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The deletion of the AcMNPV *ac124* gene resulted in a decrease in *chitinase* transcription

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ABSTRACT

The *Autographa californica multiple nucleopolyhedrovirus* (AcMNPV) *ac124* gene has been previously characterized as a viral pathogenicity factor. In this study, an *ac124*-knockout virus (vAc124KO) was generated to examine the role of the *ac124* gene in the context of the AcMNPV genome during infection. Our results showed that the absence of *ac124* does not affect the production of budded virus (BV) and occlusion bodies (OBs) in infected Sf9 cells. Western blotting analysis showed that the deletion of *ac124* does not affect the temporal expression and the relative levels of GP64, VP39, P6.9, and polyhedrin. qRT-PCR analysis showed that the transcription level of *chitinase* but not the adjacent *cathepsin* in vAc124KO infected cells was significantly lower than that of the vAcWT infected cells from 24 to 96 h p.i. Luciferase assays showed that the overexpression of Ac124 could significantly improve the ability of *chitinase* promoter to initiate reporter genes. Based on the above data, we hypothesize that Ac124 binds to the promoter of *chitinase* to regulate the expression of *chitinase* gene.

1. Introduction

Baculoviruses are rod-shaped enveloped viruses that have circular double-stranded DNA genomes (Rohrmann, 2013). *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) is the type species of *Baculoviridae* and has a genome of approximately 134 kbp that encodes 156 putative open reading frames (ORFs) (Ayres et al., 1994). During typical baculovirus replication, two genotypically identical but phenotypically distinct progeny virion forms are produced, the budded virion (BV) and the occlusion-derived virion (ODV). BVs are produced during the early phase of infection. Nucleocapsids assemble within a subnuclear structure called the virogenic stroma (VS), leave the cell nucleus, and budd through the plasma membrane modified by a number of virus-encoded proteins to form BVs. Since BVs are responsible for transmitting infection between susceptible insect tissues and between cells in cell culture, they are essential for establishing systemic infection in an infected insect. During the late phase of infection, nucleocapsids are retained in the nucleus and acquire viral induced intranuclear membrane-derived microvesicles to form ODVs. ODVs are subsequently embedded within a crystalline matrix composed mainly of the protein polyhedrin to form occlusion bodies (OBs). When a dead host releases OBs into the environment, OBs are ingested by another host and initiate a new round of infection (van Oers and Vlak, 2007).

Baculoviruses are specifically pathogenic to insects infecting insects of mainly the orders Lepidoptera, Hymenoptera, and Diptera (Herniou et al., 2003). The *Baculoviridae* can be divided into four genera: *Alphabaculovirus* (lepidopteran nucleopolyhedroviruses [NPVs]), *Betabaculovirus* (lepidopteran granuloviruses [GVs]), *Gammabaculovirus* (hymenopteran NPVs), and *Deltabaculovirus* (dipteran NPVs) (Jehle et al., 2006). The members of the *Alphabaculovirus* genus can be further subdivided into Group I and Group II (Jehle et al., 2006), which differ significantly in gene content. The viruses of Group II show more variation than Group I viruses, suggesting that Group I is a more recent lineage than Group II (Lange et al., 2004). There are 12 genes that are found in only Group I NPVs: *ac1* (*ptp*), *ac5*, *ac16* (*bv/odv-e26*), *ac27* (*iap-1*), *ac30*, *ac72*, *ac73*, *ac114*, *ac124*, *ac128* (*gp64*), *ac132*, and *ac151* (*ie2*) (Rohrmann, 2013). Some of these genes have been characterized. *ac1* encodes a RNA 5′-triphosphatase (Gross and Shuman, 1998; Takagi et al., 1998), which has been predicted to be involved in preparing RNA for cap formation (Changela et al., 2005). *ac5* is likely to be non-essential because when it was interrupted by insertional/deletion mutagenesis in BmNPV (*Bm134*), the virus appeared to be viable

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(Rohrmann, 2013). Ac16 is an envelope protein of BV and ODV, and it interacts with FP25 K to form a complex with cellular actin (Beniya et al., 1998). The deletion of Ac16 does not affect viral DNA synthesis or BV production, but the relative level of IE0 to IE1 is significantly increased (Nie et al., 2009). Transient-expression experiments indicated that IAP-1 (Ac27) suppresses apoptosis of *Trichoplusia ni* cells during *Helicoverpa armigera* NPV (HearNPV) infection (Zeng et al., 2009). Moreover, a recombinant HearNPV expressing IAP-1 also suppresses apoptosis, but the production of BV is not rescued (Zeng et al., 2009). Ac30 is likely to be nonessential because when the ortholog (Bm21) was interrupted by insertional/deletion mutagenesis in BmNPV, the virus appeared to be normal, but the deletion of *Bm21* extends the median survival time of infected larvae (Huang et al., 2008; Rohrmann, 2013). IE2 (Ac151) is a transcriptional regulator (Yoo and Guarino, 1994), and it may be involved in cell cycle regulation (Prikhod'ko and Miller, 1998). *ac72* was not characterized. Ac73 is BV associated in AcMNPV (Wang et al., 2010). It may be essential because insertion/deletion mutants of this gene in BmNPV (Bm59) could not be isolated (Rohrmann, 2013). In AcMNPV Ac114 appears to be an ODV and BV associated protein (Braunagel et al., 2003; Wang et al., 2010). It is likely to be nonessential, as an insertion/deletion mutation of this gene in BmNPV (*Bm94*) had no apparent effect on infectivity (Rohrmann, 2013). *ac128* encodes a low pH activated envelope fusion protein GP64 for the entry of BV into cells (Monsma et al., 1996; Roberts and Faulkner, 1989; Volkman, 1986; Whitford et al., 1989). Ac132 plays a role in nuclear entry (Fang et al., 2016). Most notably, Group I NPVs use GP64 as their BV fusion protein, whereas Group II NPVs lack GP64 and utilize a protein called F protein (Pearson and Rohrmann, 2002). The genomic sequence of *ac124*, the last of the 12 Group I specific genes described above, predicts a gene product of 248 amino acids (aa) with a putative molecular mass of 28.5 kDa (Ayres et al., 1994). Previous proteomic studies determining the protein composition of BV and ODV of AcMNPV showed that Ac124 is associated with BV, but was not detected in ODVs (Braunagel et al., 2003; Wang et al., 2010). The *ac124* deletion mutant had no striking phenotype in Sf9 cells, including BV production and viral morphogenesis. However, the larval bioassays showed that the mutant virus took approximately 20 h longer to kill *S. exigua* larvae than wild-type virus (Liang et al., 2014). Nevertheless, the exact role of Ac124 in viral replication remains obscure.

