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# *Autographa californica* multiple nucleopolyhedrovirus *ac66* is required for the efficient egress of nucleocapsids from the nucleus, general synthesis of preoccluded virions and occlusion body formation

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**Abstract**

Although *orf66* (*ac66*) of *Autographa californica* multiple nucleopolyhedrovirus (Ac*M*NPV) is conserved in all sequenced lepidopteran baculovirus genomes, its function is not known. This paper describes generation of an *ac66* knockout Ac*M*NPV bacmid mutant and analyses of the influence of *ac66* deletion on the virus replication in Sf-9 cells so as to determine the role of *ac66* in the viral life cycle. Results indicated that budded virus (BV) yields were reduced over 99% in *ac66*-null mutant infected cells in comparison to that in wild-type virus infected cells. Optical microscopy revealed that occlusion body synthesis was significantly reduced in the *ac66* knockout bacmid-transfected cells. In addition, *ac66* deletion interrupted preoccluded virion synthesis. The mutant phenotype was rescued by an *ac66* repair bacmid. On the other hand, real-time PCR analysis indicated that *ac66* deletion did not affect the levels of viral DNA replication. Electron microscopy revealed that *ac66* is not essential for nucleocapsid assembly, but for the efficient transport of nucleocapsids from the nucleus to the cytoplasm. These results suggested that *ac66* plays an important role for the efficient exit of nucleocapsids from the nucleus to the cytoplasm for BV synthesis as well as for preoccluded virion and occlusion synthesis.


*Keywords:* Baculovirus; Ac*M*NPV; ORF66; Viral replication; Budded virus; Occlusion

## Introduction

*Autographa californica* multiple nucleopolyhedrovirus (Ac*M*NPV) is the prototype member of the *Baculovirade* family, which is a group of arthropod-specific, enveloped, rod-shaped virus with a circular covalently closed DNA genome of 80 to 180 kb that are replicated in the nuclei of host cells (Ayres et al., 1994; Herniou et al., 2003). The family is divided into two genera, *Nucleopolyhedrovirus* (NPV) and *Granlovirus* (GV), which differ in occlusion body morphology as well as the cytopathology they induce (Williams and Faulkner, 1997). During the infection cycle, baculovirus produces two viral forms, budded virus (BV) and occlusion-derived virus (ODV). These two viral forms play different roles in the viral life cycle. ODV is responsible for horizontal transmission between insect hosts, whereas BV is responsible for the systemic spread through the insect host and propagation in tissue culture (Williams and Faulkner, 1997). BV and ODV are identical in nucleocapsid structure and genetic information, but differ in the source of virion envelopes (Funk et al., 1997; Braunagel and Summers, 1994). Nucleocapsids egress from the nucleus and then move to the plasma membrane from which they bud forming BV (Williams and Faulkner, 1997). Late in Ac*M*NPV infection, nucleocapsids acquire an envelope to form preoccluded virions (POV) in the nucleus, and the resulting virions are subsequently embedded into a paracrystalline matrix consisting mainly of the polyhedrin protein to form ODV (Williams and Faulkner, 1997). However, limited knowledge is known on how the nucleocapsids are transported to the cell membrane and how the phenotype switches from BV to ODV is determined.

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During the baculovirus infection cycle, gene transcription can be divided into early, late, and very late phases. By the host RNA polymerase II, the early gene transcription is initiated from a highly conserved TATA motif which is found in most early gene promoters (Garcia-Maruniak et al., 2004; Friesen, 1997). In addition, the transcription of several early genes is also found to initiate from other *cis*-acting regulatory elements, such as the initiator element CAGT motif (Blissard et al., 1992; Pullen and Friesen, 1995). The late and very late gene transcription is mediated by virus-encoded RNA polymerase and initiated from a highly conserved motif containing the sequence (A/G/T) TAAG that is also found in most late and very late gene promoters (Lu and Miller, 1997).

The Ac*M*NPV *orf66* (*ac66*) encodes a protein of 808 amino acids with a molecular mass of 97 kDa and possesses only a typical late and very late gene promoter motif GTAAG. Sequence-based queries performed with InterProScan program shows that Ac66 homologs are related to a protein family called viral Desmoplakin N-terminus family (IPR009615). Viral Desmoplakin N-terminus represents the N-terminus of viral desmoplakin but many family members are hypothetical. Desmoplakin is a component of mature desmosomes which are the main adhesive junctions in epithelia and cardiac muscle, and also essential for the maturation of adherens junction. No function has been ascribed to this putative Desmoplakin N-terminus motif in baculoviruses.

Previous report has shown that *ac66* does not appear to affect late gene expression in a transient-expression assay system (Li et al., 1999). With a custom-made baculovirus DNA microarray, *ac66* was expressed throughout the infection indicating that there might be other motifs involved in the regulation of the early transcription or that the mRNA might be stable (Jiang et al., 2006). Additionally, *ac66* was convincingly indentified within ODV by a very detailed proteomic analysis of ODV structure proteins (Braunagel et al., 2003). However, the role it plays in Ac*M*NPV life cycle is still unknown.

In this study, we generated an *ac66* knockout Ac*M*NPV mutant by homologous recombination in *Escherichia coli*. Our data shows that the deletion of *ac66* significantly decreases budded virus yields as well as POV and occlusion synthesis in Sf-9 cells, while *ac66* is not required for viral DNA replication. Electron microscopy analysis revealed that the number and appearance of nucleocapsids appearing in the nuclei of cells transfected with the *ac66*-null mutant bacmid were similar with that of cells transfected with wild-type bacmid. However, the number of nucleocapsids present in the cytoplasm from *ac66* knockout bacmid-transfected cells was significantly decreased.

## Results

### *RT-PCR analysis of the ac66 transcript*

The temporal transcription pattern of *ac66* was determined by RT-PCR which produced a specific 673-bp fragment and the product was detected in Ac*M*NPV-infected *Spodoptera frugiperda* Sf-9 cells as early as 3 h postinfection (p.i.) and remained detectable up to 72 h p.i. (Fig. 1). We took advantage of

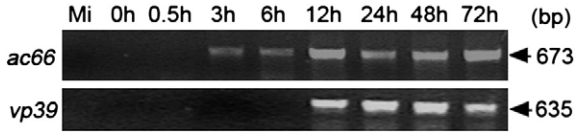


Fig. 1. RT-PCR analysis of *ac66* transcription. Total RNA was extracted from Ac*M*NPV-infected Sf-9 cells at 0, 0.5, 3, 6, 12, 24, 48, and 72 h p.i. or mock-infected (Mi) cells. Sizes of PCR products are indicated in bp.

Ac*M*NPV *vp39* gene as the control for the late gene. As expected, a 635-bp transcript was detected from 12 to 72 h p.i. No *ac66* and *vp39* RT-PCR products were detected in mock-infected cells (Fig. 1) or using the same templates as those used in RT-PCR analysis without reverse transcriptase added prior to the PCR step, indicating no possible contamination of Ac*M*NPV DNA in the RT-PCR analysis (data not shown). This result suggests that *ac66* is transcribed as an early, late, and very late gene. Because *ac66* possesses only a late promoter motif, future studies are required to distinguish the new early promoter motif.

