Characterization of AcMNPV with a deletion of *ac69* gene

Jianhao Ke,^{1,2} Jinwen Wang,¹
Riqiang Deng,¹ Lin Lin,^{1,3} Bei Jinlong,^{1,2}
Liao Yaoping,² Xunzhang Wang¹

¹State Key Laboratory of Biocontrol, School of Life Sciences, Sun Yat-sen University, Guangzhou; ²Rice Research Institute, Guangdong Academy of Agricultural Sciences, Guangzhou; ³Shenzhen Academy of Metrology and Quality Inspection, Shenzhen, China

Abstract

ORF69 (Ac69) of *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) is conserved in some baculovirus genomes. Although it has been shown that Ac69 has cap 0-dependent methyltransferase activity and is not required for budded virus production in *Spodoptera frugiperda* Sf-9 cells, its role in occlusion-derived virus synthesis and virus oral infectivity is not known. This paper describes generation of an *ac69* knockout AcMNPV bacmid mutant and analyses of the influence of *ac69* deletion on the viral infectivity in Sf-9 cells and *Trichoplusia ni* larvae so as to investigate the role of *ac69* in the viral life cycle. Results indicated that *ac69* deletion has little effect on the production rates and morphogenesis of budded virus and occlusion-derived virus in Sf-9 cells. In addition, animal experiment revealed that the deletion mutant did not affect AcMNPV infectivity for *Trichoplusia ni* larvae in LD₅₀ and LT₅₀ bioassay when administered orally. These results suggest that *ac69* may be dispensable for viral infectivity both *in vitro* and *in vivo*.

Introduction

Autographa californica multiple nucleopolyhedrovirus (AcMNPV) is the most intensively studied member of the *Baculoviridae* family, which encompasses a group of arthropod-specific DNA viruses with a circular covalently closed genome of 80 to 180 kb.¹⁻³ During baculovirus infection cycle, gene transcription is characteristic of 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 or from other cis-acting regulatory elements, such as the initiator element CAGT motif.⁴⁻⁶ By the virus-encoded RNA polymerase, the late and very late gene tran-

scription is 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.⁷

Two viral forms, budded virus (BV) and occlusion-derived virus (ODV), are produced during the viral infection cycle.⁸ ODV is responsible for horizontal transmission between insect hosts, whereas BV is responsible for systemic spread through the insect host and propagation in tissue culture.⁸ Based on the nature of the viral genome, the genes encoded by baculovirus can be divided into two broad groups, essential genes and auxiliary genes.⁹ The former is essential for viral replication, while the latter is not required for viral propagation and just provide virus with some selective advantage in nature.

orf69 (*ac69*) gene of AcMNPV encodes a protein of amino acids with a molecular mass of 30.355kDa. Among the forty-four baculoviruses that have been sequenced to date, thirty-seven nucleopolyhedroviruses (NPVs) but no granulovirus (GVs) have an Ac69 homolog. The Ac69 protein is highly conserved in AcMNPV, *Rachiplusia ou* NPV, *Bombyx mori* NPV and *Maruca vitrata* NPV. The amino acid identities of Ac69 among these four NPVs are 87-100%, while the amino acid identities between Ac69 and its homologs of other NPVs are 33-62%.

InterProScan program shows that Ac69 homologs constitute a protein family called FtsJ-like methyltransferase family (IPR009615). This family consists of FtsJ from various bacterial and archaeal sources. FtsJ is a methyltransferase, but actually has no effect on cell division. FtsJ's substrate is the 23S rRNA. The 1.5Å crystal structure of FtsJ in complex with its cofactor S-adenosylmethionine revealed that FtsJ has a methyltransferase fold. This family also includes the N terminus of flaviviral NS5 protein. It has been hypothesised that the N-terminal domain of NS5 is a methyltransferase involved in viral RNA capping.

Previous report has shown that *ac69* can stimulate late gene expression in a transient-expression assay system.¹⁰ Wu and coworkers showed that Ac69 which was synthesized during the late phase of infection had cap 0-dependent methyltransferase activity.¹¹ Characterization of a virus lacking *ac69* indicated that it was infectious when infected into Sf-9 cells and single-step viral growth curves revealed that *ac69* deletion did not affect budded virus production in tissue culture. However, the function of Ac69 in ODV formation and virus oral infectivity has not been determined.