In this study, an *ac124*-knockout virus (vAc124KO) was generated to investigate the role of *ac124* in the AcMNPV life cycle. We found that *ac124* is not essential for infectious BV and OB production. Further investigations showed that the *ac124* deletion did not affect the expression of the early genes, late genes, and very late genes. However, our results showed that the deletion of the AcMNPV *ac124* gene resulted in a decrease in *chitinase* transcription.

2. Materials and methods

2.1. Cell lines and viruses

The Sf9 cell strain, which was derived from *Spodoptera frugiperda* (Vaughn et al., 1977), was cultured at 27 °C in TNM-FH medium (Invitrogen) supplemented with 10% fetal bovine serum, 100 μg/ml penicillin, and 30 μg/ml streptomycin. The bacmid bMON14272 (Invitrogen), containing an AcMNPV genome, was propagated in the *E. coli* strain DH10B as previously described (Luckow et al., 1993). BV titers were determined with a 50% tissue culture infective dose ($TCID_{50}$) endpoint dilution assay in Sf9 cells (O'Reilly et al., 1992). Time zero was defined as the time when the viral inoculum was added during infection or replaced with fresh medium during transfection.

2.2. Construction of an ac124-knockout bacmid

An *ac124*-knockout AcMNPV bacmid was generated via ET homologous recombination as previously described (Wu et al., 2006) in which the *ac124* locus region was replaced with the chloramphenicol resistance gene (*Cm*) for antibiotic selection in the *E. coli*. A 1,182-bp PCR fragment was amplified using pUC18-ac132UCD (Fang et al., 2016) as the template and primers Ac124D1 (5′-AAAAATGAAGTTTC CCTATGTTGCGTTGTCGTACATCAACGTGACGCTGTCCCTTTCGTCTTC GAATAAATA-3′) and Ac124D2 (5′-CAATTAGTTTCGTTTT CAAACACT CTAAACCCATAACAATAGTAACACTCTAAACCAGCAATAGACATA AGC-3′). This fragment contains the 1,038-bp *Cm* cassette and 72 bp of *ac124* flanking regions at each end. Purified PCR fragments were transformed into electrocompetent DH10Bac cells, and the recombinant *ac124*-knockout bacmid was selected and PCR analyzed as previously described (Yuan et al., 2011). The resulting *ac124*-knockout bacmid was designated as bAc124KO.

2.3. Construction of an ac124-knockout virus

To facilitate the examination of viral infection, the enhanced green fluorescence protein gene (*egfp*, referred to as *gfp* in this study) and the AcMNPV polyhedrin gene (*polh*) were transposed into bAc124KO to generate an *ac124*-knockout virus (vAc124KO) as previously described (Yuan et al., 2011). Using the same method above, the *polh* and *gfp* genes were transposed into bMON14272 to generate a wild-type control virus (vAcWT).

To generate an *ac124*-repaired virus tagged with FLAG, a donor plasmid (pFB1-Ac124:Flag-PG) was constructed as follows. Using primers ac124RU (5′-CGAGCTCAATAGTCGCTCAACATGTACTC-3′ [the SacI site is underlined]) and ac124RFlag (5′-CGGGATCCTTACTTATC GTCGTCATCCTTGTAATCTTTT TTATTTTTTATGTCATCCA-3′ [the BamHI site is underlined]), a DNA fragment containing the *ac124* native promoter and the *ac124* ORF tagged with a FLAG epitope was PCR amplified from bMON14272. The PCR products were cloned into the pUC18-SV40 plasmid (Cai et al., 2012) to generate pUC18-Ac124:Flag-SV40. pUC18-Ac124:Flag-SV40 was digested with SacI and XbaI, and the resulting fragment was inserted into pFB1-PH-GFP (Wu et al., 2006) to generate the donor plasmid pFB1-Ac124:Flag-PG. pFB1-Ac124:Flag-PG was then transformed into electrocompetent DH10B cells containing the helper plasmid pMON7124 and bAc124KO to generate the *ac124*-repaired virus (vAc124:Flag).

2.4. Western blot analysis

To analyze the expression of Ac124 in infected cells, Sf9 cells (1.0×10^6) were infected with vAc124:Flag at a multiplicity of infection (MOI) of 10 $TCID_{50}$/cell. At different time points p.i., cells were collected by centrifugation at 3000 × *g* for 10 min. Western blotting was performed using a mouse monoclonal anti-FLAG antibody (1:1000; Abmart) as the primary antibody. A goat anti-mouse horseradish peroxidase (HRP)-conjugated antibody (1:3000; Pierce) was used as the secondary antibody. Proteins were visualized using an enhanced chemiluminescence system (ECL; Amersham Biosciences) according to the manufacturer's instructions.

