernatant was filtered with a 0.45 μm filter and used to purify BVs according to the method described by Wang *et al*. (2010). Polyhedra particles were purified from silkworm cadavers using the method described by Braunagel and Summers (1994). ODV were purified from polyhedra (IJkel *et al*., 2000). Purified BV and ODV were lysed in SDS-PAGE sample buffer and submitted to Western blot analysis as described previously (Du *et al*., 2006).

Western blot analysis

For the time course analysis of Bm35, infected cells were harvested at the designated time points p.i. (see above). After being washed three times in PBS, the cells were lysed in SDS-PAGE sample buffer by boiling for 5 min, centrifuged for 10 min at 12,000 rpm to remove cell debris. Western blot analysis was performed as described by Du *et al*. (2006).

Results

Sequence analysis of Bm35

Using the MotifScan tool on the website http:// myhits.isb-sib.ch/cgi-bin/motif_scan, one N-glycosylation site (aa 51–54), two casein kinase II phosphorylation sites (aa 24–27, 103–106) and two protein kinase C phosphorylation sites (aa 35–37, 59–61) were predicted in the Bm35 protein. A putative typical RING finger motif, Cys-X_2-Cys-X_n-Cys-$X_{1\text{-}3}$-His-$X_{2\text{-}3}$-Cys-X_2-Cys-X_n-Cys-X_2-Cys (C_3HC_4) was also found at aa 78–119 (Fig. 1). No signal peptide, transmembrane regions, mitochondrial targeting sequences, nuclear localization signals or membrane retention signals were found by any of motif search engines employed.

Search in the databases revealed that the Bm35 is encoded by a rare gene whose homologues were found only in the genomes of some group-I nucleopolyhedroviruses. Sequence alignment of Bm35 and its homologues was shown in Fig. 1. The Bm35 shares a high level of sequence identity (86–99%) with homologues from *Bombyx mandarina* nucleopolyhedrovirus (BomaNPV), *Autographa californica* multiple nucleopolyhderovirus (AcMNPV), *Plutella xylostella* multiple nucleopolyhderovirus (PlxyMNPV), *Rachiplusia ou* multiple nucleopolyhedrovirus (RoMNPV) and *Maruca vitrata* multiple nucleopolyhedrovirus (MaviNPV). However, Bm35 is lower identical (39–42%) to the homologues from the other group-I nucleopolyhedroviruses.