### *Construction of ac66 knockout, repair, and wt AcMNPV bacmids containing gfp (green fluorescent protein) and polyhedrin*

To determine if *ac66* is essential for viral replication, a recombinant Ac*M*NPV bacmid (Ac-*ac66*-KO) in which a 1965-bp fragment of *ac66* coding region (nt 55,556–57,520) was replaced with a Zeocin-resistant gene was constructed by homologous recombination (Fig. 2A). Two hundred sixty-four base pairs of 5′ end and 198 bp of the 3′end of *ac66* coding region were retained so that the knockout of *ac66* from Ac*M*NPV bacmid genome would not affect the transcription of DNA polymerase gene (Vanarsdall et al., 2005) and *lef-3* gene (Li et al., 1993) (Fig. 2A). The knockout of *ac66* from the genome was confirmed by PCR using the same primers as those used in PCR analysis and the results were shown in Figs. 2B and C. Primers ZeoP1 and ConfirmP2 produced a 1657-bp PCR product in Ac-*ac66*-KO, but no PCR product in wt bacmid. Primers RTP1 and RTP2 produced no PCR product in Ac-*ac66*-KO, but a 673-bp PCR product in wt bacmid. Primers REP1 and REP2 produced a 2132-bp PCR product in Ac-*ac66*-KO, but a 3305-bp PCR product in wt bacmid. Primers ORFP1 and ORFP2 produced an 1172-bp PCR product in Ac-*ac66*-KO, but a 2445-bp PCR product in wt bacmid. The PCR results indicated that *ac66* was successfully replaced by Zeocin-resistant gene and no intact *ac66* gene cassette existed in the Ac-*ac66*-KO.

To facilitate the observation of BV transmission and occlusion morphogenesis, two marker genes, *polyhedrin* and *gfp*, were introduced into *ac66* knockout bacmid polyhedrin locus by Tn7 mediated transposition (Fig. 2D). The resulting bacmid was named Ac-*ac66*-KO-GP. To confirm the phenotype resulting from the deletion of *ac66*, a rescue bacmid (Ac-*ac66*-REP-GP) was constructed by introducing the *ac66* under the control of its native promoter as well as two marker genes, *polyhedrin* and *gfp*, into the polyhedrin locus of Ac-*ac66*-KO by transposition (Fig. 2D). Ac-GP, the positive control bacmid,

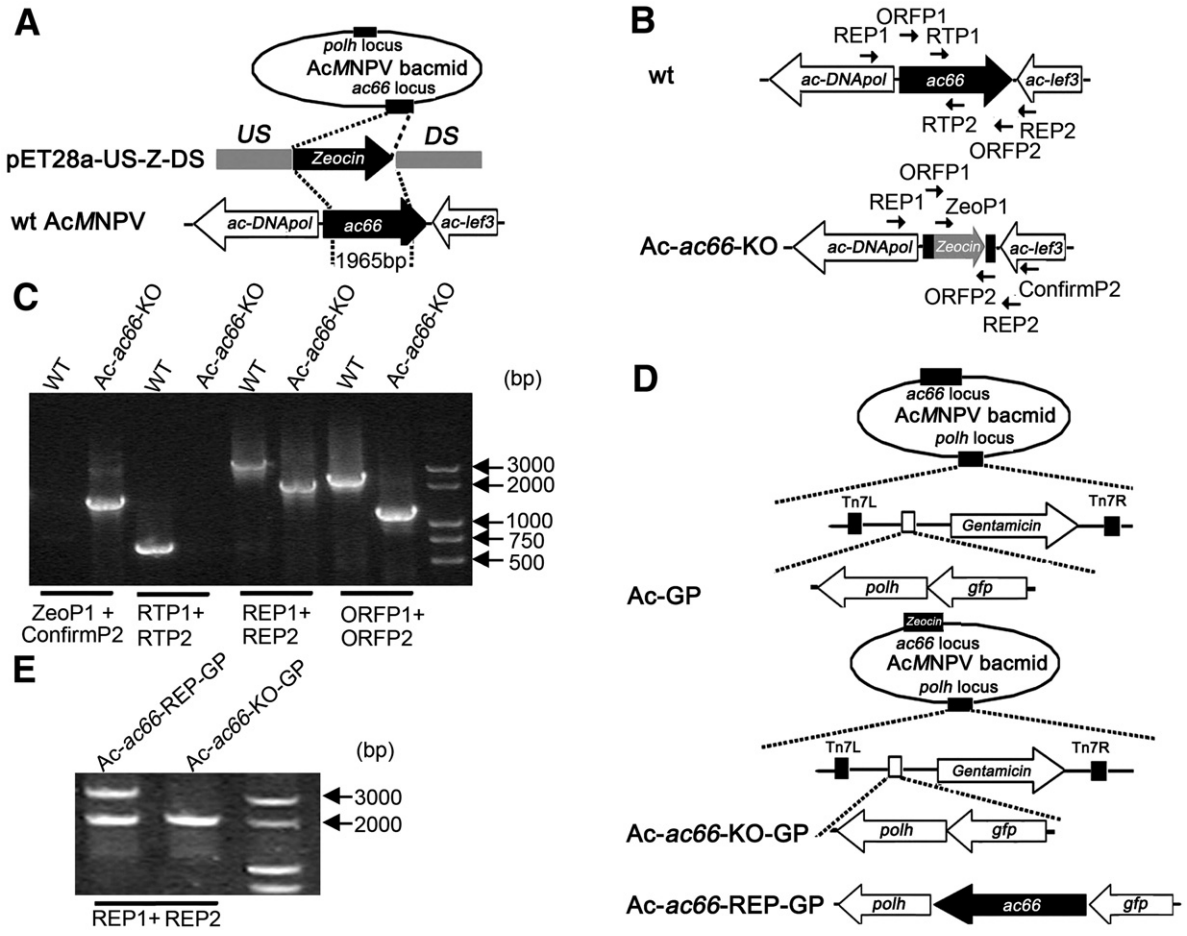


Fig. 2. Construction of *ac66* knockout, repair, and wt Ac*M*NPV bacmids containing *gfp* and *polyhedrin*. (A) Schematic diagram for construction of an *ac66* knockout bacmid containing a deletion of the *ac66* gene by recombination in *E. coli*. A 1965-bp fragment of the *ac66* orf was deleted and replaced by the Zeocin-resistant gene under the control of the EM7 promoter. (B) Diagram indicating the positions of primer pairs used in the confirmation of disruption of *ac66* and the correct insertion of Zeocin-resistant gene in *ac66* locus. (C) Confirmation by PCR analysis of the presence or absence of sequence modifications in Ac-*ac66*-KO or wt Ac*M*NPV bacmids. The virus templates are shown above each lane, and the primer pairs used are shown below. (D) Schematic diagram of three viruses, Ac-GP, Ac-*ac66*-KO-GP and Ac-*ac66*-REP-GP, showing the *polyhedrin* and *gfp* genes inserted into the polyhedrin locus by Tn7-mediated transposition. (E) Confirmation by PCR analysis of the presence sequence modifications in Ac-*ac66*-KO and Ac-*ac66*-REP-GP using REP1 and REP2 primers.

was also generated by introducing two marker genes into the polyhedrin locus of the wt bacmid by transposition (Fig. 2D). The successful transposition of *ac66* was confirmed by PCR with primers REP1 and REP2 and the result is shown in Fig. 2E. Primers REP1 and REP2 produced a single fragment of 3305 bp in Ac-*ac66*-KO-GP, but two fragments of 3305 bp and 2132 bp in Ac-*ac66*-REP-GP. Transposition event of *polyhedrin* and *gfp* marker genes was confirmed later by *gfp* expression and occlusion body formation in bacmid DNA-transfected Sf-9 cells as described below.