Therefore, to continue the functional investigation of Ac69, we generated an *ac69* knockout AcMNPV virus to examine the role of this gene using the AcMNPV bacmid system. The results described herein demonstrate that *ac69* deletion mutant had no striking pheno-

Correspondence: Xunzhang Wang, State Key Laboratory of Biocontrol, School of Life Sciences, Sun Yat-sen University, 510275 Guangzhou, China.

Fax +86.20.84113964 - Tel: +86.20.84113964.

E-mail: wxz@mail.sysu.edu.cn

Key words: Baculovirus, *orf69*, AcMNPV, Auxiliary gene.

Acknowledgments: we thank Dr. Yi Luo for providing useful suggestion during the course of the study. This work was funded by the Natural Science Foundation of Guangdong Province under Grant 4203388 and President Foundation of Guangdong Academy of Agricultural Sciences under Grant 20090102.

Received for publication: 20 June 2011.

Revision received: 2 July 2011.

Accepted for publication: 7 July 2011.

This work is licensed under a Creative Commons Attribution NonCommercial 3.0 License (CC BY-NC 3.0).

©Copyright J. Ke et al., 2011
Licensee PAGEPress, Italy
Microbiology Research 2011; 3:e4
doi:10.4081/mr.2011.e4

type in *Spodoptera frugiperda* Sf-9 cells, including BV and ODV production, cytological changes and viral morphogenesis, but also in *Trichoplusia ni* (*T. ni*) larvae, including 50% lethal dose (LD₅₀) and 50% lethal time (LT₅₀) bioassays.

Materials and Methods

Cells, viruses and primers

The insect *Spodoptera frugiperda* cells line Sf-9 cells 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 AcMNPV genome, can propagate in *Escherichia coli* strain DH10B as described previously.¹² All sequences of the primers used in this study are shown in Table 1.

Construction of *ac69* knockout AcMNPV bacmid

Strategy for construction of an *ac69*-null bacmid lacking intact *ac69* gene is shown in Figure 1A. With primers ac69U1 and ac69U2, a 579-bp fragment (at AcMNPV genome position 58841-59399) homologous to the upstream sequence of *ac69* was PCR amplified from AcMNPV genome. The resulting product was

digested with *HindIII/NotI* and then cloned into *HindIII/NotI* digested vector pET28a-Z¹³ to generate the recombinant plasmid pET28a-US-Z. With primers *ac69D1* and *ac69D2*, a 727-bp fragment (at AcMNPV genome position 59902-60609) homologous to the downstream sequence of *ac69* was PCR amplified from AcMNPV genome. The resulting product was digested with *BamHI/XbaI* and then cloned into *BamHI/XbaI* digested vector pET28a-US-Z to generate the recombinant plasmid pET28a-US-Z-DS. The pET28a-US-Z-DS was digested with *NotI/XbaI*. The resulting linear 2098-bp fragment containing Zeocin and *ac69* flank regions was gel purified and suspended in distilled water. Next, an AcMNPV *ac69* knockout bacmid was generated by using a modification of the λ phage Red recombinase system as described previously.¹³ The resulting AcMNPV *ac69* knockout bacmid was named Ac-*ac69*-KO.

The deletion of *ac69* from the AcMNPV bacmid genome was confirmed by PCR with two primer pairs. Primers REP1 and REP2 were used to confirm the partial deletion and the insert of Zeocin gene within *ac69* coding region, while Primers ConfirmP1 and ZeoP2 were used to confirm the correct junction between Zeocin and upstream. The relative positions of these two primer pairs in Ac-*ac69*-KO bacmid were illustrated in Figure 1B.

