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Open reading frame 60 of the *Bombyx mori* nucleopolyhedrovirus plays a role in budded virus production

Zhong-Jian Guo[a], Li-Hua Qiu[a], Shi-Heng An[b,1], Qin Yao[a], Enoch Y. Park[c], Ke-Ping Chen[a,*], Chuan-Xi Zhang[b,**]

[a] Institute of Life Science and School of Finance and Economics, Jiangsu University, 301 Xuefu Road, Zhenjiang 212013, Jiangsu, PR China
[b] Institute of Insect Science, Zhejiang University, 268 Kaixuan Road, Hangzhou 310029, Zhejiang, PR China
[c] Department of Applied Biological Chemistry, Faculty of Agriculture, Shizuoka University, 836 Ohya, Shizuoka 422-8529, Japan

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ABSTRACT

Open reading frame 60 (*bm60*) of the *Bombyx mori* nucleopolyhedrovirus (BmNPV) is a conserved gene among group I and some group II NPVs. *bm60* encodes a late expressed protein that localizes to both the cytoplasm and nucleus of infected cells. This paper describes the characterization of a BmNPV mutant (vbm60-Null) lacking functional *bm60*. It was observed that the production of budded virus (BV) was reduced by nearly an order of magnitude relative to wt virus in vbm60-Null-infected BmN cells and *B. mori* larvae. Quantitative real-time PCR assay showed that the viral DNA replication was affected in infected cells due to disruption of *bm60*. Larval bioassays showed that the speed of kill of vbm60-Null virus was greatly reduced, as it took approximately 28–36 h longer to kill the fifth instar *B. mori* larvae. These results suggest that BmNPV *bm60* is not essential for viral replication, but required for efficient BV production.

1. Introduction

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

As a member of proposed genus *Alphabaculovirus*, the BmNPV is an important silkworm pathogen. Since the complete genome of BmNPV (T3 strain) has been sequenced (Gomi et al., 1999), the functions of individual genes such as *ptp* (Kamita et al., 2005), *vfgf* (Katsuma et al., 2006), *Bm67* (Ge et al., 2008), *bm118* (Yang et al., 2008), *bmvp80* (Tang et al., 2008), *bm33* (Katsuma et al., 2008), *bm21* (Huang et al., 2008), *bm9* (Yang et al., 2009), *bm56* (Xu et al., 2008) and *bm41* (Tian et al., 2009) have been well-studied. BmNPV *bm60* encodes a protein of 268 amino acid residues with a predicted molecular weight of 31.0 kDa, and was found to express a protein that localizes to both the cytoplasm and nucleus of infected cells at late stages of the infection cycle (Du et al., 2006). Homologues of *bm60* are found in the genomes of all Group I and some Group II NPVs, but are not present in hymenopteran or dipteran NPVs or GVs. A homologue of *bm60*, ORF74 of the Group I NPV, *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV), was found to encode a protein associated with ODV (Braunagel et al., 2003). Interestingly, the product of a different homologue, ORF68 of the Group II NPV *Helicoverpa armigera* nucleopolyhedrovirus (HaNPV), was not associated with ODV (Deng et al., 2007). These findings implied that homologues of Bm60 may be required for a specific role in the virus life cycle.

In this study, a *bm60*-disrupted BmNPV bacmid was generated and characterized *in vitro* and *in vivo*. It was found that *bm60* disruption reduced the production of budded virus (BV) by nearly one order of magnitude relative to wild-type virus *in vitro* and *in vivo*. Larval bioassays showed that the speed of kill of the mutant virus was greatly affected, as it took approximately 28–36 h longer than did wild-type virus to kill fifth instar *Bombyx mori* larvae. Our results suggest that *bm60* plays an important role in BV production in cultured cells as well as in larvae.

* Corresponding author. Tel.: +86 511 88791923; fax: +86 511 88791923.
** Corresponding author. Tel.: +86 571 86971697; fax: +86 571 86971697.
E-mail addresses: gzh762677@ujs.edu.cn (K.-P. Chen), chxzhang@zju.edu.cn (C.-X. Zhang).
[1] Present address: College of Plant Protection, Henan Agricultural University, 95 Wenhua Road, Zhengzhou 450002, Henan, PR China.

