

The effect of *Bm67* gene deletion on *Bombyx mori* nuclear polyhedrosis virus replication

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Summary. – Homologs of *Bombyx mori* nuclear polyhedrosis virus (BmNPV) *Bm67* gene ORF67 have been found in the genome of all lepidopteran nuclear polyhedrosis viruses, but their function is still not very clear. In order to analyze it we employed a bacmid harboring the complete BmNPV genome including the *Bm67* gene and expressing infectious virus (wtBacmid) for the construction of its *Bm67*-deficient variant (Bm67-KO-Bacmid) using the Red recombination system and the *Bm67*-repaired variant (Bm67-Re-Bacmid) using the Bac-to-Bac system. By transfecting BmN cells with these bacmids we demonstrated that the *Bm67*-deficient virus did not generate infectious virus, while the repaired virus restored its infectivity, indicating that the *Bm67* gene is essential for the formation of infectious budding virus (BV). Electron microscopy of BmN cells transfected with the abovementioned bacmids showed many mature rodshaped virus particles in both wtBacmid- and Bm67-Re-Bacmid-transfected cells but none in Bm67-KO-Bacmid-transfected ones. Moreover, the real-time RT-PCR showed that the deletion of *Bm67* from wtBacmid significantly reduced the levels of viral genomic DNA and transcripts of viral early genes *dnapol*, *ie-1* and *lef-3* but not those of transcripts of late gene *vp39* and very late gene *p10*. The finding that the *Bm67*-deficient virus generated reduced levels of infectious virus and transcripts of early *dnapol* gene but not those of late genes indicates that the *Bm67* gene is essential for BmNPV replication.

Keywords: *Bombyx mori* nuclear polyhedrosis virus; *Bm67* gene; virus replication; genome replication; genome transcription

Introduction

Baculoviridae comprise the largest known group of insect viruses. Baculoviruses are specialized pathogenic microorganisms parasitic to arthropods, and their hosts are mainly among the *Lepidoptera*, *Diptera*, *Hymenoptera*, *Coleoptera*, and *Trichoptera* of the class *Insecta*. According to

the morphological characteristics of baculovirus infection, including the presence of inclusion bodies, baculoviruses can be divided into two genera, *Granulovirus* and *Nucleopolyhedrovirus* (Bulach *et al.*, 1999).

The first baculovirus genome fully sequenced was *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) (Ayres *et al.*, 1994). Its genome is composed of 133,894 bases with 154 open reading frames (ORFs) encoding 150 or more genes. As of 2009, 47 different baculovirus genomes have been sequenced, ranging in size from 80 to 180 kb, in which 90% of the genes encode proteins and very few genes overlap. The genome of the T3 strain *Bombyx mori* nuclear polyhedrosis virus (BmNPV) was sequenced by Gomi *et al.* (1999), and is 128,413 bp in length, with 135 ORFs, each encoding 60 or more amino-acid residues. Com-

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Abbreviations: AcMNPV = *Autographa californica* multiple nuclear polyhedrosis virus; BmNPV = *Bombyx mori* nuclear polyhedrosis virus; BV = budded virus; ORFs = open reading frames; p.t. = post transfection

parative genomic studies suggest that the genome is similar in structure to AcMNPV, with 115 highly conserved ORFs with greater than 90% homology (Yu *et al.*, 2013).

AcMNPV, the model species for baculoviruses infecting Lepidoptera, is the most studied baculovirus. AcMNPV gene expression is strictly regulated at the transcriptional level, and AcMNPV genes can be divided into those expressed in the early, late, and very late stages of viral replication (Parsarelli *et al.*, 2007). Early gene expression is regulated by the host RNA polymerase, and the expression products are predominantly proteins important for DNA replication and late gene expression. So far, 19 viral genes have been shown to be associated with late viral gene expression, and designated “late expression factors” (LEFs) (Rapp *et al.*, 1998; Hang *et al.*, 1995). Nine of these genes, *ie-1*, *ie-2*, *dnapol*, *p143*, *p35*, *lef-1*, *lef-2*, *lef-3*, and *lef-7*, affect viral DNA replication (Iyengar *et al.*, 1999; Vanarsdall *et al.*, 2006). Several other genes, including *lef-4*, *lef-5*, *lef-6*, *lef-8*, *lef-9*, *lef-10*, *lef-12*, *p47*, and *pp31*, are also considered to be involved in late viral gene transcription (Lu *et al.*, 1995).

All the sequenced baculovirus genomes include *Bm67*, one of the core baculovirus genes; its homologous gene in AcMNPV is ORF81 (Ge *et al.*, 2008). The role of this protein in viral replication is as yet unclear, the understanding the biological function of *Bm67*, however, would clarify the mechanism of transcriptional regulation in the insect nuclear polyhedrosis viruses. *Bm67* is located between genes *gp41* and *Bm68*, both of which are late viral genes. *gp41*, one of the 30 core baculovirus genes, is highly conserved. During viral budding, GP41, an occlusion-derived virus membrane glycoprotein, mediates the release of the nucleocapsid from the nucleus and promotes nucleocapsid trafficking to the cell membrane (Olszewski *et al.*, 1997). *Bm68* encodes a structural protein of the budded virus (BV), and its deletion reduces the viral budding rate, leading to delayed viral DNA replication (Iwanaga *et al.*, 2002). Although *Bm67* is essential to early viral DNA replication and *Bm67*-deleted viruses do not form infectious virus particles (Ge *et al.*, 2008), the role of *Bm67* in viral gene transcription has not yet been determined.