### *Analysis of knockout, rescue, and wt AcMNPV bacmids replication in transfected Sf-9 cells*

To examine the effect of *ac66* deletion on virus replication, Sf-9 cells were transfected with Ac-GP, Ac-*ac66*-KO-GP, and Ac-*ac66*-REP-GP, respectively. Fluorescence due to the expression of *gfp* in transfected cells was monitored. No difference among the three viruses was observed before 24 h posttranfection (p.t.) (Fig. 3A), all exhibiting comparable transfection efficiencies of approximately 20%. Fluorescence was observed in almost all Ac-GP or Ac-*ac66*-REP-GP-transfected cells by 72 h p.t. (Fig. 3A), indicating the rapid spread of virus among cells. In sharp contrast to that, the number of Ac-*ac66*-KO-GP-transfected cells showed only a slight increase by 72 h p.t. (Fig. 3A), and only approximately 30% cells appeared to fluoresce even by 96 h p.t., indicating very little spread of the virus among cells.

Microscopic analysis showed significant differences between Ac-*ac66*-KO-GP and Ac-GP or Ac-*ac66*-REP-GP-transfected cells. Occlusion bodies appeared in approximately 15 to 20% of the Ac-GP and Ac-*ac66*-REP-GP-transfected cells at 48 h p.t. (data not shown), and appeared in nearly all of the Ac-GP and Ac-*ac66*-REP-GP-transfected cells by 96 h p.t (Fig. 3B). In contrast, however, even by 96 h p.t., only very few (approximately 0.0005%) of Ac-*ac66*-KO-GP-transfected cells appeared occlusion bodies (Fig. 3B), and the cell number did not correspond to that of the cells initially transfected.

To access the effect of *ac66* knockout on BV replication, a virus growth curve analysis was performed. Ac-GP and Ac-*ac66*-

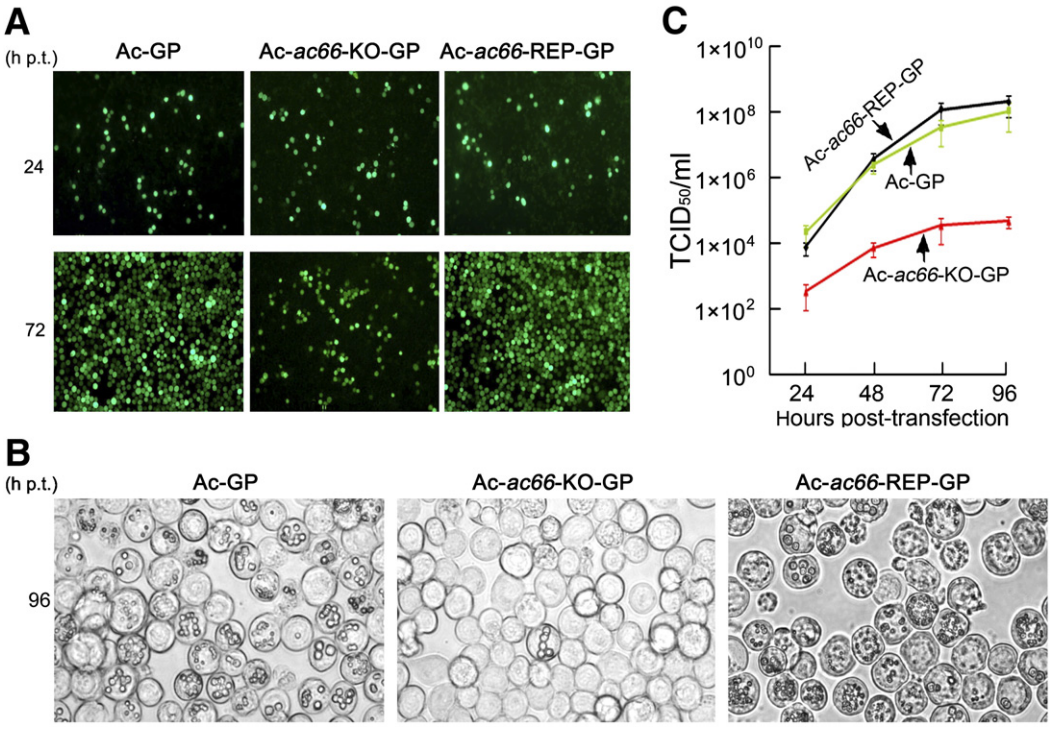


Fig. 3. Analysis of viral replication in virus bacmid DNA-transfected Sf-9 cells. (A) Fluorescence microscopy of Ac-GP, Ac-*ac66*-KO-GP or Ac-*ac66*-REP-GP-transfected Sf-9 cells at 24 and 72 h p.t. (B) Light microscopy of Ac-GP, Ac-*ac66*-KO-GP or Ac-*ac66*-REP-GP-transfected Sf-9 cells at 96 h p.t. (C) Virus growth curve generated from Ac-GP, Ac-*ac66*-KO-GP or Ac-*ac66*-REP-GP-transfected Sf-9 cells. Sf-9 cells were transfected with 2.0 μg of bacmid DNA from each virus. Cells culture supernatants were harvested at the selected time points and assayed for the production of infectious virus by $TCID_{50}$ assay. Each datum point represents the average titer derived from three independent transfections. Error bars represent standard errors.

REP-GP-transfected cells showed a similar increase in virus titers, reaching $2.2\times10^6$ and $3.4\times10^6$ $TCID_{50}$/ml respectively at 48 h p.t., and keeping at least 5000-folds higher even at 96 h p.t. than that of Ac-*ac66*-KO-GP, which showed only $6.8\times10^3$ $TCID_{50}$/ml at 48 h p.t. (Fig. 3C). This result corresponded to the conclusion that Ac-*ac66*-KO-GP caused an over 99% reduction in BV production in comparison to Ac-GP or Ac-*ac66*-REP-GP in bacmid-transfected cells. Thus, these initial results showed that the deletion of *ac66* had a dramatic effect on synthesis of BVs and occlusion bodies.

To verify the initial results obtained by bacmid DNA transfection, the virus was passaged with the BV produced from bacmid-transfected cells using a very low MOI of 0.001. The result showed no difference among the three viruses before 24 h p.i., only a few isolated cells appearing fluorescence (data not shown). Near 100% of the cells infected with Ac-GP or Ac-*ac66*-REP-GP exhibited fluorescence by 84 h p.i. (Fig. 4A), indicating rapid spread of virus among the cells. In contrast, however, a single-cell infection phenotype that persisted up to 84 h p.i. was observed in Ac-*ac66*-KO-GP-infected cells (Fig. 4A), indicating that BV infection was restricted primarily to the cells initially infected.