To evaluate the effects of *ac124* deletion on viral protein expression, Sf9 cells were infected with vAc124KO or vAcWT at an MOI of 10 $TCID_{50}$/cell. The cells were collected, lysed in RIPA buffer (Thermo Scientific) for 30 min on ice, and centrifuged for 10 min at 14,000 × g at 4 °C. The supernatants were collected and subjected to immunoblotting. Western blotting was performed as described previously (Yuan et al., 2011). The primary antibodies used were the following: (i) mouse monoclonal AcMNPV GP64 antibody (1:1000; eBioscience), (ii) rabbit polyclonal AcMNPV P6.9 antibody (1:1000), (iii) rabbit polyclonal AcMNPV VP39 antibody (1:1000), (iv) rabbit polyclonal AcMNPV POLH antibody (1:1000), and (v) mouse monoclonal GADPH antibody (1:1000; Abmart). The anti-rabbit horseradish peroxidase (HRP) antibody (1:5000; GE) or goat anti-mouse HRP secondary antibodies (1:3000; Amersham Biosciences) were used. The signals were detected using the ECL System (Amersham Biosciences) according to

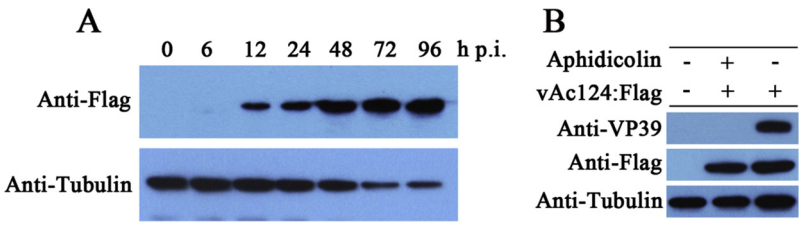


Fig. 1. *ac124* is an early gene. (A) Temporal expression analysis of Ac124. Cells were mock-infected (Mi) or infected with vAc124:Flag at an MOI of 10 $TCID_{50}$/cell. At the indicated time points p.i., cells were harvested, and total cellular proteins were detected using Western blotting with an anti-FLAG antibody. An anti-tubulin antibody was used as a loading control. (B) Cells were infected with vAc124:Flag in the presence (+) or absence (-) of the DNA synthesis inhibitor aphidicolin. Total cellular proteins were detected using Western blotting with anti-FLAG, anti-VP39, and anti-tubulin antibodies to detect FLAG-tagged Ac124, VP39, and Tubulin, respectively.

the manufacturer's instructions.

2.5. Analysis of viral growth

Sf9 cells (1.0×10^6) were transfected with 1.0 μg of the constructed bacmid (vAcWT, vAc124KO, or vAc124:Flag) using the Cellfectin liposome reagent (Invitrogen) in triplicate. At the indicated time points posttransfection (p.t.), cells were monitored by fluorescence microscopy, and supernatants were harvested to determine titers.

2.6. Assays for OB production

Sf9 cells (1.0×10^6) were grown to confluence in a 35 mm dish and then infected with vAcWT or vAc124KO. For quantification of total produced polyhedra, 1% (w/v) SDS was added to the cell pellets at the indicated time points p.i., and the cell pellets were incubated at 37℃ for 30 min to release polyhedra. The numbers of polyhedra were counted using a hemocytometer.

2.7. RNA extraction and qRT-PCR

Sf9 cells (1.0×10^6) were grown to confluence in a 35 mm dish and then infected with vAcWT or vAc124KO. At different time points p.i., cells were collected by centrifugation at 3000 × *g* for 10 min. Total RNA isolation and cDNA synthesis were performed as previously described (Cai et al., 2012). Quantitative reverse transcription-PCR (qRT-PCR) was performed using TransStart Green qPCR SuperMix (TransGen Biotech) according to the manufacturer's protocol. Six genes associated with viral proliferation or larval death (*vp39*, *gp64*, *polh*, *fgf*, *p10*, and *chitinase*) were chosen to investigate the transcriptional levels. The qPCR data of viral genes were normalized to host 18S rRNA (Li et al., 2015), and the relative expression of genes was calculated using the $2^{\triangle\triangle CT}$ method (Schmittgen and Livak, 2008).

2.8. Plasmid constructions

A 600-bp fragment containing the promoter of *chitinase* was PCR amplified from bMON14272 (nt 112,414 to 113,013) using the primers ChitinasePU (5′-CTCGAGGGCTTCGAACGCTG TGTGCA-3′ [the XhoI site is underlined]) and ChitinasePD (5′-AAGCTTTTTAATT TATCTTA ATTTTAA-3′ [the HindIII site is underlined]). The PCR products were ligated into a luciferase reporter plasmid pGL3-Basic (Wei et al., 2013) to generate a plasmid pGL3-Chitinase-Pro. The fragment containing the *ac124* ORF was PCR amplified from bMON14272 (nt 109,269 to 110,012) using the primers Ac124PU (5′-GGATCCATGGGTTTGTTTGC GTGTTG-3′ [the BamHI site is underlined]) and Ac124PD (5′-GAATT CTTATTTTTTATTTTTTATGTCATCCA-3′ [the EcoRI site is underlined]). The PCR fragment was inserted downstream of the immediate early 2 promoter on plasmid pIBV5-His to a transient expression plasmid named pIB-Ac124.

2.9. Luciferase assays

Sf9 cells (4×10^4) were transfected with 0.1 μg of the DNA indicated below using Cellfectin (Invitrogen) as the transfection reagent following the manufacturer's protocol. Cell extracts were prepared 48 h after transfection using a passive lysis buffer. Luciferase activities were evaluated with a Dual-Luciferase Assay System (Promega, Madison, WI) according to the manufacturer's recommendations using Varioskan LUX plate reader (Thermo Fisher Scientific, Waltham, MA). Background luminescence was determined using untransfected cells and the background readings were then subtracted from the resulting luminescence readings before being normalized to Renilla luminescence and presented as relative luminescence unit. The luciferase activity data (average ± standard deviation) were collected from triplicate assays of three independent transfections and are presented as the number of relative light units (RLUs) per microgram protein.