Red recombination technology is a genetic engineering technology developed in recent years (Copeland *et al.*, 2001). The λ phage Red recombinase, an exogenous gene sandwiched between two sequences homologous to the target gene, undergoes homologous recombination with the target gene. This effectively inserts the foreign gene into the position of the target gene in the host genome, efficiently achieving gene knockout (Costantino *et al.*, 2003). Red recombination technology has been widely applied to the functional analysis of the genes of AcMNPV and BmNPV (Guarino *et al.*, 2002; Dickison *et al.*, 2012). To investigate the function of *Bm67*, we generated a *Bm67*-deficient virus and studied the impact of this deletion on infectious virus

production and viral genome replication and transcription, using wild type (encodes a complete BmNPV) and *Bm67*-repair viruses as controls.

Materials and Methods

Cells and reagents. BmN cells, *Escherichia coli* strains TG1, DH10Bac and BW25113, plasmids pFastBac1 and pKD3 are all stored in our laboratory (Yu *et al.*, 2013). l-Arabinose was purchased from Promega (USA). SOC liquid medium was purchased from Biocolor BioScience & Technology Company; TRIzol® Reagent was purchased from Invotrigen (USA). A 1-kb DNA ladder marker, DL2000 DNA ladder marker, T4 ligase, the pMD18-T vector, and restriction endonucleases *Bam*HI, *Eco*RI and *Hind*III were purchased from TaKaRa Bio (Japan). The gel extraction kit was from Axygen (USA). *Taq* DNA polymerase and the corresponding PCR reactions reagents were from the Beijing Dingguo Biotechnology Company (China). KOD Plus high-fidelity DNA polymerase and the corresponding reaction reagents were purchased from Toyobo (Japan). DNA transfection reagent was purchased from SignaGen (USA). All primers were synthesized by the Shanghai Shengon Biotechnology Company (China).

Construction of the *Bm67* gene-deficient *Bacmid*. To produce a *Bm67* gene-deficient bacmid, a linear fragment targeting *Bm67* was constructed (Fig. 1a). The PCR amplification primers and all other primers sequences are shown in Table 1. A targeting fragment of around 1100 bp, designated Bm67-C, was amplified by PCR using the pKD3 plasmid as the template and Bm67-CF and Bm67-CR as primers. It was transformed into competent DH10Bac cells containing the pKD46 plasmid. After l-Arabinose was added to induce the expression of λ Red recombinase, the linear fragment Bm67-C recombined with *Bm67* in the viral genome (Yu *et al.*, 2013). Positive clones were selected on plates containing chloramphenicol and kanamycin. The primers Bm67F/Bm67R and catF/catR were used to confirm the recombination and *Bm67* deletion.

Construction of the *Bm67* gene-repaired *Bacmid*. To generate a *Bm67* gene-repaired bacmid, the pFastBac1-Bm67 plasmid was generated by inserting the *Bm67* gene downstream of the polyhedrin promoter using the Bac-to-Bac® Baculovirus Expression System, as shown in Fig. 1b. The primers were based on the *Bm67* sequence, with *Bam*HI and *Eco*RI restriction sites introduced, together with 297 bp of the *Bm67* promoter sequence (Table 1). The digested PCR products were used to generate the plasmid pFastBac1-Bm67, which was transformed into DH10Bac cells containing the *Bm67* gene-deficient bacmid. The positive clones were screened on plates containing Kanamycin, gentamicin, tetracycline, IPTG and 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal). The primers for identification of the *Bm67* presence were M13, pFB-Bm67F, and pFB-Bm67R (Table 1).

Real-time PCR assay of *gp41* DNA. Real-time quantitative PCR (qPCR) was used to assess the rate of viral DNA replication. To eliminate the impact of the sample volume, the levels of β -actin were used