Temporal transcription analysis of *bm35* in BmNPV-infected BmN cells

Temporal transcription of *bm35* was investigated by RT-

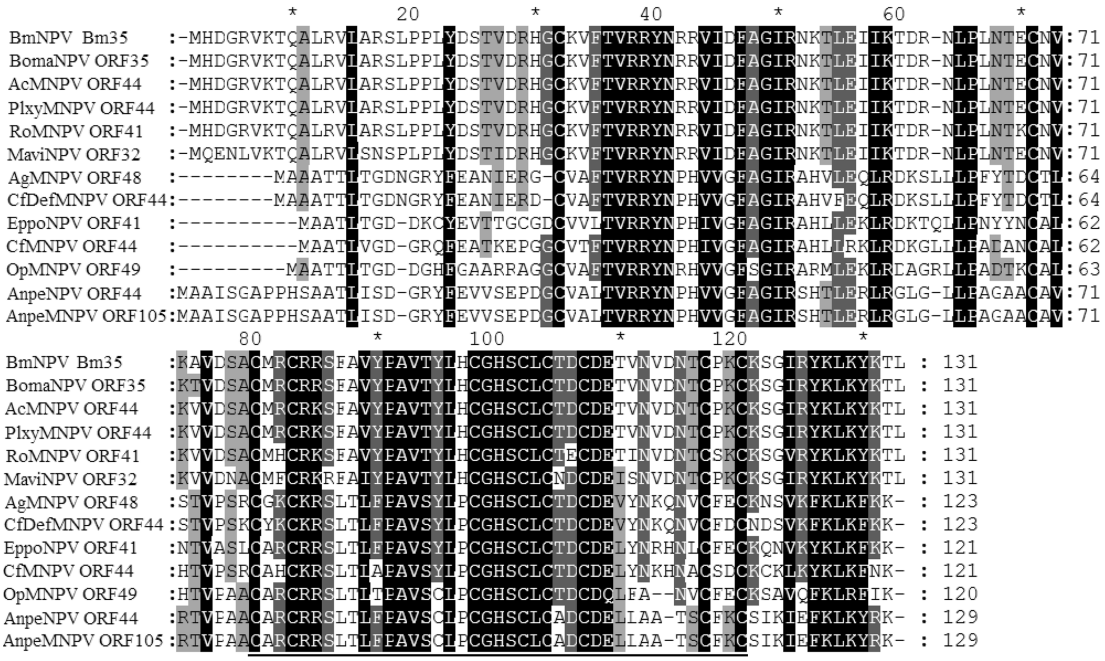

Fig. 1. Multiple alignment of amino acid sequence of Bm35 and its homologues from other NPV. Two shading levels were set as black for 100% identity and grey for 80% identity. The putative RING finger motif was underlined. Viruses in abbreviation were BmNPV: *Bombyx mori* NPV (Gomi *et al*., 1999), BomaNPV: *Bombyx mandarina* NPV (Xu *et al*., 2010), AcMNPV: *Autographa californica* multiple NPV (Ayres *et al*., 1994), PlxyMNPV: *Plutella xylostella* multiple NPV (Harrison and Lynn, 2007), RoMNPV: *Rachiplusia ou* multiple NPV (Harrison and Bonning, 2003), MaviNPV: *Maruca vitrata* NPV (Chen *et al*., 2008), AgMNPV: *Anticarsia gemmatalis* multiple NPV (Oliveira *et al*., 2006), CfDefNPV: *Choristoneura fumiferana* DEF multiple NPV (Lauzon *et al*., 2005), EppoNPV: *Epiphyas postvittana* NPV (Hyink *et al*., 2002), OpMNPV: *Orgyia pseudotsugata* multiple NPV (Ahrens *et al*., 1997), AnpeMNPV: *Antheraea pernyi* multiple NPV (Fan *et al*., 2007), CfMNPV: *Choristoneura fumiferana* multiple NPV (de Jong *et al*., 2005), AnpeNPV: *Antheraea pernyi* NPV (Nie *et al*., 2007).

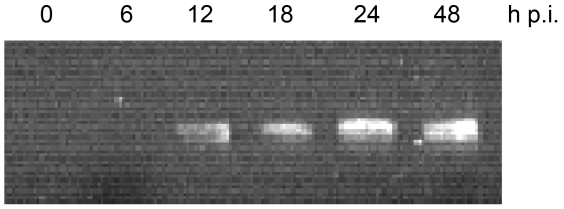

Fig. 2. Transcription analysis of *bm35* in BmNPV-infected BmN cells by RT-PCR.

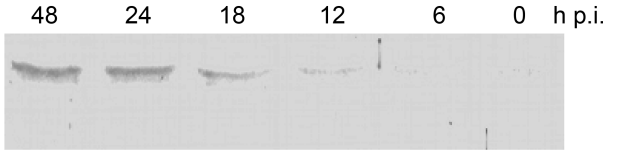

Fig. 3. Temporal expression analysis of Bm35 protein in BmNPV-infected BmN cells by Western blot analysis.

PCR using total RNA isolated from infected BmN cells at different time points. A single transcription band about 400 bp in length was observed at 12 h p.i., and remained detectable until 48 h p.i. (Fig. 2).

Time course analysis of Bm35 expression in BmNPV-infected BmN cells

Initially, the Bm35 protein was highly expressed in *E. coli* in frame with $(His)_6$ tag and purified using Ni^{2+}-NTA agarose beads. The size of fusion protein His-Bm35 was approximately 21.0 kDa, similar to theoretical size of Bm35 plus tag $(His)_6$, indicating that Bm35 was expressed completely (Data not shown). Purified His-Bm35 was used to immunize rabbit to produce anti-Bm35 polyclonal antiserum.

To detect the expression of Bm35, BmNPV-infected cells were harvested at different time points and probed with Bm35 polyclonal antibody by Western blot. A faint immunoreactive band of approximately 15 kDa was first found at 12 h p.i. and remained detectable until 48 h p.i. (Fig. 3). Together with the results from temporal analysis of *bm35* transcription, it was confirmed that Bm35 is synthesized in the late stage of BmNPV infection cycle.