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Table 1
Primers used in this study.

Primer name	Primer sequence [a](5′ → 3′)
60-F	ATG AAA ATA AAT TTG TGC AAT
60-R	TCA TGT TTC TTT TTT GAA AAC T
IE-1-Promoter-F	G CTCGAG ATC AAT GTC TTT GTG AT
IE-1-Promoter-R	A CTCGAG TTG GTT GTT CAC GAT C
EGFP-F	A CTCGAG ATG GTG AGC AAG GGC G
EGFP-R	A AAGCTT TTA CTT GTA CAG CTC GTC
HSV-tk-F	A GAATTC CTA TGG CAG GGC CTG
HSV-tk-R	A GTCGAC GGG GGA GGC TAA CTG
60-Repair-R	A GTCGAC TTA TGA AGA GTG GCT
60-Repair-F	C TCTAGA TCC GCA TTA TAA GCA
Bm60352F	CGTAGTGATAGTAATCGCCGC
Bm60452R	AGTCGAGTCGCGTCGCTTT

[a] Restriction sites are underlined.

2. Materials and methods

2.1. Cells, insects, bacmids, plasmids, bacterial strains and primers

The *B. mori* cell line BmN was cultured at 27 °C in TC-100 insect medium (Sigma, St. Louis, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Gibco-BRL, Gaithersburg, USA). *B. mori* larvae (F1 hybrid JingSong × HaoYue) were reared on fresh mulberry leaves at 25 °C with a 12:12 h light/dark photoperiod.

Escherichia coli strains BW25113 (containing plasmid pKD46 encoding the genes for λ Red recombination system) and BW25141 (harboring pKD3 encoding a chloramphenicol acetyltransferase gene (*cat*)) were kindly provided by Dr. Mary K.B. Berlyn of the *E. coli* Genetic Stock Center, Yale University, USA (Datsenko and Wanner, 2000). The *E. coli* strain BmDH10Bac (containing the BmNPV genome bacmid and a helper plasmid pMON7124 encoding a transposase) was provided by Dr. Enoch Y. Park of the Department of Applied Biological Chemistry, Shizuoka University, Japan (Motohashi et al., 2005). *E. coli* DH10Bac, DH10B and plasmid pFastBac1 were purchased from Invitrogen Life technologies (Carlsbad, CA, USA). All primers used in this study are shown in Table 1.

2.2. Construction of donor plasmids

To generate wild-type, *bm60*-disrupted BmNPV virus, a donor plasmid was constructed as follows. Initially, plasmid pFastBac1 was digested with *Bam*HI and *Bst*1107I to remove the *polyhedrin* gene promoter of the AcMNPV. The resulting adhesive end was treated with Klenow polymerase. Self-ligation of the blunt fragment resulted in plasmid pFast. An *Xho*I-*Hin*dIII fragment containing the enhanced green fluorescence protein ORF (*egfp*) was amplified with primers EGFP-F and EGFP-R using the plasmid pEGFP-C1 (BD Biosciences Clontech, San Jose, CA, USA) as a template. The fragment was inserted into *Xho*I-*Hin*dIII-digested pFast to produce pFast-egfp. Finally, an *Xho*I fragment containing the *ie1* promoter was PCR amplified from BmNPV genomic bacmid with the primer pair IE-1-Promoter-F/IE-1-Promoter-R, and cloned into *Xho*I-digested pFast-egfp, to obtain pFast-Pie1-egfp.