Table 1. Primer sequences for PCR amplification

Primer name	Primer sequence (5'→3')
Bm67-CF ^a	Forward: 5'- <u>AAGTACACGTGTCCAATGGGTACTCGTTTGAATTCATCCAGGCAGTCAA</u> GTGTAGGCTGGAGCTGCTTC-3'
Bm67-CR ^a	Reverse: 5'- <u>CGGCGCAAGTCATCAGCACCGTTTGAAAACATGATGCGCCGACACAAAAT</u> GATGGGAATTAGCCATGGTCC-3'
Bm67F Bm67R	Forward: 5'-ATGACGACGACGACGACGACGAAGACGA-3' Reverse: 5'-TCATCTGTCATACTTTTATTGTGA-3'
catF catR	Forward: 5'-CACGTTTAAATCAAACTGGTG-3' Reverse: 5'-CAATATGGACAACCTCTTCG-3'
β-actinF β-actinR	Forward: 5'-GCGCGGCTACTCGTTCACTACC-3' Reverse: 5'-TGCCGCAAGCTTCCATACCC-3'
M13F M13R	Forward: 5'-GTTTTCCTCCAGTCACGAC-3' Reverse: 5'-CAGGAAACAGCTATGAC-3'
pFB-Bm67F pFB-Bm67R	Forward: 5'-CGCGGATCCAATATTACTTAAAAAACGGGGCGT-3' Reverse: 5'-CCGGAATTCTCATCTGTCATACTTTTATTGTGA-3'
gp41F gp41R	Forward: 5'-CGTAGTGTTAGTAATCGCCGC-3' Reverse: 5'-AGTCGAGTCGCGTCGCTTT-3'
Bmie-1F Bmie-1R	Forward: 5'-CGAGACGGCTGCACAAA-3' Reverse: 5'-TGCCCAAAAGAAACCCACA-3'
Bmlf-3F Bmlf-3R	Forward: 5'-TCGGATGACCGTTCTACCTCTT-3' Reverse: 5'-CTTCCAGCAGCATTGAGATTG-3'
Bmvp39F Bmvp39R	Forward: 5'-AGACACCACAAACCCGAACAC-3' Reverse: 5'-TTGATCGCCAACACCACCT-3'
BmvP10F BmvP10R	Forward: 5'-TTGATCGCCAACACCACCT-3' Reverse: 5'-CGATTCTTCCAGCCCGTTT-3'
BmdnapoIF BmdnapoIR	Forward: 5'-GCCGATTGCGTTTTTTGCC-3' Reverse: 5'-GATTGCCATTTGTGCTTGTT-3'

^aThese two primers were composed of 50-bp *Bm67* homology arms (underlined) and a 20-bp *cat* homologous zone. Letters in boxes represent the restriction enzymes *Bam*H' and *Eco*R'.

as an internal control, amplified with primers β-actinF and β-actinR. BmN cells in the logarithmic growth phase (1×10^6 /35mm dish) were transfected with 1 μg of Bm67-KO-Bacmid, the wild-type bacmid (wt-Bacmid), or Bm67-Re-Bacmid DNA using DNA transfection reagent (SignaGen) according to the manufacturer's instructions, and the cells were harvested 6, 12, 24, 36, and 48 hr after transfection. Total DNA was extracted, digested with *Dpn*I for 5 hr to remove any exogenous methylated bacmid DNA, and analyzed with qPCR using the *gp41* gene-specific primers gp41F and gp41R (Vanarsdall *et al.*, 2005).

Real-time RT-PCR assay of transcripts of the *dnapol*, *ie-1*, *lef-3*, *vp39* and *p10* genes. The timing and extent of viral gene transcription was assessed with qPCR. BmN cells were transfected with bacmids as described above and their DNA was harvested 6, 12, 24, 36, and 48 hr later. Total RNA was extracted with TRIzol® Reagent and treated with DNaseI to remove any residual BmNPV genomic DNA. cDNA was synthesized with reverse transcriptase and an oligo(dT)₁₈ primer and amplified with BmNPV *ie-1*, *lef-3*, *vp39*, *dnapol*, or *p10* gene-specific primers. The primer pairs Bmie-1F/Bmie-1R, Bmlf-3F/Bmlf-3R, Bmvp39F/Bmvp39R, BmvP10F/BmvP10R, and BmdnapoIF/BmdnapoIR were used (Table 1). β-Actin was amplified as the control, as described above.

Virus titration. BmN cells were transfected with bacmids as described above. Cell culture supernatants were collected 12, 18, 24,

48, 72, and 96 hr after transfection, and the 50% tissue culture infectious dose (TCID₅₀) was calculated. BmN cells in the logarithmic growth phase were seeded in 96-well plates at 1×10^3 cells/well. The viral supernatant was diluted and loaded into each well, and the cells were cultured for five days. The viral titer (TCID₅₀) was calculated with the method of Reed and Muench (Reed *et al.*, 1938).

Electron microscopy. BmN cells (1×10^5 /35 mm dish) were transfected with 1 μg of Bm67-KO-Bacmid, wtBacmid, or Bm67-Re-Bacmid DNA, and the cells were collected 24 hr after transfection. The cells were fixed in 2.5% glutaraldehyde solution at 4 °C overnight and processed according to the standard procedures. 70–90 nm thin sections were cut and observed and analyzed with a transmission electron microscope (HitachiH-7650).