3. Results

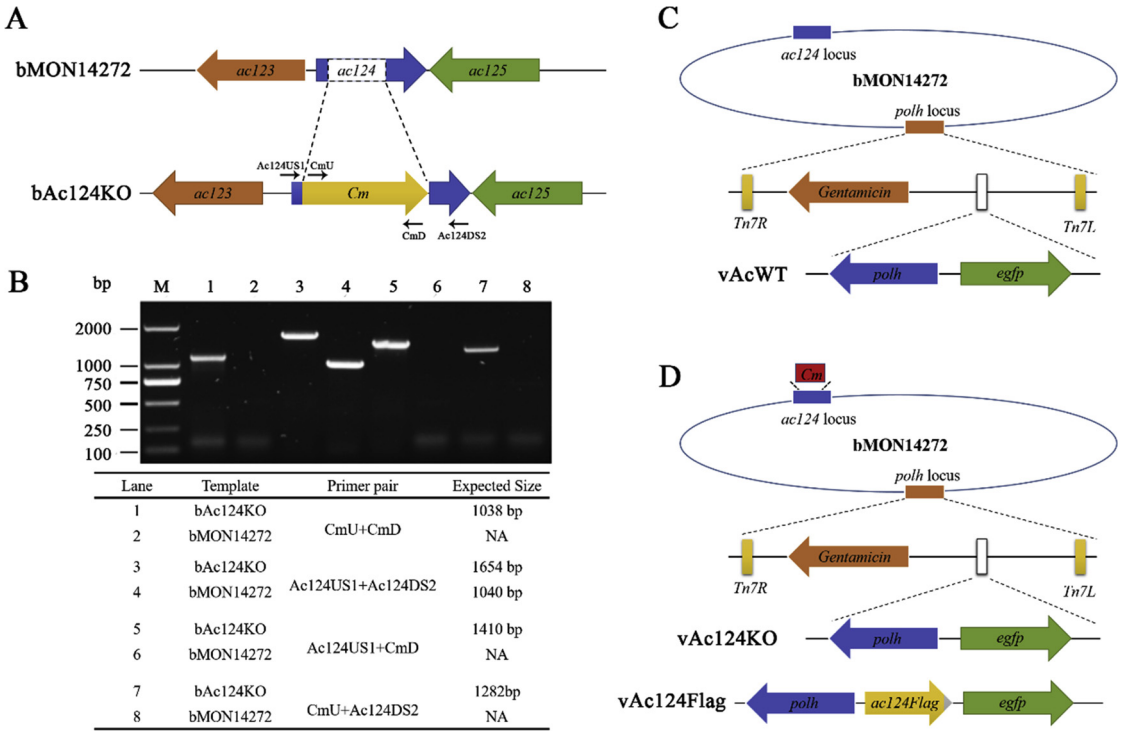
3.1. ac124 is an early gene

To analyze the temporal expression of Ac124, Sf9 cells were infected or mock-infected with vAc124:Flag at an MOI of 10. At designated time points p.i., vAc124:Flag-infected cells were collected and were analyzed by Western blotting using an anti-FLAG antibody. As shown in Fig. 1A, a major FLAG-immunoreactive band of approximately 30 kDa was first detected at 12 h p.i., and it could still be detected at 96 h p.i. To assess whether Ac124 synthesis required prior DNA replication, the DNA synthesis inhibitor aphidicolin was added to vAc124:Flag-infected cells. As shown in Fig. 1B, Ac124 was detected in aphidicolin-treated cells. As a control to test the efficiency of the drug treatment, the same membrane was stripped from antibodies and reprobed with an anti-VP39 antibody to detect the late viral protein VP39. As expected, the synthesis of VP39 was not detected in aphidicolin-treated cells (Fig. 1B). Taken together, these results indicated that *ac124* is an early gene.

3.2. Construction of an ac124-knockout bacmid and confirmation of its construction by PCR

bAc124KO was constructed by deleting the central portion of *ac124* and retaining 100 nt from the 5′ end and 244 nt from the 3′ end of the *ac124* coding region to ensure the expression of the neighboring genes *ac123* and *ac125* (Fig. 2A). The 400-bp fragment of the *ac124* ORF was replaced with a 1,038-bp *Cm* cassette between nt 109,369 and nt 109,768 of the AcMNPV genome to generate the *ac124*-knockout bacmid (Fig. 2A).

The deletion of *ac124* from the *ac124* locus of bMON14272 and the insertion of the *Cm* cassette were confirmed by PCR (Fig. 2B). Primer pair CmU/CmD (Fig. 2A) was used to confirm the insertion of the *Cm* cassette. Primers Ac124US1 and Ac124DS2 (Fig. 2A) were used to confirm the deletion of 400 bp within the *ac124* coding region and its replacement with the 1,038-bp *Cm* cassette. Primer pair Ac124US1/CmD and CmU/Ac124DS2 (Fig. 2A) were used to confirm the recombination junctions upstream and downstream of *ac124*, respectively. The sizes of the PCR-amplified products obtained were as predicted following successful recombination (Fig. 2B).



Lane	Template	Primer pair	Expected Size
1	bAc124KO	CmU+CmD	1038 bp
2	bMON14272		NA
3	bAc124KO	Ac124US1+Ac124DS2	1654 bp
4	bMON14272		1040 bp
5	bAc124KO	Ac124US1+CmD	1410 bp
6	bMON14272		NA
7	bAc124KO	CmU+Ac124DS2	1282bp
8	bMON14272		NA

Fig. 2. Construction of recombinant bacmids used in this study. (A) Strategy for construction of bAc124KO. The 400-bp fragment of the *ac124* ORF was replaced with a 1,038-bp *Cm* cassette between nt 109,369 and nt 109,768 of the AcMNPV genome to generate the *ac124*-knockout bacmid (bAc124KO). (B) PCR confirmation of bAc124KO. The table below the agarose gel shows the templates, primers, and expected product sizes. NA, no amplified product expected. (C) Schematic diagram of the wild-type control virus (vAcWT). The vAcWT was generated by inserting the *polh* and *gfp* genes inserted into the *polh* locus by Tn7-mediated transposition. (D) Schematic diagram of vAc124KO and vAc124:Flag, showing *polh* and *gfp* inserted into the *polh* locus of bAc124KO by Tn7-mediated transposition.

3.3. Construction of AcBAC recombinants containing the polh and egfp genes, vAcWT, vAc124KO, and vAc124:Flag

To determine whether the deletion of *ac124* had any effect on OB morphogenesis and to facilitate the examination of viral infection, the *polh* gene of AcMNPV and *egfp* were inserted into the *polh* locus of bAc124KO via Tn7-mediated transposition as described previously (Fang et al., 2016). Thus, an *ac124*-knockout AcMNPV, which we designated vAc124KO, was constructed (Fig. 2D). The repair bacmid was constructed to rescue and confirm the phenotype resulting from the deletion of *ac124*, vAc124:Flag, carrying the *polh* gene, *egfp*, and *ac124* with a FLAG tag at the C terminus (Fig. 2D). vAcWT, bMON14272 carrying the *polh* and *egfp* genes, was used as a positive control (Fig. 2C). All constructs were confirmed by PCR, the expression of *egfp*, and the formation of occlusion bodies.