Microscopic analysis revealed that occlusion bodies were observed in approximately 80% of Ac-GP or Ac-*ac66*-REP-GP-infected cells by 96 h p.i., but in none of Ac-*ac66*-KO-GP infected cells (Fig. 4B), which laced occlusion bodies even by 120 h p.i. when almost 100% of Ac-GP or Ac-*ac66*-REP-GP-infected cells appeared to contain occlusion bodies (data not shown). In addition, a second growth curve analysis revealed a similar proficiency between Ac-GP and Ac-*ac66*-REP-GP in virus production (Fig. 4C), indicating the successful rescue of the Ac-*ac66*-KO-GP defective phenotype by reinsertion of *ac66*, suggesting that the defective phenotype of Ac-*ac66*-KO-GP was directly due to the knockout of *ac66*. BV titers of Ac-GP or Ac-*ac66*-REP-GP rapidly reached peak levels at approximately 96 h p.i. In contrast, BV titers of Ac-*ac66*-KO-GP reached only 81$TCID_{50}$/ml even at 120 h p.i., at least $10^6$ lower than that of Ac-GP or Ac-*ac66*-REP-GP. These results supported the above conclusion that deletion of *ac66* decreased significantly the production of BVs and occlusion bodies.

### *ac66 deletion does not affect viral DNA replication*

To detect whether the knockout of *ac66* affects the viral DNA replication, a real-time PCR analysis was performed to compare the initiation and viral DNA replication level among Ac-GP, Ac-*ac66*-REP-GP, and Ac-*ac66*-KO-GP-transfected cells. The result revealed that the three viruses had the same initiation time of viral replication by 0–12 h p.t., and with a similar increase of DNA levels by 24 h p.t. (Fig. 5), indicating that *ac66* is not essential for the viral DNA replication. After 24 h p.t., great differences were observed between Ac-*ac66*-KO-GP and Ac-GP or Ac-*ac66*-REP-GP bacmid. The DNA levels generated by Ac-*ac66*-REP-GP and Ac-GP bacmid showed a similarly steady increase from 24 to 96 h p.t. (Fig. 5). However, the DNA levels generated by Ac-*ac66*-KO-GP

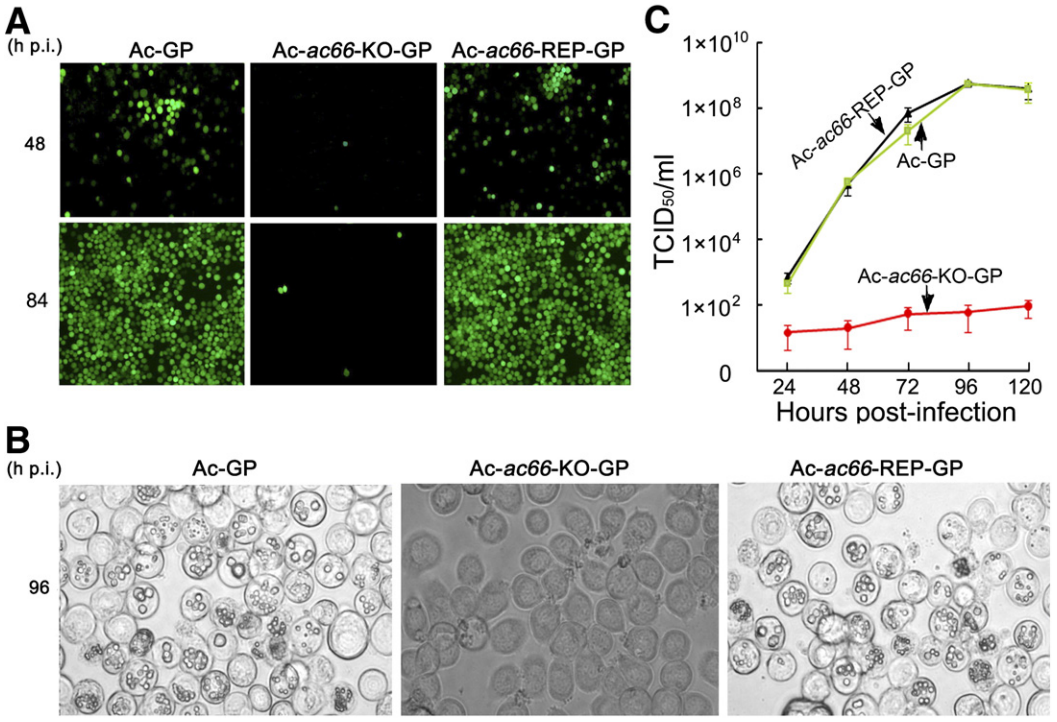


Fig. 4. Analysis of viral replication in virus-infected Sf-9 cells. (A) Fluorescence microscopy of Ac-GP, Ac-*ac66*-KO-GP or Ac-*ac66*-REP-GP-infected Sf-9 cells at 48 and 84 h p.i. (B) Light microscopy of Ac-GP, Ac-*ac66*-KO-GP or Ac-*ac66*-REP-GP-infected Sf-9 cells at 96 h p.i. (C) Virus growth curve generated from Ac-GP, Ac-*ac66*-KO-GP or Ac-*ac66*-REP-GP-infected Sf-9 cells. Sf-9 cells were infected at an MOI of 0.001 from each virus. Cells culture supernatants were harvested at the selected time points and assayed for the production of infectious virus by $TCID_{50}$ assay. Each datum point represents the average titer derived from three independent infections. Error bars represent standard errors.

bacmid showed only a slight increase from 24 to 96 h p.t. (Fig. 5). Based on the equal number of cells collected at designated time points, these data suggested that the increase of DNA levels generated by Ac-*ac66*-REP-GP or Ac-GP was due to the secondary infection, whereas the replication of Ac-*ac66*-KO-GP DNA was restricted primarily to the initially transfected cells. Hence, real-time PCR analysis indicated that *ac66* is not required for viral DNA replication in Sf-9 cells.

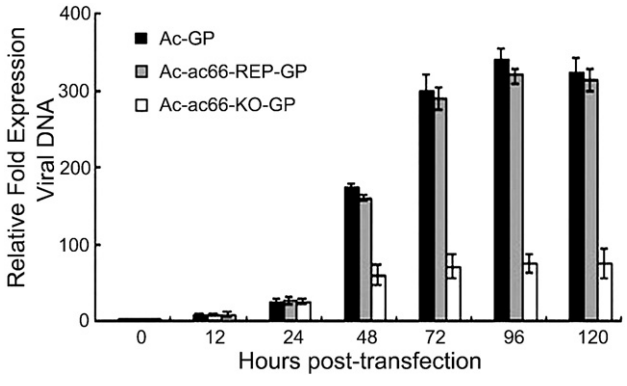


Fig. 5. Real-time PCR analysis of viral DNA replication in transfected Sf-9 cells. Sf-9 cells were transfected in triplicate with 2.0 μg of Ac-GP, Ac-*ac66*-KO-GP or Ac-*ac66*-REP-GP bacmid. At the designated time points, total cellular DNA was isolated from each virus bacmid DNA-transfected Sf-9 cells, digested with the restriction enzyme DpnI to eliminate input bacmid, and analyzed by real-time PCR. The bars heights indicate the average and the error bars represent the standard deviation.