Results

Construction of Bm67-deficient and Bm67-repaired bacmids

Following the recombination in *E.coli* to delete the *Bm67* gene by replacement with a linear fragment Bm67-C cas-

sette, a single clone was randomly selected from agar plates containing antibiotics and cultured overnight. Bacmid DNA was confirmed with PCR using wtBacmid as the control and the following primer pairs: Bm67F/catR, catF/Bm67R, and Bm67F/Bm67R. As shown in Fig. 2a, although no band was detected in the control wtBacmid-transfected cells (data not shown), the sizes of the amplification products were about 660 bp, 990 bp, and 1600 bp when primer pairs Bm67F/catR, Bm67R/catF, and Bm67F/Bm67R were used, respectively. These results suggest that the *Bm67* gene was successfully deleted from the bacmid genome, and this virus was designated “Bm67-KO-Bacmid”.

Following recombination to repair the Bm67-KO-Bacmid and re-introduce the *Bm67* gene, a single clone was randomly selected from the agar plates containing antibiotics, and cultured overnight. The bacmid DNA was confirmed with PCR using the following primer pairs: M13F/M13R, M13F/pFB-Bm67R, and pFB-Bm67F/M13R. As shown in Fig. 2b, the sizes of the amplification products when primer pairs M13F/M13R, M13F/pFB-Bm67R, or pFB-Bm67F/M13R were used were roughly 3,700 bp, 2,500 bp, and 1,400 bp, respectively, which is consistent with the calculated size, suggesting that the *Bm67* gene was successfully repaired. This virus was designated “Bm67-Re-Bacmid”.

Effect of *Bm67* gene deletion on replication of viral genomic DNA

To analyze the effects of *Bm67* gene deletion on *BmNPV* DNA replication, BmN cells were transfected with Bm67-KO-Bacmid, wtBacmid, or Bm67-Re-Bacmid DNA, and the cells were harvested 6, 12, 24, 36, and 48 hr after transfection. As illustrated in Fig. 3, the viral DNA copy number in the Bm67-KO-Bacmid-transfected cells was lower at 12 hr than in cells transfected with wtBacmid or Bm67-Re-Bacmid ($P < 0.05$) and significantly lower at 24–48 hr ($P < 0.01$). The viral DNA copy numbers in the wtBacmid- or Bm67-Re-Bacmid-transfected cells increased significantly between 24 and 48 hr post transfection (p.t.). In contrast, although the viral DNA copy number in cells transfected with the *Bm67*-deletion virus also increased during this period, it was significantly lower than in control cells, suggesting that the *BmNPV Bm67* gene is essential for genome replication.

Effect of *Bm67* gene deletion on infectious virus replication

The supernatants of BmN cells transfected with Bm67-KO-Bacmid, wtBacmid, or Bm67-Re-Bacmid were collected 12, 18, 24, 48, 72, and 96 hr p.t., and the viral titers determined (Fig. 4). The supernatant of the cells transfected with Bm67-KO-Bacmid was not infectious, indicating that Bm67-KO-Bacmid did not produce infectious viral particles.

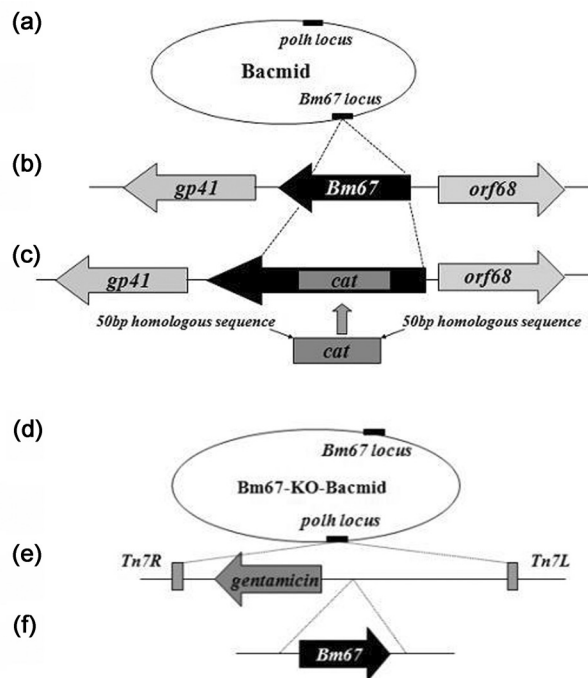


Fig. 1

Construction of *Bm67*-deficient and *Bm67*-repaired bacmids

(a) Location of *Bm67* in the Bm67-KO-Bacmid genome; (b) recombination between *cat* and *Bm67*; (c) PCR amplification of the target fragment using the pKD3 plasmid as the template and primers Bm67-CF/Bm67-CR; (d) location of *polh* in the Bm67-Re-Bacmid genome; (e) insertion of *Bm67*, with its promoter, into the transposon; (f) PCR amplification of *Bm67* and its promoter using the wild-type bacmid as the template.