3.4. ac124 is not essential for infectious BV production

To determine the effect of *ac124* deletion on viral replication, vAcWT, vAc124KO, or vAc124:Flag was transfected into Sf9 cells. Cells were monitored by fluorescence microscopy at the indicated time points p.t. At 24 h p.t., no obvious differences in the number of fluorescent cells were observed among these three viruses, indicating that the transfection efficiencies were comparable (Fig. 3A). Furthermore, at 48 h p.t., a significant increase in the number of fluorescent cells was observed for all three viruses (Fig. 3A). At 72 h p.t., fluorescence was observed in almost all cells transfected with vAcWT, vAc124KO, or vAc124:Flag (Fig. 3A). Light microscopy analysis showed that OBs could be detected in cells transfected with vAcWT, vAc124KO, or vAc124:Flag at 96 h p.t. (Fig. 3A). These results suggested that the deletion of *ac124* does not affect the generation of infectious BV and the production of OBs.

To further assess the effect of *ac124* deletion on virus replication, a virus growth curve experiment was performed. Sf9 cells were transfected with each bacmid DNA, individually. Supernatants were harvested at the designated time point p.t., BV titers were determined using a $TCID_{50}$ endpoint dilution assay. As expected, the levels of BV production from vAc124KO, vAc124:Flag, and vAcWT were similar, indicating that infectious BVs were produced in all three transfected cells (Fig. 3B). In conclusion, these findings indicated that *ac124* is not essential for infectious BV production.

3.5. The deletion of ac124 does not affect OB production

In order to determine whether the deletion of *ac124* affects the production and numbers of OBs, Sf9 cells were infected with vAc124KO or vAcWT, and monitored by fluorescence microscopy at the indicated time points p.i. No difference was observed between the two viruses at 24 h p.i., and the infection efficiencies were comparable (approximately 100%) (Fig. 4A). Light microscopy showed that OBs with a normal appearance formed in cells transfected individually with the two viruses and that the number of cells containing OBs did not differ between the two viruses at 72 h p.i. (Fig. 4A). At 120 h p.i., most of the cells infected with vAc124KO or vAcWT contained OBs (Fig. 4A). In order to investigate whether the numbers of OBs in the cells infected by the two viruses is comparable, OBs were collected from vAcWT- or vAc124KO-infected cells and counted using a hemocytometer. As shown in Fig. 4B, from 72 to 120 h p.i., there was no significant difference in the numbers of OBs produced by the two viruses. These results suggested that the deletion of *ac124* does not affect OB production.

3.6. The deletion of ac124 reduces the transcription level of chitinase

As demonstrated above, *ac124* is an early gene; therefore, it is possible that Ac124 functions as a regulatory protein. To test this hypothesis, Sf9 cells infected with vAcWT or vAc124KO were subjected to

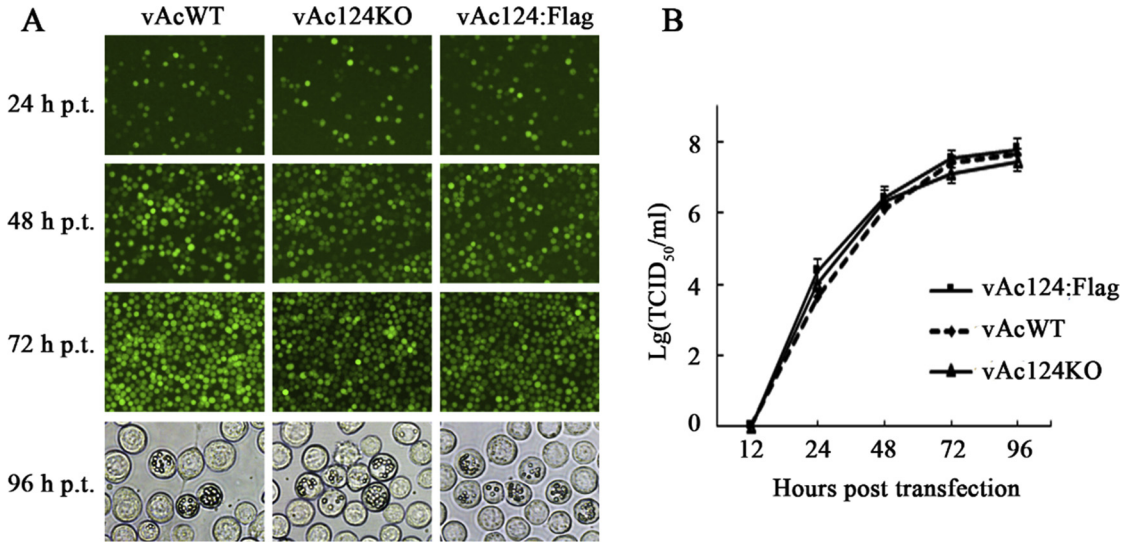


Fig. 3. The deletion of *ac124* does not affect infectious BV production. (A) Microscopy of Sf9 cells transfected with bacmid DNA. Fluorescence microscopy of Sf9 cells transfected with vAcWT, vAc124KO, or vAc124:Flag from 24 to 72 h p.t. showed the progression of viral infection. Light microscopy showed the formation of OBs in Sf9 cells transfected with vAcWT, vAc124KO, or vAc124:Flag at 96 h p.t. (B) Virus growth curves of vAcWT, vAc124KO, and vAc124:Flag in Sf9 cells as determined with $TCID_{50}$ endpoint dilution assays. Error bars represent standard deviations.

immunoblotting. The results showed that the early-late gene product GP64 was detected beginning at 6 h p.i. in vAc124KO- and vAcWT-infected cells (Fig. 5). In addition, the late proteins P6.9 and VP39 were detected at 18 h p.i. in vAc124KO- or vAcWT-infected cells (Fig. 5). As shown in Fig. 5, there were no obvious differences in either the amount or onset of the expression of POLH proteins between vAcWT- and vAc124KO-infected cells. These results indicated that the deletion of *ac124* did not affect the GP64, VP39, P6.9, and POLH expression.