### *ac66 deletion does not affect polyhedrin transcription level*

As described above, *ac66* deletion caused significant reduction of occlusion formation. To rule out the possibility that reduced occlusion body formation was due to the inefficient polyhedrin transcription, polyhedrin transcription levels were analyzed by RT-PCR and compared between Ac-GP and Ac-*ac66*-KO-GP-transfected cells. The result revealed similar polyhedrin transcription levels at 24 h p.t. (Fig. 6), prior to the onset of BV secondary infections. No RT-PCR product was detected in mock-transfected cells or using the same templates as those used in RT-PCR analysis without reverse transcriptase added prior to the PCR step, indicating no possible contamination of Ac*M*NPV DNA in the RT-PCR analysis (data not shown). Based on the equal number of cells collected at designated time points, these data suggested that *ac66* deletion did not affect polyhedrin transcription. The difference in polyhedrin transcription level between the two viruses at 48 h p.t. was due to the rapid spread of Ac-GP virus

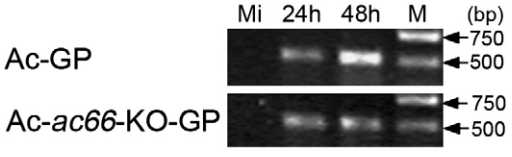


Fig. 6. Transcription analysis of polyhedrin in Ac-GP or Ac-*ac66*-KO-GP-transfected Sf-9 cells. Total RNA was extracted from each bacmid (Ac-GP, Ac-*ac66*-KO-GP)-transfected Sf-9 cells at 24 and 48 h p.t. or mock-transfected (Mi) cells. M: DNA size marker.

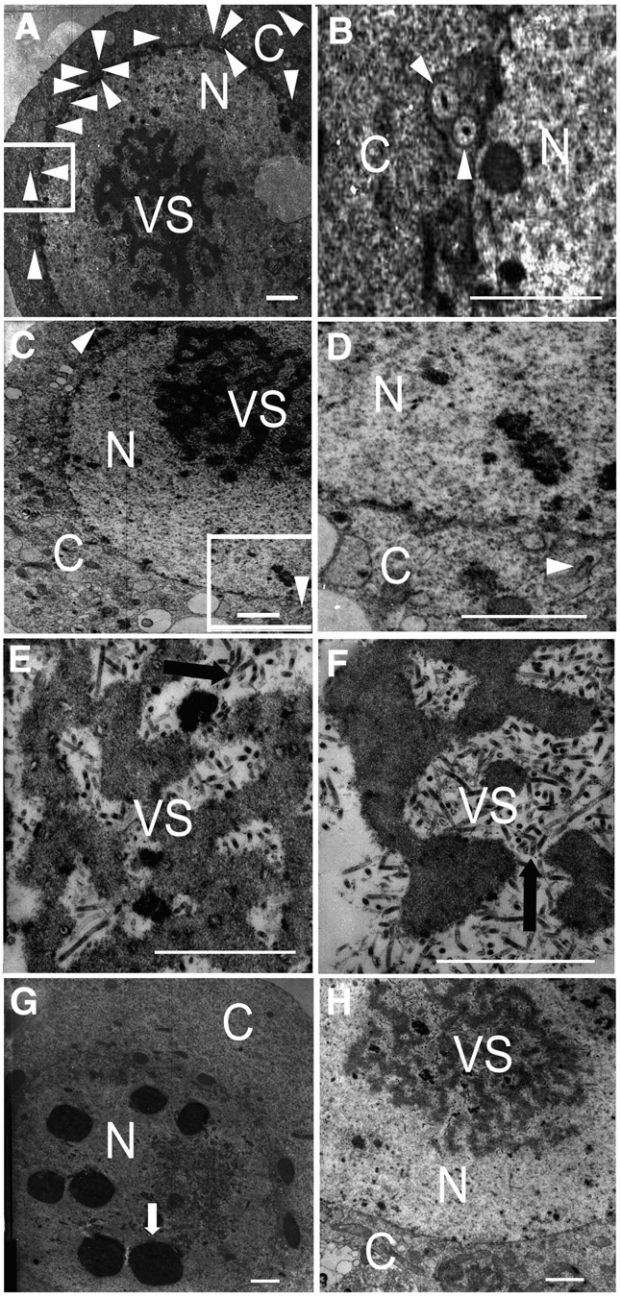


Fig. 7. Electron microscopic analysis of Sf-9 cells transfected with either Ac-GP (A, B, E, G) or Ac-*ac66*-KO-GP (C, D, F, H) at 36 h p.t. (A, B, C, D, E, F) or 96 h p.t. (G, H). B and D are higher magnification of the rectangles in A and C respectively. The nucleus (N), cytoplasm (C), and virogenic stroma (VS) regions are indicated. Nucleocapsids in the cytoplasm or budding at cytoplasmic membrane are indicated with Triangles. Black arrow indicates nucleocapsids in the virogenic stroma. White arrow indicates occlusion within the nucleus. For A, B, C, D, E, F, G, and H, the bar represents 1 μm.

among cells and the restriction of Ac-*ac66*-KO-GP virus primarily to the initially transfected cells (Fig. 6).

### *Electron microscopic analysis of Ac-GP and Ac-ac66-KO-GP-transfected cells*

To further analyze how *ac66* blocks both BV and occlusion body production, the Ac-GP and Ac-*ac66*-KO-GP bacmids-transfected cells were examined by electron microscopy at 36 and 96 h p.t. (Fig. 7).

The result showed that typical virogenic stroma and an extensive numbers of rod-shaped nucleocapsids appeared in the nuclei of both Ac-GP and Ac-*ac66*-KO-GP bacmids-transfected cells. No distinguishable difference of nucleocapsids phenotype, i.e., normal length nucleocapsids with an electron-dense nucleoprotein core, was observed between the two viruses bacmid-transfected cells with even an analysis of 100,000-fold nucleocapsids magnification (data not shown), suggesting that the knockout of *ac66* did not affect the nucleocapsid assembly.

Nucleocapsids were frequently observed in the process of penetrating the nuclear membrane, within the cytoplasm, or budding from the cytoplasmic membrane in all Ac-GP-transfected cells examined, but very few were observed in examined cells transfected with *ac66* knockout bacmid. On the other hand, more nucleocapsids from Ac-*ac66*-KO-GP-transfected cells were observed to be associated with virogenic stroma (VS) regions than those from Ac-GP-transfected cells, suggesting *ac66* may be required for nucleocapsids to be released from the virogenic stroma.