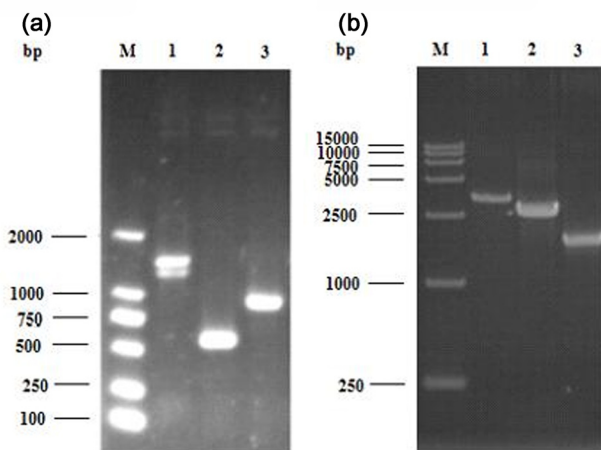


Fig. 2

Validation of *Bm67*-deficient (a) and *Bm67*-repaired viruses (b) by PCR

Lane 1: primers Bm67F and Bm67R were employed; lane 2: primers Bm67F and catR were employed; lane 3: primers Bm67R and catF were employed; lane 4: The primers M13F and M13R were employed; lane 5: primers M13F and pFbBm67R were employed; lane 6: primers pFbBm67F and M13R were employed.

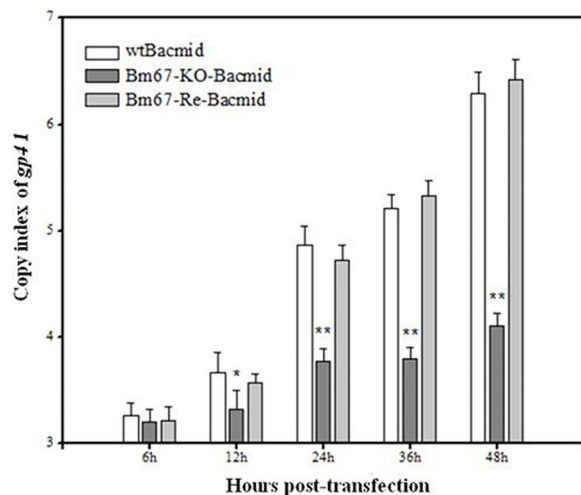


Fig. 3

Virus copy number in cells transfected with bacmids

The cells transfected with the bacmids were harvested at 6–48 hr p.t. and assayed for *gp41* by real-time PCR. * $P < 0.05$, ** $P < 0.01$ vs wtBacmid and Bm67-Re-Bacmid. Values are expressed as means \pm SEM. Similar results were obtained in three independent experiments. P value was calculated by SPSS software, comparing the average copy level of Bm67-KO-Bacmid against the average copy levels of wtBacmid and Bm67-Re-Bacmid.

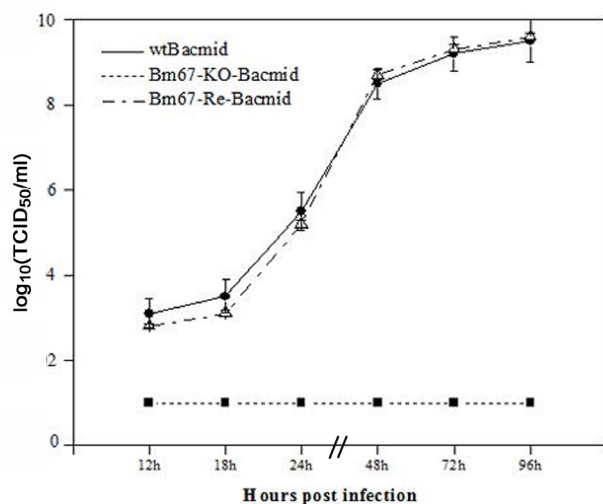
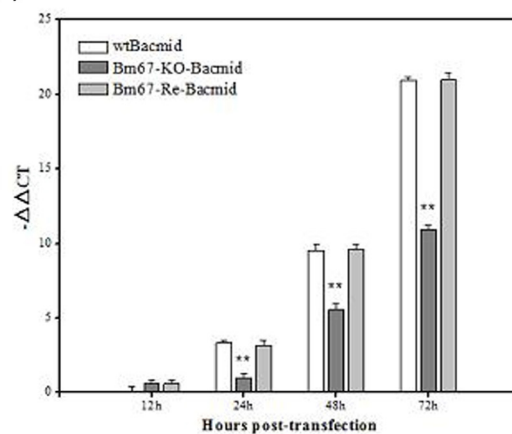


Fig. 4

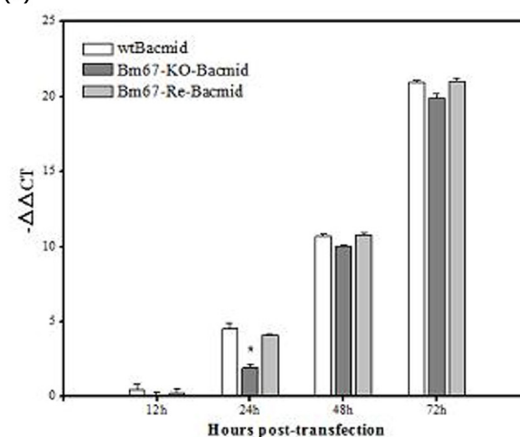
Virus titers in cells transfected with bacmids

The cells transfected with the bacmids were harvested at 6–48 hr p.t. and assayed for the virus titers.