To further determine whether the deletion of *ac124* affected the transcription of *chitinase*, qRT-PCR was performed to analyze the transcription kinetics of AcMNPV genes in *chitinase*. As shown in Fig. 6A, the transcription level of *chitinase* gene in cells infected with vAc124KO or vAcWT were low but comparable at 12 and 18 h p.i. However, the transcription level of *chitinase* gene in vAc124KO infected cells was 3.52 fold lower than that of the vAcWT infected cells at 24 h p.i. (Fig. 6A). This lower level of transcription continues until 96 h p.i. (Fig. 6A). Since the *chitinase* gene and the *cathepsin* gene are adjacent genes, and the two have a 45 bp overlapping region upstream of their respective initiation codons we also measured *cathepsin* gene expression. As shown in Fig. 6B, the transcription level of *cathepsin* in cells infected with vAc124KO or vAcWT were comparable from 12 to 96 h p.i. These results suggested that the deletion of *ac124* does not affect the transcription level of *cathepsin*. In order to investigate the mechanism by which Ac124 reduces the transcription level of *chitinase*, luciferase assays were performed. Sf9 cells were cotransfected with pIB-Ac124 expressing Ac124, pGL3-Chitinase-Pro with the *chitinase* promoter driving firefly luciferase, and pRL-TK expression *Renilla* luciferase or cotransfected with pIBV5-His, pGL3-Chitinase-Pro, and pRL-TK. At 48 h p.t., Cell extracts were prepared and luciferase activities were evaluated with a Dual-Luciferase Assay System. As shown in Fig. 7, the overexpression of Ac124 could significantly improve the ability of the *chitinase* promoter to initiate expression of reporter genes. Taken together, these observations indicated that Ac124 can bind to the promoter region of the *chitinase* gene and enhance the transcription level of the *chitinase* gene. When the *ac124* gene is absent in the vector control, the transcription level of the *chitinase* is significantly decreased.

4. Discussion

In this report, we studied the function of AcMNPV *ac124*, one of the Group I NPV-specific 12 genes that are found only in Group I NPV genomes sequenced to date. To enable this, we generated an *ac124*-knockout virus via ET homologous recombination. Following transfection with vAc124KO, viral replication was not restricted to the initially transfected cells, indicating that the production of infectious BVs was not affected (Fig. 3). These results were consistent with previous findings where the deletion of *ac124* also did not affected the production of infectious BVs (Liang et al., 2014). Previous studies have demonstrated that *ac124* is not required for viral replication, but it accelerates the killing of infected larvae (Liang et al., 2014). Baculovirus infection is initiated after the ingestion of food contaminated with OBs containing the infectious enveloped virions. OBs dissolve in the insect midgut and release the embedded virions (Granados and Lawler, 1981). Due to the important role of OBs in the infection of larvae, assays were performed to investigate whether the deletion of *ac124* affects OB production. In this study, we found that the deletion of *ac124* does not affect the production and numbers of OBs. These results suggest that the delay in larval death time is not due to a decrease in OB production.

Baculovirus gene expression involves sequential and coordinated expression of immediate-early, early, late, and very late genes. Following viral entry and translocation of the viral DNA to the nucleus, immediate-early gene expression is followed by early gene expression. After the initiation of viral DNA replication, late genes are expressed. In this study, we found that the expression of Ac124 was detected from 18 to 96 h p.i. (Fig. 1A) implying that *ac124* is an early gene. To assess if Ac124 synthesis required prior DNA replication, the DNA synthesis inhibitor aphidicolin was added to vAc124:Flag infected cells. As shown in Fig. 1B, the expression of Ac124 is not blocked by aphidicolin. Together, these results suggested that *ac124* is an early gene. The products of the early genes function to both accelerate viral DNA replication and to prepare the host cell for viral multiplication (Friesen, 1997). The late phase of gene expression requires a number of early gene products and viral DNA replication (Lu et al., 1997; Passarelli and Miller, 1993). In order to investigate whether the deletion of the early gene *ac124* affected the transcription or expression of the early, late, and very late genes, four representative viral genes, *gp64*, *p6.9*, *vp39*, and *polh*, were selected. *gp64* is an early gene that is transcribed by the host RNA polymerase II. *vp39* and *p6.9* are late genes, and *polh* is the very late genes; these three genes are transcribed by a viral RNA polymerase. The results showed that the deletion of *ac124* did not affect GP64, VP39, P6.9, and POLH expression (Fig. 5). Surprisingly, the deletion of *ac124* reduces the transcription level of *chitinase*, which was approximately