To obtain the *bm60*-repaired BmNPV virus, another plasmid, called pFast-Pie1-egfp-bm60Re was constructed as follows. First, a *Herpes simplex* virus (HSV) thymidine kinase (TK) poly(A) signal harboring *Eco*RI-*Sal*I restriction sites was amplified from vector pEGFP-C1 (BD Biosciences Clontech, San Jose, CA, USA) with primers HSV-tk-F and HSV-tk-R. This fragment was introduced into corresponding restriction sites of pFast-Pie1-egfp to yield pFast-Pie1-egfp-tk. Another *Xba*I-*Sal*I fragment containing the *bm60* coding region and its own promoter was amplified with the primers 60-Repair-F and 60-Repair-R, and cloned into pFast-Pie1-egfp-tk, which was digested with *Xba*I and *Sal*I to obtain the vector pFast-Pie1-egfp-bm60Re.

2.3. Construction of wild-type, bm60-disrupted and repaired BmNPV viruses

In a previous study, we reported the construction of a *bm60*-disrupted BmNPV bacmid (Wang et al., 2007). This bacmid was extracted from *E. coli* BW25113 and transformed into electrocompetent *E. coli* DH10B by electroporation. Kanamycin-resistant, *lacZ*-positive colonies were selected. After identity confirmation by PCR using the primer pair 60-F/60-R, the helper plasmid pMON7124 isolated from *E. coli* DH10Bac, was introduced into the selected DH10B. Kanamycin- and tetracycline-resistant colonies were obtained and referred to as *E. coli* BmDH10B-bm60Null.

The donor plasmids pFast-Pie1-egfp and pFast-Pie1-egfp-bm60Re were transformed into *E.coli* BmDH10B-bm60Null to integrate the *bm60* repair fragment and *egfp* into the BmNPV genome by Tn7-mediated transposition (Luckow et al., 1993). Colonies were selected on medium containing kanamycin, tetracycline, gentamicin, X-gal and IPTG. Positive colonies were screened by PCR with M13 forward and reverse primers. The obtained bacmid contained the *egfp* gene under the control of the *ie1* promoter and was designated vbm60-Null. The bacmid containing the *egfp* gene, *bm60* coding region and its own promoter was designated vbm60-Repair. To construct the wild-type bacmid, the vector pFast-Pie1-egfp was transformed into *E. coli* BmDH10Bac. The bacmid isolated from a positive colony was called vbm60-Wt. Extraction of the bacmids from *E. coli* and transfection into BmN cells were performed according to the methods described in the BAC-TO-BAC Baculovirus Expression System instruction manual (Invitrogen, Carlsbad, CA).

2.4. Transfection-infection assay

A transfection-infection assay was performed to determine the replication efficiency of vbm-Wt, vbm60-Null or vbm60-Repair in BmN cells (Lin and Blissard, 2002a,b). Briefly, BmN cells were transfected with these bacmids. At 5 days p.t., supernatants were collected and added to a second group of freshly plated BmN cells. These cells were incubated further and examined daily via a fluorescent microscopy.

2.5. BV production assay

To assess whether *bm60* is required for BV production, a viral growth curve analysis was carried out. For this experiment, 2×10^6 BmN cells were transfected with each bacmid or infected with BVs at an MOI of 0.5 or 5.0 pfu and incubated at 27 °C. After 8 h for transfection or 1 h for infection, 2 ml fresh medium was added after three washes with serum-free medium. At selected time points, a small amount of culture medium was harvested. To investigate BV production in the hemolymph of *B. mori* larvae, fifth instar larvae were injected with BV of vbm-Wt, vbm60-Null and vbm60-Repair (1000 pfu/larva). Hemolymph was collected at 5 days p.i. and added to a 0.1× TE (10 mM Tris, pH 7.5, 1.0 mM EDTA) solution containing 5 mM phenylthiocarbamide to inhibit prophenoloxidase activity. Exactly 50 μl of hemolymph per larva was collected at a proleg wound; 30 larvae were used for each virus. The obtained hemolymph was centrifuged for 10 min at 5000 × *g* and filtered through a 0.45 μm filter. The titers of these samples were determined by end point dilution and were expressed as $TCID_{50}$/ml (King and Possee, 1992). One-way ANOVA followed by Dunnett's test (two-tailed test) was used to compare the resulting titers (SPSS, v17.0).