Construction of knockout, repair and wt AcMNPV bacmids with polyhedrin and green fluorescent protein

As described previously,¹³ two marker genes, *polyhedrin* and *gfp*, were introduced into the polyhedrin locus of *ac69* knockout, repair and wt bacmids by Tn7 mediated transposition (Figure 1C). With Primers REP1 and REP2, a 1454-bp *XbaI/XhoI* fragment (at AcMNPV genome position 59112-60547) containing the wt *ac69* 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*¹⁴ to generate pFB-*ieGP-ac69*. 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-*ac69*-KO bacmid and the helper plasmid pMON7124 were transformed with pFB-*ieGP* and pFB-*ieGP-ac69* to generate the *ac69* knockout bacmid (Ac-*ac69*-KO-GP) and the *ac69* repair bacmid (Ac-*ac69*-REP-GP), respectively. Strategy for confirmation of the all constructs by PCR analysis is shown in Figure 1D. Primers ConfirmP1 and ZeoP2 produced a single 1898-bp band in Ac-*ac69*-KO-GP and Ac-*ac69*-REP-GP but no product in Ac-GP. Primers REP1 and REP2 produced single fragments of 1454 bp and 1746 bp for Ac-GP and Ac-*ac69*-KO-

GP, respectively, but both fragments for Ac-*ac69*-REP-GP. Transposition event of *polyhedrin* and *gfp* marker genes was confirmed later by GFP expression and occlusion body formation in virus-infected Sf-9 cells as described below. 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 analysis of virus growth curve, Sf-9 cells (2×10^6 /35-mm-diameter dish) were transfected in triplicate with 2.0 μ g of each bacmid DNA (Ac-GP, Ac-*ac69*-KO-GP and Ac-*ac69*-REP-GP)

using Cellfectin liposome reagent (Invitrogen Life Technologies). Virus supernatant was collected at 96 h post-transfection (p.t.). The BV titers were determined by TCID₅₀ end point dilution assay in Sf-9 cells. 15 Sf-9 cells were subsequently infected in triplicate with each virus at an MOI of 5 and at designated time points the titers were determined by a TCID₅₀ end-point dilution assay in Sf-9 cells.

Electron microscopy analysis

2×10^6 Sf-9 cells were infected with each virus (Ac-GP and Ac-*ac69*-KO-GP) at an MOI of 5. At 24 and 72 h post-infection (p.i.), cells were dislodged and pelleted at 1000 g for 5 min. Cells were fixed, dehydrated, embedded,

Table 1. Primers used in this study.

Primers	Sequences ^a	Restriction enzymes
ZeoP1	5'GACAAGCTTAATCTAAGGGGCGGTGT	<i>HindIII</i>
ZeoP2	5'CATGGATCCTGCTGCTCATGTTGGTCT	<i>BamHI</i>
ac69U1	5'TATGCGGCCGCGCTACTTTCTCCAGACAAA	<i>NotI</i>
ac69U2	G 5'GTCAAGCTTAACGCCAACATCTAGGTCAG	<i>HindIII</i>
ac69D1	5'CATGGATCCTTCTTCTCG GCCTGCCAATTC	<i>BamHI</i>
ac69D2	5'GACTCTAGAGGTTGATTGCAAACTTGGCAGC	<i>XbaI</i>
REP1	5'ACGTCTAGACAACGAGAGAATAAGAGCGG	<i>XbaI</i>
REP2	3'5'TTACTCGAGCGAAGGACAATGACCATCAATCG	<i>XhoI</i>
ConfirmP1	5'CACCTCATTGATGTCGTCAGA	-

^aThe restriction sites are underlined.

Figure 1. Construction of *ac69* knockout, repair, and wt AcMNPV bacmids containing *gfp* and *polyhedrin*. (A) Schematic diagram for construction of an *ac69* knockout bacmid containing a deletion of the *ac69* gene by recombination in *E. coli*. A 503-bp fragment of the *ac69* orf was deleted and replaced by the Zeocin resistance gene under the control of the EM7 promoter. (B) Diagram indicating the positions of primer pairs used in the confirmation of disruption of *ac69* and the correct insertion of Zeocin resistant gene in *ac69* locus. (C) Schematic diagram of three viruses, Ac-GP, Ac-*ac69*-KO-GP and Ac-*ac69*-REP-GP, showing the polyhedrin and *gfp* genes inserted into the polyhedrin locus by Tn7-mediated transposition. (D) Confirmation by PCR analysis of the presence or absence of sequence modifications among each bacmid (Ac-GP, Ac-*ac69*-KO-GP and Ac-*ac69*-REP-GP). The virus templates are shown above each lane, and the primer pairs used are shown below.

sectioned, and stained as described previously.¹⁴ Samples were observed under a JEM-100CXII transmission electron microscope operation at 80 kilovolts.