Detection of Bm35 in BV and ODV particle

To explore whether the Bm35 protein is a structural com-

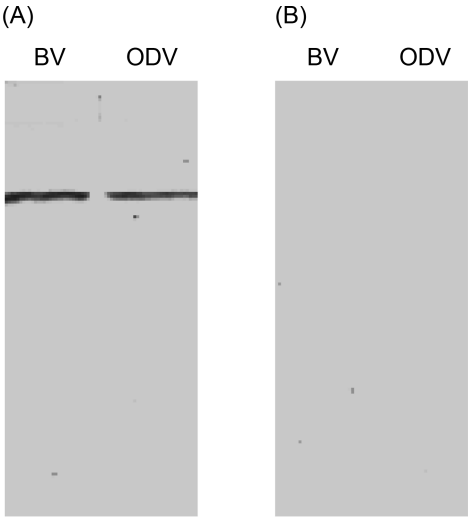

Fig. 4. Structural localization analysis of Bm35 protein in purified BV and ODV. Western blot analysis was performed using antibodies specific to VP80 (A) and Bm35 (B).

ponent, Western blot analysis was performed on purified BmNPV BV and ODV using Bm35 polyclonal antibody, and no positive bands could be detected (Fig. 4B). To compare the absence of Bm35 protein to other structural component, total proteins of BV and ODV were reacted with antibody specific to VP80, a capsid-associated protein (Müller *et al*., 1990). A band was observed in BV and ODV samples (Fig. 4A). Thus, these results suggested that Bm35 is a non-structural protein associated with BmNPV BV and ODV.

Discussion

A recent proposed reclassification has expanded family *Baculoviridae* to four genera: *Alphabaculovirus* (lepidopteran NPVs), *Betabaculovirus* (lepidopteran GVs), *Gammabaculovirus* (hymenopteran NPVs) and *Deltabaculovirus* (dipteran NPVs) (Jehle *et al*., 2006). Based on phylogeny, the proposed *Alphabaculovirus* viruses are subdivided into group I and group II (Zanotto *et al*., 1993; Bulach *et al*., 1999). The Bm35 is a rare protein whose homologues are encoded by some group-I NPVs (Fig. 1), suggesting that Bm35 and its homologues may play a specific role related to its unique host in individual virus life cycle. The BmNPV is a group-I virus and potentially encodes 136 proteins (Gomi *et al*., 1999). Among these 136 genes, six (*iap1*, *orf35*, *iap2*, *cg30*, *ie2*, and *pe38*) are predicted to encode proteins containing a RING finger motif (Imai *et al*., 2003). In the present study, the transcription, expression and viral particle localization of BmNPV Bm35 were characterized. Bm35 is not a structural component associated with the BV and ODV particle. The transcription and expression of Bm35 are detected at 12 h p.i., and remained detectable until 48 h p.i.

The gene expression of baculovirus can be divided into 4 phases: immediate-early, delayed-early, late and very late. After infection, the immediate-early and delayed-early genes are transcribed by host RNA polymerase II and most of them are thought to be involved in viral replication and late gene expression (Todd *et al*., 1996). However, expression of delayed-early genes appears to be dependent on the presence of immediate-early gene products (Kelly *et al*., 2007). Late and very-late genes are transcribed by viral RNA polymerase whose expression is regulated by the early gene products. Late gene expression is dependent on the replication of virus DNA and late proteins are frequently involved in the regulation of late and very late gene expression (Hefferon, 2004). Very late protein expression is characterized by the prominent synthesis of p10 and polyhedrin. These very-late proteins are often involved in the processes of occlusion and cell lysis (Hasnain *et al*., 1997). Together with the results we obtained previously (Guo *et al*., 2010), it seems that Bm35 expression synchronizes with the replication of BmNPV DNA. So, it is postulated that Bm35 may be associated with late and very-late gene expression.

Although some basic characteristics of Bm35 were described in this paper, much information about this gene keeps unknown. The function of *bm35* is being further investigated by deletion and mutagenesis studies using a BmNPV bacmid.

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References

Acharya A, Gopinathan KP (2002) Transcriptional analysis and preliminary characterization of ORF Bm42 from *Bombyx mori* nucleopolyhedrovirus. Virology 299, 213-224.