(a)



(b)



(c)

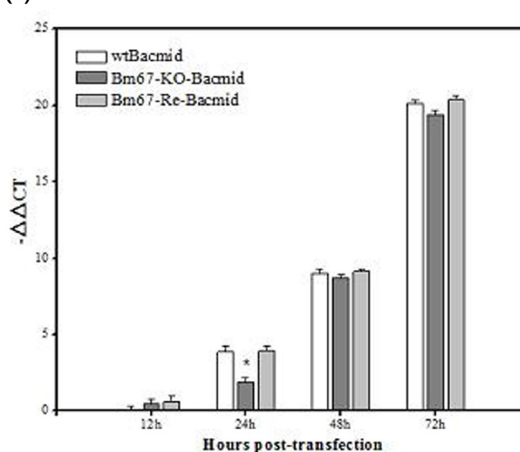


Fig. 5

Levels of transcripts of early genes *dnapol* (a), *ie-1* (b) and *lef-3* (c) in cells transfected with bacmids

Real-time RT-PCR was employed. * $P < 0.05$ vs wtBacmid and Bm67-Re-Bacmid. Values are expressed as means \pm SEM. Similar results were obtained in three independent experiments. P value was calculated by SPSS software, comparing the average transcriptional copy level of Bm67-KO-Bacmid against the average transcriptional copy levels of wtBacmid and Bm67-Re-Bacmid.

The supernatant of the cells transfected with wtBacmid or Bm67-Re-Bacmid yielded infectious virus, and the viral titers increased rapidly, almost reaching their maxima at 48 hr.

Effect of Bm67 gene deletion on viral gene transcription

Because gene expression in *BmNPV* is mainly regulated at the transcriptional level, early viral genes can be transcribed by the host RNA polymerase, whereas the late viral genes can be transcribed by viral RNA polymerase (Todd *et al.*, 1996). To study the effects of *Bm67* deletion on *BmNPV* gene transcription, we measured the levels of several early genes essential to viral replication. Three early genes, *dnapol*, *ie-1*, and *lef-3*, were analyzed in cells transfected with Bm67-KO-Bacmid, wtBacmid, or Bm67-Re-Bacmid. As shown in Fig. 5, the level of *dnapol* transcripts in cells transfected with Bm67-KO-Bacmid was only 10% of that detected in the wtBacmid- and Bm67-Re-Bacmid-transfected cells 24 hr after transfection, indicating a significant reduction in *dnapol* expression. Although the transcription of *dnapol* in cells transfected with Bm67-KO-Bacmid had increased at 48 and 72 hr p.t., it was always significantly lower than that detected in the wtBacmid- and Bm67-Re-Bacmid-transfected cells ($P < 0.01$). Similarly, 24 hr after transfection, the transcription of *ie-1* and *lef-3* in cells transfected with Bm67-KO-Bacmid were 40% and 45%, respectively, of that detected in cells transfected with Bm67-Re-Bacmid or wtBacmid ($P < 0.05$). The transcription levels of *ie-1* and *lef-3* in cells transfected with Bm67-KO-Bacmid had increased 48 and 72 hr p.t., and were only slightly lower than those detected in the wtBacmid- and Bm67-Re-Bacmid-transfected cells ($P > 0.05$).

To determine the impact of *Bm67* on the transcription of late and very late genes, we analyzed the expression of the late gene *vp39* and very late gene *p10* in cells transfected with Bm67-KO-Bacmid, wtBacmid, or Bm67-Re-Bacmid. As shown in Fig. 6, the timing and levels of transcription of the late gene *vp39* and the very late gene *p10* in the Bm67-KO-Bacmid-transfected cells did not differ significantly from those in the cells transfected with wtBacmid or Bm67-Re-Bacmid ($P > 0.05$).

Effect of Bm67 gene deletion on virus assembly

To analyze the impact of *Bm67* deletion on virus particle assembly, ultrathin sections were generated from wtBacmid-, Bm67-KO-Bacmid-, and Bm67-Re-Bacmid-transfected BmN cells and observed with a transmission electron microscope. As shown in Fig. 7, there were no viral particles in the Bm67-KO-Bacmid-transfected cell nuclei, suggesting that the loss of *Bm67* affected virus assembly. However, the wtBacmid- and Bm67-Re-Bacmid-transfected cells showed the typical symptoms of baculovirus infection, including the enlarged nuclei and the formation in the cell nucleoli