Bioassays

Bioassays were performed to evaluate whether *ac69* has any effect on the infectivity of AcMNPV. 2×10^6 Sf-9 cells were infected in triplicate with each virus (Ac-GP and Ac-*ac69*-KO-GP) at an MOI of 5. Polyhedrin inclusion bodies (PIBs) were harvested at 120 h p.i. and then purified by centrifugation and resuspended in double-distilled water as described previously.¹⁵ Newly molted third-instar *T. ni* larvae were starved for 6 h and then were individually reared in 12-well plates with artificial diet containing each dosage (0, 25, 50, 100 and 200 PIBs per larvae, respectively) for the 50% lethal does (LD_{50}) analysis or containing each dosage (0 and 200 PIBs per larvae, respectively) for the 50% lethal time (LT_{50}) analysis. Only the larvae that completely ingested the diet with virus within 12 h were further reared. Thirty-six larvae per dosage were used in the infection experiments and the experiments were repeated for three times. For LT_{50} analysis, mortality was recorded every 12h after infection until larvae died or pupated. The LD_{50} and LT_{50} values were determined using the probit analysis.

Results

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

To examine the effect of *ac69* deletion on viral propagation and occlusion synthesis, Sf-9 cells were infected with each virus (Ac-GP, Ac-*ac69*-KO-GP and Ac-*ac69*-REP-GP) at an MOI of 5. Fluorescence due to the expression of *gfp* in infected cells was monitored (Figure 2A). No difference among the three viruses was observed by 12 h p.i. Fluorescence was observed in almost all Ac-GP, Ac-*ac69*-KO-GP or Ac-*ac69*-REP-GP-infected cells by 48 h p.i., indicating all these viruses were able to produce infectious budded virus.

Microscopic analysis revealed that occlusion bodies were observed in approximately 40% of Ac-GP, Ac-*ac69*-KO-GP or Ac-*ac69*-REP-GP-infected cells by 36 h p.i., and appeared in nearly all of Ac-GP, Ac-*ac69*-KO-GP and Ac-*ac69*-REP-GP-infected cells by 72 h p.i. (Figure 2B), implying that *ac69* deletion did not affect occlusion body synthesis.

To access the effect of *ac69* knockout on BV replication, a virus growth curve analysis was performed. The results revealed that Sf-9 cells

infected with Ac-GP, Ac-*ac69*-KO-GP or Ac-*ac69*-REP-GP showed steady increase in virus production and the slope of the growth curves of the three viruses had no significant difference (Fig. 2C), indicating *ac69* deletion had little effect on the efficiency of BV synthesis in Sf-9 cells.

Electron microscopy analysis of *ac69*wt, knockout and mock-infected Sf-9 cells

To further analyze whether *ac69* deletion has any effect on virus morphogenesis, Sf-9 cells infected with Ac-GP or Ac-*ac69*-KO-GP were examined by electron microscope. Results revealed that the nuclei of cells infected with either Ac-GP or Ac-*ac69*-KO-GP exhibited the typical baculovirus infection symptoms, i.e., enlarged nuclei, a typically reorganized electron-dense virogenic stroma (the putative site of nucleocapsids assembly) enriched with nucleocapsids, bundles of nucleocapsids aligning with envelopes and their subsequent envelopment to form ODV, and occlusion bodies containing ODVs appearing

within the ring zone which is a significant and morphologically distinct peristomal compartment of nucleoplasm and yields along with maturation of the virogenic stroma, but no such structure in mock-infected cells. In addition, no distinguishing differences of nucleocapsids and occlusion bodies were observed between Ac-GP and Ac-*ac69*-KO-GP-infected cells. Electron microscopic analysis of Ac-*ac69*-KO-GP-infected Sf-9 cells is shown in Figure 3. These observations indicated that *ac69* deletion did not affect morphogenesis of BVs and ODVs in Sf-9 cells.