Ahrens CH, Russell RL, Funk CJ, Evans JT, Harwood SH, Rohrmann GF (1997) The sequence of the *Orgyia pseudotsugata* multinucleocapsid nuclear polyhedrosis virus genome. Virology 229, 381-399.

Ayres MD, Howard SC, Kuzio J, Lopez-Ferber M, Possee RD

(1994) The complete DNA sequence of *Autographa californica* nuclear polyhedrosis virus. Virology 202, 586-605.

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.

Bulach DM, Kumar CA, Zaia A, Liang B, Tribe DE (1999) Group II nucleopolyhedrovirus subgroups revealed by phylogenetic analysis of polyhedrin and DNA polymerase gene sequences. J Invertebr Pathol 73, 59-73.

Chen HQ, Chen KP, Yao Q, Guo ZJ, Wang LL (2007) Characterization of a late gene, ORF67 from *Bombyx mori* nucleopolyhedrocirus. FEBS Lett 581, 5863-5842.

Chen YR, Wu CY, Lee ST, Wu YJ, Lo CF, Tsai MF, Wang CH (2008) Genomic and host range studies of *Maruca vitrata* nucleopolyhedrovirus. J Gen Virol 89, 2315-2330.

Daimon T, Katsuma S, Shimada T (2007) Mutational analysis of active site residues of chitinase from *Bombyx mori* nucleopolyhedrovirus. Virus Res 124, 168-175.

de Jong JG, Lauzon HAM, Dominy C, Poloumienko A, Carstens EB, Arif BM, Krell PJ (2005) Analysis of the *Choristoneura fumiferana* nucleopolyhedrovirus genome. J Gen Virol 86, 929-943.

Du MF, Yin XM, Guo ZJ, Zhu LJ (2006) Characterization of a late gene, ORF60 from *Bombyx mori* nucleopolyhedrovirus. J Biochem Mol Biol 39, 737-742.

Fan Q, Li S, Wang L, Zhang B, Ye B, Zhao Z, Cui L (2007) The genome sequence of the multinucleocapsid nucleopolyhedrovirus of the Chinese oak silkworm *Antheraea pernyi*. Virology 366, 304-315.

Gomi S, Majima K, Maeda S (1999) Sequence analysis of the genome of *Bombyx mori* nucleopolyhedrovirus. J Gen Virol 80, 1323-1337.

Grace TDC (1967) Establishment of a line of cells from the silkworm *Bombyx mori*. Nature 216, 613.

Guo ZJ, Qiu LH, An SH, Yao Q, Park EY, Chen KP, Zhang CX (2010) Open reading frame 60 of the *Bombyx mori* nucleopolyhedrovirus plays a role in budded virus production. Virus Res 151, 185-191.

Harrison RL, Bonning BC (2003) Comparative analysis of the genomes of *Rachiplusia ou* and *Autographa californica* multiple nucleopolyhedroviruses. J Gen Virol 84, 1827-1842.

Harrison RL, Lynn DE (2007) Genomic sequence analysis of a nucleopolyhedrovirus isolated from the diamondback moth, *Plutella xylostella*. Virus Genes 35, 857-873.

Hasnain SE, Jain A, Habib S, Ghosh S, Chatterji U, Ramachandran A, Das P, Venkaiah B, Pandey S, Liang B, Ranjan A, Natarajan K, Azim CA (1997) Involvement of host factors in transcription from baculovirus very late promoters – a review. Gene 190, 113-118.

Hefferon KL (2004) Baculovirus late expression factors. *J Mol Microbiol Biotechnol* 7, 89-101.

Hyink O, Dellow RA, Olsen MJ, Caradoc-Davies KMB, Drake K, Herniou EA, Cory JS, O'Reilly DR, Ward VK (2002) Whole genome analysis of the *Epiphyas postvittana* nucleopolyhedrovirus. J Gen Virol 83, 957-971.

IJkel WFJ, Westenberg M, Goldbach RW, Blissard GW, Vlak JM, Zuidema D (2000) A novel baculovirus envelope fusion protein with a proprotein convertase cleavage site. Virology 275, 30-41.

Imai N, Matsuda N, Tanaka K, Nakano A, Matsumoto S, Kang W (2003) Ubiquitin ligase activities of *Bombyx mori* nucleopolyhedrovirus RING finger proteins. J Virol 77, 923-930.