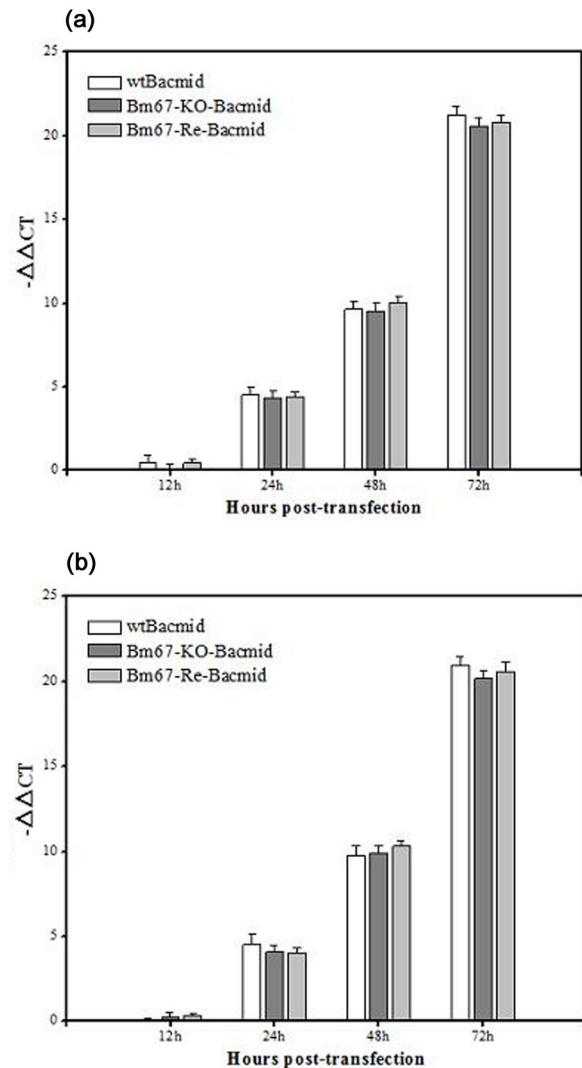


Fig. 6

Levels of transcripts of late genes *vp39* (a) and *p10* (b) in cells transfected with bacmids

Real-time RT-PCR was employed. Values are expressed as means \pm SEM. Similar results were obtained in three independent experiments.

of virogenic stroma, and a large number of nucleocapsids were observed within the nuclei. This is consistent with the measurements of viral titers.

Discussion

In this study, the Red recombination technology was used to generate a *Bm67*-deletion virus, Bm67-KO-Bacmid. To construct a *Bm67*-repaired virus, Bm67-Re-Bacmid, the *Bm67* gene and promoter were inserted downstream from the polyhedrin promoter in Bm67-KO-Bacmid using the

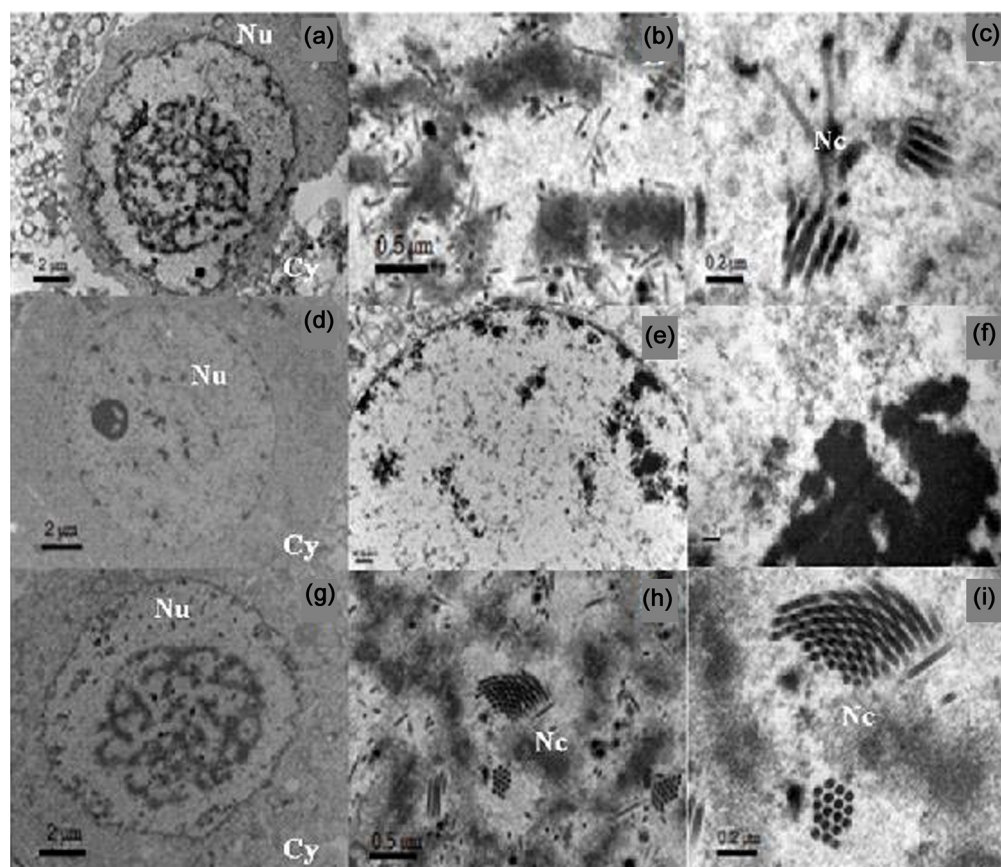


Fig. 7

Electron microscopic analysis of cells transfected with the bacmids

Cells analyzed 24 hr p.t. wtBacmid: magnification 5,000 \times (a), 20,000 \times (b) and 50,000 \times (c); Bm67-KO-Bacmid: magnification 5,000 \times (d), 20,000 \times (e) and 50,000 \times (f); Bm67-Re-Bacmid: magnification 5,000 \times (g), 20,000 \times (h) and 50,000 \times (i). Nc, nucleocapsid; Nu, nucleus; Cy, cytoplasm. Bars indicate the scale.