Effect of *ac69* deletion of AcMNPV on infectivity for *Trichoplusia ni* larvae

The infectivities of Ac-GP and Ac-*ac69*-KO-GP were determined for newly molted three-instar *T. ni* larvae in 50% lethal dose (LD_{50}) and 50% lethal time (LT_{50}) bioassays when administered orally (Tables 2 and 3). No significant difference in LD_{50} and LT_{50} was observed between the two viruses, suggesting *ac69* is not required for viral infectivity in *T. ni* larvae

Figure 2. Analysis of viral replication in virus-infected Sf-9 cells. (A) Fluorescence microscopy of Ac-GP, Ac-*ac69*-KO-GP or Ac-*ac69*-REP-GP-infected Sf-9 cells at 12 and 48 h p.i. (B) Light microscopy of Ac-GP, Ac-*ac69*-KO-GP or Ac-*ac69*-REP-GP-infected Sf-9 cells at 36 and 72 h p.i. (C) Virus growth curve generated from Ac-GP, Ac-*ac69*-KO-GP or Ac-*ac69*-REP-GP-infected Sf-9 cells. Sf-9 cells were infected at an MOI of 5 from each virus. Cells culture supernatants were harvested at the selected time points and assayed for the production of infectious virus by TCID₅₀ assay. Each datum point represents the average titer derived from three independent infections. Error bars represent standard errors.

Discussion

The AcMNPV *ac69* gene, which encodes a protein that contains an S-adenosyl methionine (AdoMet)-dependent methyltransferase signature motif, was first demonstrated to have cap 0-dependent methyltransferase activity by Wu and coworkers,¹¹ who postulated that it is one of AcMNPV auxiliary genes, which are not essential for virus replication but nonetheless provide it with some selective advantage, based on the existing *ac69* homologs for methylation of viral mRNAs in host and the unaffected budded virus replication level in the *ac69*-null virus-infected Sf-9 cells. While the hypothesis that *ac69* is an auxiliary gene has been partially confirmed in experimental tissue culture systems, it is still not known if *ac69* deletion has any effect on ODV synthesis *in vitro* and virus infectivity *in vivo*. This study, by comprehensive analyses of an *ac69* knockout virus, provides primary evidence that *ac69*, as a cap 0-dependent methyltransferase, is dispensable for ODV production in Sf-9 cells and virus oral infectivity in *T.ni* larvae.

In the present study, we describe the use of ET-mediated recombination technology to delete *ac69* gene from the AcMNPV genome. This allowed us to investigate the role of AcMNPV *ac69* during the AcMNPV virus life cycle. We found that *ac69* gene deletion have little effect on the production rates and morphogenesis of budded virus and occlusion body in Sf-9 cells, as evidenced by similar slope of virus growth curve among *ac69* wt, knockout, and rescue viruses, and subsequent electron microscopy observation, i.e., the normal rod-shaped nucleocapsids associating with electron-dense edges of the virogenic stroma and polyhedra containing ODVs in *ac69* knockout virus-infected cells in comparison with that in wt virus-infected cells. In addition, LD₅₀ and LT₅₀ bioassays performed with *T.ni* larvae revealed no significant difference between the *ac69* knockout virus and wt virus. Together, these results support the above hypothesis that *ac69* is an auxiliary gene and suggest that *ac69* may be dispensable for viral virulence or infectivity both *in vitro* and *in vivo*.