To study this observation in a quantitative manner, twenty Sf-9 cells transfected with Ac-GP or Ac-*ac66*-KO-GP bacmids were randomly chosen for electron microscopy analysis and the total numbers of nucleocapsids in the nucleus, exiting the nuclear membrane, in the cytoplasm or budding from the cytoplasmic membrane were summarized (Table 1). The result showed a total of 6556 and 480 nucleocapsids within the nucleus and in the pathway of budded virus synthesis (inner nuclear membrane, the cytoplasm, and the cytoplasmic membrane) respectively in the 20 Ac-GP-transfected cells, compared to a total of 6757 nucleocapsids within the nucleus, and only 21 nucleocapsids in the pathway of BV synthesis in Ac-*ac66*-KO-GP-transfected cells, implying that *ac66* it is not required for the production or assembly of nucleocapsids but for the efficient egress of nucleocapsids from the nucleus to cytoplasm.

Normal occlusion bodies and plenty of POVs were present in the nuclei of Ac-GP-transfected cells at 96 h p.t., but no occlusion bodies and very few POV were observed in Ac-*ac66*-KO-GP-transfected cells examined. On the other hand, a total of 6676 nucleocapsids were still observed within the nuclei of the 20 Ac-*ac66*-KO-GP-transfected cells by 96 h p.t., similar to that at 36 h p.t., suggesting the requirement of *ac66* for the intranuclear envelopment of nucleocapsids to form POV.

Table 1
Summary of numbers of the nucleocapsids in the nucleus and in the BV pathway from 20 Sf-9 Ac-GP or Ac-*ac66*-KO-GP-transfected cells at 36 h p.t.

| Virus | Nucleus | Nuclear membrane | Cytoplasm | Cytoplasmic membrane | Total[a] budding |
|---|---|---|---|---|---|
| Ac-GP | 6556 | 4 | 361 | 115 | 480 |
| Ac-*ac66*-KO-GP | 6757 | 0 | 18 | 3 | 21 |

[a] Total budding refers to the nucleocapsids in the pathway of budded virus synthesis including those associated with or exiting the nuclear membrane, within the cytoplasm and budding from the cytoplasmic membrane.

## Discussion

In this paper, the role of Ac*M*NPV *ac66* was investigated by generating an *ac66* knockout bacmid and the results show that *ac66* is required for the efficient egress of nucleocapsids from the nucleus to cytoplasm and then the efficient production of BV, as well as for the general POV and occlusion synthesis.

BV production was shown to be almost completely blocked by deletion of *ac66* from Ac*M*NPV bacmid. The mutant virus (Ac-*ac66*-KO-GP) was primarily restricted to the initially bacmid-transfected cells. This phenotype was further confirmed by passage assay, virus growth curve, and viral DNA replication analyses. Rescue of the wild-type phenotype by reinsertion of *ac66* into the polyhedrin locus of the mutant bacmid confirmed that the observed phenotype was directly due to the deletion of *ac66* from Ac*M*NPV bacmid DNA. Therefore, *ac66* is essential for high level BV production, though it is not strictly essential for BV synthesis. This result is similar to that in other studies (Milks et al., 2003; Dai et al., 2004; Ge et al., 2007).

Examination of *ac66* knockout virus-transfected cells by electron microscopy revealed that the inefficient BV production was due to inefficient egress of nucleocapsids from the nucleus to cytoplasm, which may be further due to inefficient release of nucleocapsids from virogenic stroma, as evidenced by more normal nucleocapsids associating with the virogenic stroma in *ac66*-null mutant transfected cells in comparison to that in wild-type virus-transfected cells and only sporadic both single and bundled *ac66* knockout virus nucleocapsids appearing between the perimeter of the virogenic stroma and the nuclear membrane. Thus, this study suggests that *ac66* may play a key role in nucleocapsids release from virogenic stroma and their subsequent transport for BV production.

To date, a number of genes have been shown to involve in the process of nucleocapsids formation and trafficking. For example the Ac*M*NPV P78/83 and VP1054 (Olszewski and Miller, 1997b; Possee et al., 1991), which are nucleocapsid proteins of both budded virus and occlusion-derived virion have been demonstrated as essential for nucleocapsids assembly. The Ac*M*NPV 38K, localized to the virogenic stroma of nucleus, has been shown to be required for the assembly of normal nucleocapsids interrupting both BV and ODV formation but has not shown to be a nucleocapsids protein (Wu et al., 2006). VLF-1 of Ac*M*NPV is both a putative tyrosine recombinase and a component of nucleocapsid (Yang and Miller, 1998; Vanarsdall et al., 2004). Immunoelectron microscopic analysis revealed that the VLF-1 knockout virus was defective in producing mature capsids in Sf-9 cells (Vanarsdall et al., 2006). Interestingly, rescuing the VLF-1 knockout bacmid construct with a copy of VLF-1 that carries a mutation of a highly conserved tyrosine (Y355F) was sufficient to restore the production of nucleocapsids with a normal appearance, whereas abundant nucleocapsids particles within the virogenic stroma and no nucleocapsids could be seen dispersed between the perimeter of the virogenic stroma and the nuclear membrane, suggesting VLF-1 may play another role in mediating nucleocapsids to be released from the virogenic stroma (Vanarsdall et al., 2006). Similar mutant phenotype was observed in present study. Perhaps there is a functional interaction between Ac66 and VLF-1 for facilitating nucleocapsids to be released from the virogenic stroma and further study addressing this possibility will lead to a better understanding on how nucleocapsids are released from the virogenic stroma. Noticeably, it is possible that indistinguishable malformation phenotype of *ac66* knockout virus nucleocapsids would have not been transported, and perhaps this would change the overall conclusion about the function of *ac66*.

Thus far no mechanism by which nucleocapsids enter the cytoplasm from the nucleus is proposed. It has been shown that nucleocapsids may exit the nucleus through nuclear pores, the nuclear membrane or migration into endoplasmic reticulum (Hess and Falcon, 1978; Fraser and Hink, 1982; Williams and Faulkner, 1997). Exiting through the nuclear membrane is the most common method of egress observed in electron microscopic studies, by which nucleocapsids acquire a double membrane vesicle derived from the nuclear membrane (Williams and Faulkner, 1997). Two other Ac*M*NPV viral proteins, GP41 and EXON0, have been shown to be essential for the egress of nucleocapsids from the nucleus. GP41 is expressed at the late and very late phases of viral infection cycle, and is an *O*-glycosylated and ODV-specific proteins (Whitford and Faulkner, 1992). A GP41 temperature-sensitive mutant failed to produce BV when infected cells at the nonpermissive temperature of 33 °C (Olszewski and Miller, 1997a). Electron microscopy showed that GP41 was not required for the nucleocapsid production but for the egress of nucleocapsids from the nucleus to cytoplasm (Olszewski and Miller, 1997a). EXON0, transcribed as a late gene and including a Ring finger motif as well as a predicted leucine-rich coiled-coil domain, has been shown to be required for the efficient production of BV in a previous report (Dai et al., 2004). In a recent report, fractionation of BV into nucleocapsid and envelope components revealed that EXON0 was the twelfth identified nucleocapsid protein (Fang et al., 2007), along with P78/83 (Russell et al., 1997), VP1054 (Olszewski and Miller, 1997b), FP25 (Braunagel et al., 1999), VLF-1 (Yang and Miller, 1998), VP39 (Thiem and Miller, 1989), BV/ODV-C42 (Braunagel et al., 2001), P87 (Lu and Carstens, 1992) , P24 (Wolgamot et al., 1993), Ac101 (Vanarsdall et al., 2007), Ac142 (Vanarsdall et al., 2007), and Ac144 (Vanarsdall et al., 2007), which have been found to associate with both Ac*M*NPV BV and ODV. In the nucleocapsids cellular localization study, EXON0 was shown to be localized to both the cytoplasm and nuclei of infected Sf-9 cells throughout the infection. Electron microscopy revealed that EXON0 was not required for the production of equal numbers and normal appearing nucleocapsids within the nucleus but for the efficient egress of nucleocapsids from the nucleus to the cytoplasm in the pathway of BV synthesis (Fang et al., 2007). Therefore, Ac66 is the protein identified along with GP41 and EXON0, which are not required for the nucleocapsids assembly, but for the egress of Ac*M*NPV nucleocapsids from nucleus to cytoplasm. It has been shown that egress is a complex procedure that involves many viral and cellular proteins for some large DNA animal viruses, e.g., herpesvirus (Fuchs et al., 2002; Reynolds et al., 2002). Thus, there could be a functional interaction among these three proteins, perhaps together with other proteins such as VLF-1, facilitating