Bac-to-Bac[®] Baculovirus Expression System. BmN cells transfected with the *Bm67*-deleted virus did not produce infectious viral particles. However, this phenotype was rescued by the ectopic expression of the *Bm67* gene, suggesting that this gene is critical for the formation of infectious viral particles. These findings are consistent with those published by Ge *et al.* (2008) who demonstrated that the deletion of *Bm67* reduces viral nucleocapsid assembly, and hinders the formation of BV in enveloped viruses. Our transmission electron microscopy results also confirmed that *Bm67* deletion affects the capacity for virus assembly, leading to a failure to form mature viral particles. However, the *Bm67*-restored virus, Bm67-Re-Bacmid, formed a large number of nucleocapsids in the nuclei.

Many processes may be inhibited in the *Bm67*-deficient virus, including viral DNA replication, the regulation of gene expression, and nucleocapsid assembly and transport (Oomens and Blissard, 1999; Guarino *et al.*, 2002; Milks *et al.*, 2003; Yamagishi *et al.*, 2007; Ke *et al.*, 2008). We found

that the level of viral DNA replication was significantly lower in the Bm67-KO-Bacmid-transfected cells than in the cells transfected with wtBacmid or Bm67-Re-Bacmid, and that the cells transfected with Bm67-KO-Bacmid yielded supernatant with low infectivity. We concluded that although *Bm67* enhances BV assembly, the *Bm67*-deficient viral genome can still replicate, albeit at a slower rate. Because BV are usually produced following the second round of infection (Volkman, 2007), 24hr after transfection, the Bm67-KO-Bacmid may allow only one round of replication, leading to a limited increase in the viral genome copy number, but few infectious BV.

Previous studies have highlighted the association between certain specific replication factors and baculovirus replication (Lu *et al.*, 1995; Yu *et al.*, 2013; Vanarsdall *et al.*, 2004). For example, Vanarsdall *et al.* found that the knockout of *vlf-1*, which is expressed at the late stage of replication, inhibits viral DNA replication and prevents BV production and dissemination (Vanarsdall *et al.*, 2004). Yu *et al.* (2013) deleted

the late-expression factor *lef-10* and observed reduced viral DNA replication and the reduced transcription of early and late genes. Therefore, we hypothesized that *Bm67* may reduce the transcription levels of some replication-regulating factors. We investigated the transcription levels of several viral genes in cells transfected with the *Bm67*-deficient virus, and found that *Bm67* deletion affected the transcription levels of the early genes *ie-1*, *lef-3*, and *dnapol* to different degrees. Twenty-four hours after the cells were transfected with *Bm67*-KO-Bacmid, the transcript level of the *dnapol* genes was only 10% of that in cells transfected with wtBacmid or *Bm67*-Re-Bacmid. Similarly, the transcription levels of *ie-1* and *lef-3* were only 40% and 45%, respectively, of those in cells transfected with wtBacmid or *Bm67*-Re-Bacmid. In contrast, the transcription of the late gene *vp39* and the very late gene *p10* was barely affected by the deletion of *Bm67*. These findings suggest that *Bm67* deletion reduces the expression of the viral DNA polymerase gene (*dnapol*) and other early genes, which are required for viral DNA replication, and consequently limits viral genome replication (Okano *et al.*, 1999). Interestingly, the levels of *ie-1* and *lef-3* transcripts in the cells transfected with *Bm67*-KO-Bacmid had increased significantly 48 hr after transfection, and were restored to the levels detected in cells transfected with the wild-type virus. Therefore, we hypothesize that the deletion of *Bm67* only delayed the transcription of some early genes. In contrast, *dnapol* transcription was depressed at all the time points tested. The baculovirus-specific DNA polymerase, originally identified by Mikhailov *et al.* in *BmNPV*, has a 3'→5' exonuclease activity and is involved in the synthesis of the DNA leading strand and lagging strand, thus ensuring the fidelity of DNA synthesis (Mikhailov *et al.*, 1986). In 2005, Vanarsdall *et al.* found that the replication of the viral genomic DNA is inhibited by the knockout of *dnapol* (Vanarsdall *et al.*, 2005).

In summary, we speculate that the reduced levels of viral DNA replication that result from *Bm67* deletion are caused by the reduced transcription of viral *dnapol* and the delayed transcription of some early genes. A further in-depth study is required to determine the influence of *Bm67* deletion on the expression levels of viral early genes.

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