To date, a number of AcMNPV genes have been shown to be auxiliary genes which can be divided into two categories, genes likely to facilitate virus replication at the cellular level and genes likely to function at the organismal level.⁹ For example the AcMNPV *p10* gene, ranged into the former category at the present time, has high expression levels late in the infection process, and has been shown not to be required for viral virulence or infectivity.^{9, 16-18} Although a large number of *p10* mutant viruses have now been generated, its role during viral infection is still unclear. AcMNPV

genes *vfgf* and *ac18* have been shown not to be essential for viral propagation both *in vitro* and *in vivo*.¹⁹⁻²¹ However, lack of *vfgf* delayed the death time in two host species, *Spodoptera frugiperda* and *T.ni*, when the virus was delivered by feeding but not by intrahemocoelic injection, and disruption of *ac18* delayed the death time in *T.ni* larvae when the virus was delivered by feeding, suggesting the two genes may play a role in efficient virus infection *in vivo*. Recently, an ODV-E66 deletion mutant

was shown not to be essential for virus replication, but resulted in a 50% lethal dose that was 10³ higher than that of the repaired and control viruses when inoculated *per os*, suggesting that ODV-E66 envelope protein most likely played an important role in the oral infectivity of AcMNPV.³ In comparison with the *ac69* gene, while it has been shown to be an auxiliary gene with a cap 0-dependent methyltransferase activity, the selective advantage it offers AcMNPV virus is still unknown.

Table 2. Dose mortality of Ac-GP and Ac-*ac69*-KO-GP for third-instar *T.ni* larvae.

Virus	LD ₅₀ (PIBs per larva)	95% Fiducial limit (PIBs per larva)	
		Lower	Upper
Ac-GP	52.1	40.6	63.5
Ac- <i>ac69</i> -KO-GP	46.8	23.4	68.8

Table 3. Time mortality of Ac-GP and Ac-*ac69*-KO-GP for third-instar *T.ni* larvae.

Virus	LT ₅₀ (h)	95% Fiducial limit (h)	
		Lower	Upper
Ac-GP	83.1	81.4	84.9
Ac- <i>ac69</i> -KO-GP	80.1	78.3	81.9

Figure 3. Electron microscopic analysis of Ac-*ac69*-KO-GP-infected Sf-9 cells to characterize *ac69*-null mutant. Panels A and B show virogenic stroma enriched with nucleocapsids appearing within the nucleus at 24 h p.i. Panels C indicates the aligning of bundles of nucleocapsids with envelopes and their subsequent envelopment to form ODV. Panels D shows occlusion bodies containing ODVs appearing within the nucleus at 72 h p.i. The nucleus (N), cytoplasm (C), and virogenic stroma (VS) regions are indicated. Triangles indicate bundles of nucleocapsids, ODV in the process of becoming enveloped or fully enveloped ODV. White arrow indicates nucleocapsids in the virogenic stroma. Black arrow indicates occlusion bodies containing ODVs within the nucleus. For A, B, C, D the bar represents 1 μ m.

As a rule of thumb, viruses will not expend the cost of replication and expressing a gene unless there is some compensating benefit to the virus in terms of increased fitness. Thus it is reasonable to assume that all AcMNPV virus auxiliary genes have some significant role in the viral life cycle. However, the roles of many auxiliary genes, e.g., the AcMNPV *p10*, *ac69* and *PCNA*, are still unclear while they have been tested by a large number of experimental methods.⁹ The reasons why functions for these genes are so difficult to identify may be as follows; first, auxiliary genes are often tested for effects in only one or a small number of host species; second, with current laboratory assay, it is possible that an evolutionally significant increase in fitness might be too small to be reliably detected; and finally, in addition to some generally assayed parameters such as virulence, other parameters that contributes to virus fitness, e.g., ODV stability, host behavior and the ability of the virus to survive as a latent infection, are rarely assayed. Thus, more considerable work, e.g., testing in a larger number of host species and parameters such as ODV stability, is still required to unravel the role of the *ac69* gene in AcMNPV life cycle.

Although the exact role of *ac69* during the viral infection cycle is still unclear, the present study will lead to a better understanding of the molecular aspects of this protein, and provides a large number of experimental data for the further *ac69* gene functional analysis.