the movement of Ac*M*NPV nucleocapsids through the nucleus. Further studies addressing this possibility will lead to a better understanding on how BV nucleocapsids enter the cytoplasm from the nucleus.

Normally in the late stage of Ac*M*NPV infection, BV production is reduced in favor of intranuclear envelopment of nucleocapsids for POV synthesis in the peristromal ring zone of virogenic stroma by an undefined mechanism, and POV is subsequently embedded into polyhedrin condensation to form ODV (Williams and Faulkner, 1997), but the mechanism of the phenotype switch from BV to POV production is still unknown.

Results in this study demonstrated that the *ac66* knockout virus could not progress through the very late phase in Sf-9 cells, as evidenced by the greatly reduced POV and occlusion production in the nuclei of transfected cells, suggesting that *ac66* is also involved in POV and occlusion synthesis, or associated with phenotype switch from BV to ODV. A possible explanation for this mutant phenotype may be ascribed to the fact that the unable egress of nucleocapsids from virogenic stroma can lead to failure in subsequent POV and occlusion synthesis.

Among the forty-one baculovirus genomes that have been sequenced to date, thirty-seven lepidopteran baculoviruses contain the putative Desmoplakin N-terminus motif, including all type I and type II NPV and GV. The only four exceptions are three Neodiprion NPVs (N. abietis NPV, N. lecontei NPV, and N. sertifer NPV) and Culex NPV, which infect hymenopterans and dipteran, respectively. Although the function of this motif in baculoviruses is still unknown, the fact that it is highly conserved in baculovirus genomes indicates its importance. Therefore, it is of particular interest in future studies to know if Desmoplakin N-terminus is associated with the efficient egress of nucleocapsids to the plasma membrane for BV formation as well as POV and occlusion synthesis.

While Ac66 has been shown to be a structural protein of ODV, it is not yet clear if it is also a component of BV. Western blot analysis will be required to address this possibility. In addition, further study in which cellular localization analysis is required will help to shed light on the role of *ac66* in viral infection cycle. Therefore, although the exact role of Ac66 in the processes of nucleocapsids egress from nucleus as well as POV and occlusion synthesis is still unclear, the present study will lead to a better understanding of the molecular aspects of this protein and the molecular basis for factors governing these processes.

## Materials and methods

### *Cells and viruses*

The insect *S. frugiperda* cells line Sf-9 were cultured at 27 °C in Grace's medium (Invitrogen Life Technologies) supplemented with 10% fetal bovine serum, penicillin (100 μg/ml) and streptomycin (30 μg/ml). Bacmid bMON14272 (Invitrogen Life Technologies), containing the Ac*M*NPV genome, can propagate in *E. coli* strain DH10B as described previously (Luckow et al., 1993).

### *Transcriptional analysis of ac66*

Total RNA was isolated from $4\times10^6$ Sf-9 cells infected with Ac*M*NPV virus at MOI of 10 at 0, 0.5, 3, 6, 12, 24, 48, 72 h p.i. or mock using RNAiso Reagent (TaKaLa) according to manufacturer's instructions. After the RNA samples were treated with RNase-Free DNase (Promega), the first strand of cDNA was synthesized with AMV Reverse transcriptase (Promega). With the *ac66*-specific primers RTP1: 5′ *GCACGTAGTAAACAACGTGACC* 3′ and RTP2: 5′ *CTCGTTCAACTGGCGATTCATG* 3′ or the *VP39*-specific primers *VP39*RTP1: 5′ *TAAGCGTTCTGTCCAGCTCAC* 3′ and *VP39*RTP2: 5′ *CAAAATGACGATCGCTAGGC* 3′, PCRs were then carried out using 32 cycles of 94 °C for 60 s, 55 °C for 40 s, and 72 °C for 60 s.

### *Construction of ac66 knockout AcMNPV bacmid*

First, we constructed a transfer vector in which *ac66* was replaced by *Zeocin* for antibiotic selection in *E. coli.* as described below. A 792-bp fragment containing Zeocin-resistant gene under the control of the EM7 promoter was PCR amplified from pPICZaA (Invitrogen Life Technologies) with primers ZeoP1: 5′ *GACAAGCTTAATCTAAGGGGCGGTGT* 3′ (HindIII site was underlined) and ZeoP2: 5′ *CATGGATCCTGCTGCTCACATGTTGGTCT* 3′ (BamHI site was underlined). The PCR product was digested with BamHI/HindIII and then ligated into vector pET28a, which was digested with BamHI/HindIII to generate the recombinant plasmid pET28a-Z. With primers *ac66*U1: 5′ *TATGCGGCCGCCACAAACGTGGTGTAGCTGATG* 3′ (NotI site was underlined) and *ac66*U2: 5′ *GTCAAGCTTGATGTTGTGCGTCAACGTGTTG* 3′ (HindIII site was underlined), a 571-bp fragment homologous to the upstream sequence of *ac66* was PCR amplified from Ac*M*NPV genome. The resulting product was digested with HindIII/NotI and then cloned into vector pET28a-Z, which was also digested with HindIII/NotI to generate the recombinant plasmid pET28a-US-Z. With primers *ac66*D1: 5′ *CATGGATCCGAGATGGAGTCTATTGCAGATCAGG* 3′ (BamHI site was underlined) and *ac66*D2: 5′ *GACTCTAGATCTACGCGCATCGAATTGTGC* 3′ (XbaI site was underlined), a 683-bp fragment homologous to the downstream sequence of *ac66* was PCR amplified from Ac*M*NPV genome. The resulting product was digested with BamHI/XbaI and then cloned into vector pET28a-US-Z, which was also digested with BamHI/XbaI to generate the recombinant plasmid pET28a-US-Z-DS. The pET28a-US-Z-DS was digested with NotI/XbaI. The resulting linear 2046-bp fragment containing *Zeocin* and *ac66* flank regions was gel purified and suspended in distilled water.