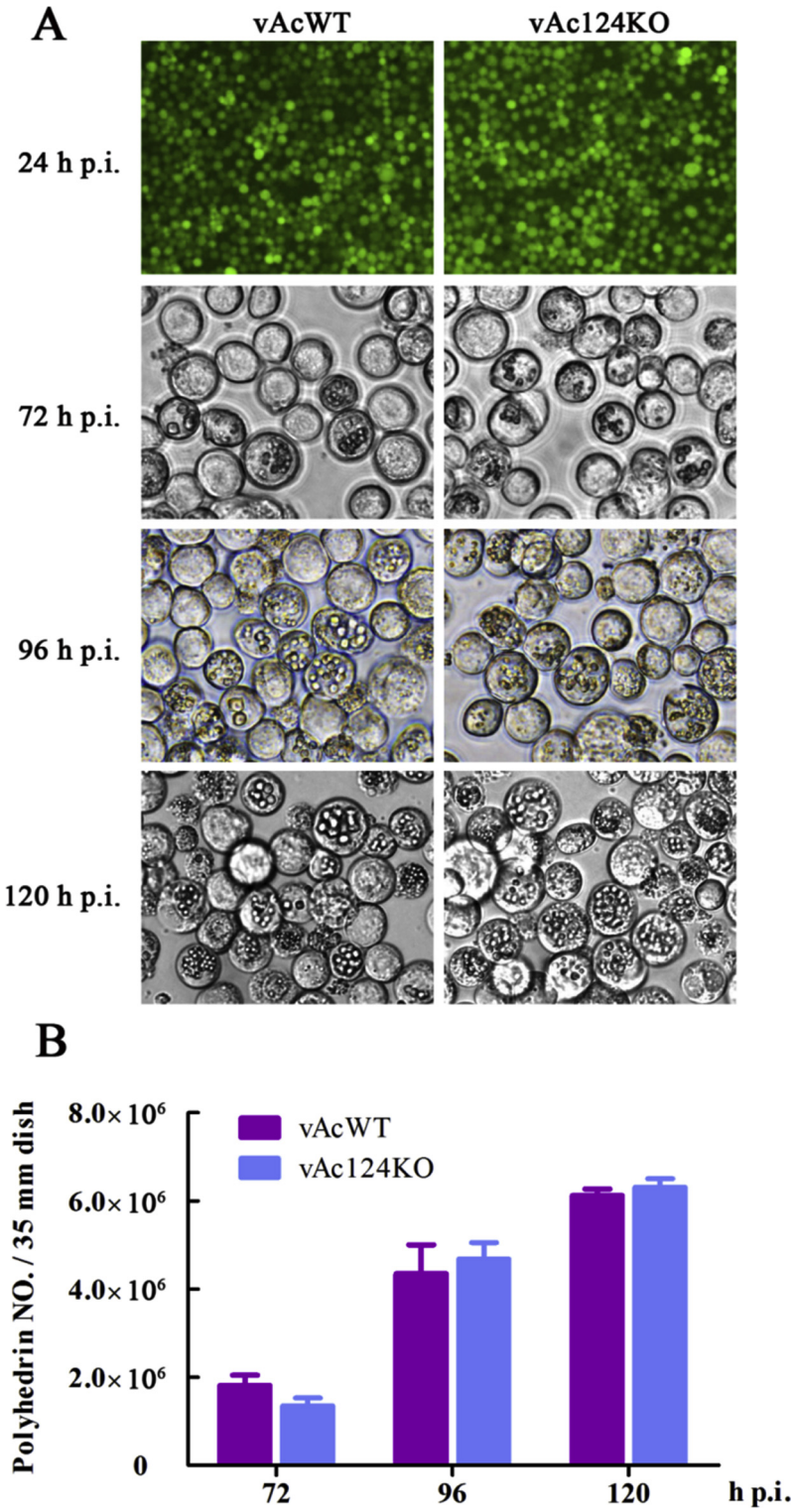


Fig. 4. The deletion of *ac124* does not affect OB production. (A) Light microscopy showed the formation of OBs in Sf9 cells transfected with vAcWT or vAc124KO from 72 to 120 h p.i. (B) Sf9 cells were infected with vAcWT or vAc124KO. At the indicated time points p.i., the OBs were collected and quantified using a hemocytometer.

3.52 times lower lower in its absence.

Chitinase is a late expressed gene and its product is localized to the endoplasmic reticulum in infected cells by KDEL, an endoplasmic reticulum retention motif (Hodgson et al., 2011; Saville, 2004; Thomas et al., 1998) and also is BV associated (Wang et al., 2010). Homologs of *chitinase* are present in genomes of all Group I (except AgMNPV), all Group II (except AdhoNPV) and four GVs (Agse, Cp-, Ha-, and XecnGV) and is phylogenetically clustered with a number of lepidopteran chitinases, e.g. it shows 63% aa sequence identity to B. *mori chitinase* (Rohrmann, 2013). In conjunction with cathepsin, chitinase participates in the liquefaction of insects late in infection (Rohrmann, 2013). When it is deleted along with cathepsin, insects remained intact for several days after death (Hawtin et al., 1997). Combine with the previous report by Liang et al (Liang et al., 2014) with the results of this

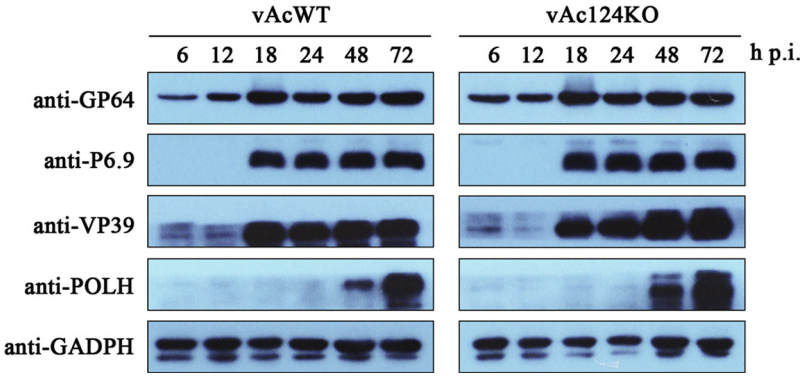


Fig. 5. Analysis of the effect of Ac124 deletion on viral gene expression. Time course analysis of GP64, P6.9, VP39, and POLH synthesis. Sf9 cells were infected with vAcWT or vAc124KO at an MOI of 5 $TCID_{50}$/cell and harvested at different time points. The cell lysates were subjected to immunoblotting with anti-GP64, anti-P6.9, anti-VP39, or anti-POLH antibody. An anti-GADPH antibody was used as a loading control.

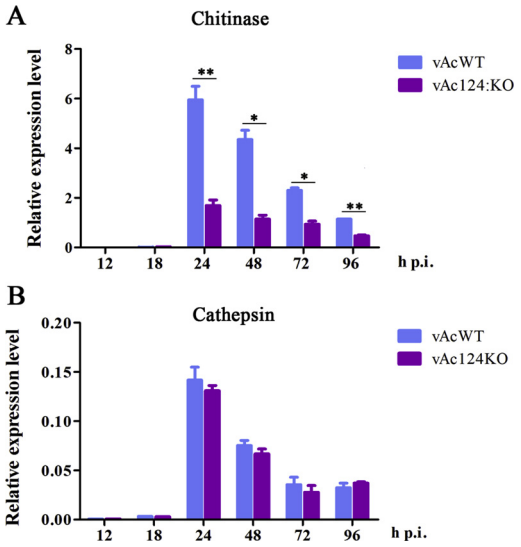


Fig. 6. qRT-PCR analysis of viral gene transcription. The effects of Ac124 deletion on the transcription of *chitinase* and *cathepsin* genes were measured with qRT-PCR at 12, 18, 24, 48, 72, and 96 h p.i. The transcript level of *chitinase* and *cathepsin* were normalized to that of the host 18S rRNA transcripts. Data were analyzed by Student's *t*-test *, $P < 0.05$; **, $P < 0.01$. The error bars represent the standard deviations from the mean.