References

1. Ayres MD, Howard SC, Kuzio J, et al. The complete DNA sequence of *Autographa californica* nuclear polyhedrosis virus. *Virology* 1994;202:586-605.
2. Jakubowska AK, Peters SA, Ziemnicka J, et al. Genome sequence of an enhancer gene-rich nucleopolyhedrovirus (NPV) from *Agrotis segetum*: collinearity with *Spodoptera exigua* multiple NPV. *J Gen Virol* 2006;87:537-55.
3. Xiang X, Chen L, Hu X, et al. *Autographa californica* multiple nucleopolyhedrovirus *odv-e66* is an essential gene required for oral infectivity. *Virus Res* 2011;158:72-8.
4. Blissard GW, Kogan PH, Wei R, Rohrmann GF. A synthetic early promoter from a baculovirus: Roles of the TATA box and conserved start site CAGT sequence in basal levels of transcription. *Virology* 1992;190:783-93.
5. Pullen SS, Friesen PD. The CAGT motif functions as an initiator element during early transcription of the baculovirus transregulator *ie-1*. *J Virol* 1995;69:3575-83.
6. Garcia-Maruniak A, Maruniak JE, Zanutto PMA, et al. Sequence analysis of the genome of the Neodiprion sertifer nucleopolyhedrovirus. *J Virol* 2004;78:7036-51.
7. Lu A, and Miller LK, in: Miller LK (Ed.). *The Baculoviruses*. New York: Plenum Press 1997; pp. 193-211.
8. Williams GV, and Faulkner P, in: Miller LK (Ed.). *The Baculoviruses*. New York: Plenum Press 1997; pp. 61-107.
9. O'Reilly DR, in: Miller LK. (Ed.). *The Baculoviruses*. New York: Plenum Press 1997; pp. 267-295.
10. Li LL, Harwood SH, Rohrmann GF. Identification of additional genes that influence baculovirus late gene expression. *Virology* 1999;255:9-19.
11. Wu X, Guarino LA. *Autographa californica* nucleopolyhedrovirus *orf69* encodes an RNA cap (nucleoside-2'-O)-methyltransferase. *J Virol*, 2003;77:3430-4.
12. Luckow VA, Lee SC, Barry GF, Olins PO. Efficient generation of infectious recombinant baculoviruses by site-specific transposon-mediated insertion of foreign genes into a baculovirus genome propagated in *Escherichia coli*. *J Virol* 1993;67:4566-79.
13. Ke J, Wang J, Deng R, Wang X. *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. *Virology* 2008;374:421-31.
14. Li Y, Wang JW, Deng RQ, et al. *vlf-1* Deletion Brought AcMNPV to Defect in Nucleocapsid Formation. *Virus Genes* 2005;31:275-84.
15. O'Reilly DR, Miller LK, Luckow VA. *Baculovirus expression vector: a laboratory manual*. New York: WH Freeman and Co.; 1992.
16. Adang MJ, Miller LK. Molecular cloning of DNA complementary to mRNA of the baculovirus *Autographa californica* nuclear polyhedrosis virus: Location and gene products of RNA transcripts found late in infection. *J Virol* 1982;44:782-93.
17. Smith GE, Vlak JM, Summers MD. In Vitro Translation of *Autographa californica* Nuclear Polyhedrosis Virus Early and Late mRNAs. *J Virol* 1982;44:199-208.
18. Vlak JM, Klinkenberg FA, Zaai KJM, et al. Functional studies on the *p10* gene of *Autographa californica* nuclear polyhedrosis virus using a recombinant expressing a *p10*- β -galactosidase fusion gene. *Gen Virol* 1988;69:765-76.
19. Detvisitsakun C, Hutfless EL, Berretta MF, Passarelli AL. Analysis of a baculovirus lacking a functional viral fibroblast growth factor homolog. *Virology* 2006;346:258-65.
20. Detvisitsakun C, Cain EL, Passarelli AL. The *Autographa californica* M nucleopolyhedrovirus fibroblast growth factor accelerates host mortality. *Virology* 2007;365:70-8.
21. Wang Y, Wu W, Li Z, et al. *ac18* is not essential for the propagation of *Autographa californica* multiple nucleopolyhedrovirus. *Virology* 2007;367:71-81.