Jehle JA, Blissard GW, Bonning BC, Cory JS, Herniou EA, Rohrmann GF, Theilmann DA, Thiem SM, Vlak JM (2006) On the classification and nomenclature of baculoviruses: a proposal for revision. Arch Virol 151, 1257-1266.

Katsuma S, Horie S, Daimon T, Iwanaga M, Shimada T (2006) In vivo and in vitro analyses of a *Bombyx mori* nucleopolyhedrovirus mutant lacking functional *vfgf*. Virology 355, 62-70.

Kelly BJ, King LA, Possee RD (2007) Introduction to baculovirus molecular biology; in *Baculovirus and Insect Cell Expression Protocols*. Murhammer DW (ed), pp. 25-53, Humana Press, Totowa, New Jersey.

King LA, Possee RD (1992) The baculovirus expression system: a laboratory guide. Chapman and Hall, London, UK.

Lauzon HAM, Jamieson PB, Krell PJ, Arif BM (2005) Gene organization and sequencing of the *Choristoneura fumiferana* defective nucleopolyhedrovirus genome. J Gen Virol 86, 945-961.

Müller R, Pearson MN, Russell RL, Rohrmann GF (1990) A capsid-associated protein of the multicapsid nuclear polyhedrosis virus of *Orgyia pseudotsugata*: genetic location, sequence, transcriptional mapping, and immunocytochemical characterization. Virology 176, 133-144.

Nie ZM, Zhang ZF, Wang D, He PA, Jiang CY, Song L, Chen F, Xu J, Yang L, Yu LL, Chen J, Lv ZB, Lu JJ, Wu XF, Zhang YZ (2007) Complete sequence and organization of *Antheraea pernyi* nucleopolyhedrovirus, a dr-rich baculovirus. BMC Genomics 8, 248-261.

Oliveira JV, Wolff JL, Garcia-Maruniak A, Ribeiro BM, de Castro ME, de Souza ML, Moscardi F, Maruniak JE, Zanotto PM (2006) Genome of the most widely used viral biopesticide: *Anticarsia gemmatalis* multiple nucleopolyhedrovirus. J Gen Virol 87, 3233-3250.

Theilmann DA, Blissard GW, Bonning BC, Jehle JA, O'Reilly DR, Rohrmann GF, Thiem SM, Vlak JM (2005) Baculoviridae; in *Virus taxonomy: the eighth report of the International Committee on Taxonomy of Viruses*. Fauquet CM, Mayo MA, Maniloff J, Desselberger U, Ball LA (eds.), pp. 177-185, Elsevier Academic Press, New York, NY.

Todd JW, Passarelli AL, Lu A, Miller LK (1996) Factors regulating baculovirus late and very late gene expression in transient-expression assays. J Virol 70, 2307-2317.

Wang D, An SH, Guo ZJ, Xu HJ, Zhang CX (2005) Characterization of *Helicoverpa armigera* nucleopolyhedrovirus *orf33* that encodes a novel budded virion derived protein, BVe31. Arch Virol 150, 1505-1515.

Wang F, Yang LR, Tang XD, Mo JC, Yang WJ, Zhang CX

(2006) The translational and transcriptional initiation sites of BmNPV *lef-7* gene. Virus Genes 33, 351-357.

Wang R, Deng F, Hou D, Zhao Y, Guo L, Wang H, Hu Z (2010) Proteomics of the *Autographa californica* nucleopolyhedrovirus budded virions. J Virol 84, 7233-7242.

Xu HJ, Yang ZN, Wang F, Zhang CX (2006) *Bombyx mori* nucleopolyhedrovirus ORF79 encodes a 28-kDa structural protein of the ODV envelope. Arch Virol 151, 681-695.

Xu YP, Ye ZP, Niu CY, Bao YY, Wang WB, Shen WD, Zhang CX (2010) Comparative analysis of the genomes of *Bombyx mandarina* and *Bombyx mori* nucleopolyhedrovirus. J Microbiol 48, 102-110.

Zanotto PMDA, Kessing BD, Maruniak JE (1993) Phylogenetic interrelationships among baculoviruses: evolutionary rates and host associations. J Invertebr Pathol 62, 147-164.