Next, an Ac*M*NPV *ac66* knockout bacmid was generated by using a modification of the λphage Red recombinase system as described below. Electro-competent DH10B cells harboring bMON14272 were first transformed with pBAD-gbaA which supplied λ Red recombination function (Muyrers et al., 1999). The resulting clone cells were then induced by the addition of L-arabinose to allow expression of the λ Red system, made competent and electro-transformed with 1 μg of the purified linear 2046-bp fragment as previously described

(Pijlman et al., 2002). The electroporated cells were incubated at 37 °C for 1 h in 1 ml SOC, 200 μ1 was subsequently spreaded onto low salt LB agar containing 25 μg/ml Zeocin, 50 μg/ml Kanamycin. Plates were incubated at 37 °C for 24 h, and colonies resistance to Zeocin and Kanamycin were selected and verified by PCR analysis. The resulting Ac*M*NPV *ac66* knockout bacmid was named Ac-*ac66*-KO. The deletion of *ac66* from the Ac*M*NPV bacmid genome was confirmed by PCR with four primer pairs. Primers RTP1 and RTP2 were used to confirm the partial deletion within *ac66* coding region. Primers ZeoP1 and ConfirmP2: 5′ *CGTGGTTCAAGTGGAATGTTCTGTC* 3′ were used to confirm the correct junction between Zeocin and downstream. Primers ORFP1: 5′ *CATGGATCCATGCAGCGATGGCCCAAATATG* 3′ (BamHI site was underlined) and ORFP2: 5′ *GACAAGCTTCTATTCGACGTTTGGTTGAACGCTGG* 3′ (HindIII site was underlined) were located to the orf of *ac66*. Primers REP1: 5′ *ACGTCTAGACGTCGATCCACCGACATAGTTA* 3′ (XbaI site was underlined) and REP2: 5′ *TTACTCGAGGTCGATCGAACAAACACGTTG* 3′ (XhoI site was underlined) were used to detect the correct junction between the upstream and downstream.

### *Construction of Knockout, repair and wt AcMNPV bacmids with polyhedrin and gfp*

To facilitate the detection of virus infection and examine if the *ac66* knockout has any effect on occlusion morphogenesis, two marker genes *polyhedrin* and *gfp* were introduced into *ac66* knockout, repair and wt bacmids. With primers REP1 and REP2, a 3323-bp XbaI/XhoI fragment containing the wt *ac66* gene with its own promoter and poly (A) tail was PCR amplified. The resulting product was digested with XbaI/XhoI and cloned into XbaI/XhoI digested pFB-ieGP (Li et al., 2005) to generate pFB-ieGP-*ac66*. The electro-competent DH10B cells harboring wt bacmid and the helper plasmid pMON7124 were transformed with pFB-ieGP to generate the wt control bacmid (Ac-GP). The electro-competent DH10B cells harboring Ac-*ac66*-KO bacmid and the helper plasmid pMON7124 were transformed with pFB-ieGP and pFB-ieGP-*ac66* to generate the *ac66* knockout bacmid (Ac-*ac66*-KO-GP) and the *ac66* repair bacmid (Ac-*ac66*-REP-GP), respectively. The successful transposition of *ac66* was confirmed by PCR with primers REP1 and REP2. The correct recombinant bacmid was isolated and electroporated back into DH10B cell, and colonies were screened for sensitivity to tetracycline to ensure that the isolated bacmid was free of the helper plasmid.

### *Virus growth curve*

For the analysis of virus growth curve, $2\times10^6$ Sf-9 cells were transfected in triplicate with 2.0 μg of each bacmid DNA (Ac-GP, Ac-*ac66*-KO-GP, Ac-*ac66*-REP-GP) using Cellfectin liposome reagent (Invitrogen Life Technologies) or infected in triplicate with BV at an MOI of 0.001. The cell monolayer was incubated for 4 h after transfection or 1 h after infection, washed 3 times by Grace's medium, subsequently replenished with 2 ml Grace's medium containing 10% fetal bovine serum, penicillin (100 μg/ml), streptomycin (30 μg/ml), and then incubated at 27 °C. Virus supernatant was collected at various times post-transfection or postinfection. BV production was determined by $TCID_{50}$ end point dilution assay in Sf-9 cells (O'Reilly et al., 1992).

### *DNA replication analysis by real-time PCR*

To assess viral DNA replication in transfected cells, $2\times10^6$ Sf-9 cells were transfected in triplicate with 2.0 μg of each bacmid DNA (Ac-GP, Ac-*ac66*-KO-GP and Ac-*ac66*-REP-GP). At designated time points, cells were harvested and each cell pellet was washed twice with PBS. Total DNA was then isolated from transfected Sf-9 cells at 0, 12, 24, 48, 72, 96, and 120 h p.t. using Universal Genomic DNA Extraction kit (TaKaRa) according to manufacturer's instructions. The total DNA was diluted to a total volume of 150 μl with sterile water. As described previously (Vanarsdall et al., 2005), with modification, 10 μl of total DNA from each time point were digested using 10 U of DpnI restriction enzyme (NEB) overnight in 50 μl total to eliminate input bacmid prior to PCR. With primers described previously (Vanarsdall et al., 2005), the real-time PCR reaction was performed using 12.5 μl of the digested DNA added to iQ™ SYBR® Green Supermix (Bio-rad) according to manufacturer's instructions in the iQ™5 machine (Bio-rad) and using following conditions: denaturation at 95 °C for 5 min, followed by 45 cycles of 95 °C for 30 s, 60 °C for 20 s and 72 °C for 20 s. Melting curve analysis was performed at the end of each PCR assay for specificity control. A standard curve was created with a twofold sample of purified Ac-GP DNA templates at 96 h p.t. used in the quantitative PCR. Five dilutions (each1:10) were prepared to cover the workable concentrations of the DNA templates.

### *Transcription analysis of polyhedrin*

For transcription analysis of polyhedrin, $2\times10^6$ Sf-9 cells were transfected with mock or 2.0 μg of each bacmid DNA (Ac-GP and Ac-*ac66*-KO-GP). As described above, total RNA was isolated from bacmid-transfected cells at 24, 48 h p.t. or mock-transfected cells. After the RNA samples were treated with RNase-Free DNase (Promega), the first strand of cDNA was synthesized. PCR was then carried out with the *polyhedrin*-specific primers PolhP1: 5′ *GTTATCAAGAACGCTAAGCGC* 3′ and PolhP2: 5′ *CCAGATGACACGATCGATGA* 3′ using 30 cycles of 94 °C for 60 s, 60 °C for 46 s, and 72 °C for 30 s.

### *Electron microscopy*

$2\times10^6$ Sf-9 cells were transfected with 2.0 μg of each bacmid DNA (Ac-GP and Ac-*ac66*-KO-GP). At 36 and 96 h p.t., cells were dislodged and pelleted at 1000 g for 5 min. Cells were fixed, dehydrated, embedded, sectioned, and stained as described previously (Li et al., 2005). Samples were observed under a JEM-100CXII transmission electron microscope operation at 80 kV.

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