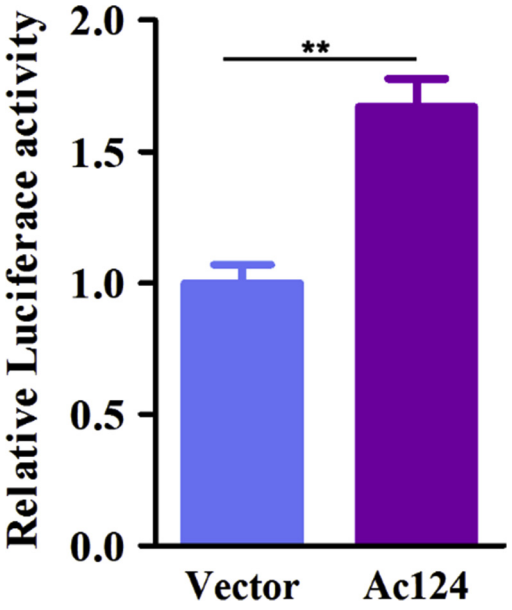


Fig. 7. Luciferase activities of *chitinase* promoters. 1 μg of each reporter plasmid was transfected into Sf9 cell. Cells were harvested for luciferase assays at 48 h after transfection. Vector represent cells cotransfected with pIBV5-His, pGL3-Chitinase-Pro with the *chitinase* promoter driving firefly luciferase, and pRL-TK expression *Renilla* luciferase. Ac124 represent cells cotransfected with pIB-Ac124 expressing Ac124, pGL3-Chitinase-Pro, and pRL-TK. Results are normalized relative to the luciferase activity of vector, which was set as 1.

study, we hypothesize that Ac124 could bind to the promoter region of the *chitinase* gene to accelerate the transcription and expression of *chitinase*. When it is deleted, the transcription level of the *chitinase* gene is also reduced, thereby delaying the killing speed in the larvae. The baculovirus late promoter initiation is highly precise, initiating at the second position of the conserved TAAG motif (Chen et al., 2013; Rohrmann, 2013). Although the *chitinase* and *cathepsin* are adjacent, the transcription direction of the two is opposite, and both contain the baculovirus late promoter motif (TAAG). The promoter of the *chitinase* gene is located 14 nt upstream of the ATG, and the promoter of the *cathepsin* is located 27 nt upstream of the ATG (Chen et al., 2013). In other words, the promoter regions of the two genes do not overlap. Therefore, we speculate that this is why the putative Ac124 binding affects only the *chitinase* promoter but not the *cathepsin* promoter activity.

In summary, this study has shown that *ac124*, an early gene, is not required for the production of BV and OB. However, the deletion of *ac124* resulted in a significant decrease in the transcription of *chitinase*.

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References

Ayres, M.D., Howard, S.C., Kuzio, J., Lopez-Ferber, M., Possee, R.D., 1994. The complete DNA sequence of *Autographa californica* nuclear polyhedrosis virus. Virology 202 (2), 586–605.

Beniya, H., Braunagel, S.C., Summers, M.D., 1998. Autographa californica nuclear polyhedrosis virus: subcellular localization and protein trafficking of BV/ODV-E26 to intranuclear membranes and viral envelopes. Virology 240 (1), 64–75.

Braunagel, S.C., Russell, W.K., Rosas-Acosta, G., Russell, D.H., Summers, M.D., 2003. Determination of the protein composition of the occlusion-derived virus of Autographa californica nucleopolyhedrovirus. Proc. Natl. Acad. Sci. 100 (17), 9797–9802.

Cai, Y., Long, Z., Qiu, J., Yuan, M., Li, G., Yang, K., 2012. An ac34 deletion mutant of Autographa californica nucleopolyhedrovirus exhibits delayed late gene expression and a lack of virulence in vivo. J. Virol. 86 (19), 10432–10443.

Changela, A., Martins, A., Shuman, S., Mondragon, A., 2005. Crystal structure of baculovirus RNA triphosphatase complexed with phosphate. J. Biol. Chem. 280 (18), 17848–17856.

Chen, Y.R., Zhong, S., Fei, Z., Hashimoto, Y., Xiang, J.Z., Zhang, S., Blissard, G.W., 2013. The transcriptome of the Baculovirus *Autographa californica* multiple nucleopolyhedrovirus in *Trichoplusia ni* cells. J. Virol. 87 (11), 6391–6405.

Fang, Z., Li, C., Wu, W., Yuan, M., Yang, K., 2016. The Autographa californica multiple nucleopolyhedrovirus Ac132 plays a role in nuclear entry. J. Gen. Virol. 97 (11), 3030–3038.

Friesen, P.D., 1997. Regulation of baculovirus early gene expression. In: Miller, L.K. (Ed.), The Baculoviruses. Plenum Press, New York, N.Y, pp. 141–166.

Granados, R.R., Lawler, K.A., 1981. In vivo pathway of Autographa californica

baculovirus invasion and infection. Virology 108 (2), 297–308.
Gross, C.H., Shuman, S., 1998. Characterization of a baculovirus-encoded RNA 5'-triphosphatase. J. Virol. 72 (9), 7057–7063.
Hawtin, R.E., Zarkowska, T., Arnold, K., Thomas, C.J., Gooday, G.W., King, L.A., Kuzio, J.A., Possee, R.D., 1997. Liquefaction of Autographa californica nucleopolyhedrovirus-infected insects is dependent on the integrity of virus-encoded chitinase and cathepsin genes. Virology 238 (2), 243–253.
Herniou, E.A., Olszewski, J.A., Cory, J.S., O'Reilly, D.R., 2003. The genome sequence and evolution of baculoviruses. Annu. Rev. Entomol. 48 (1), 211–234.
Hodgson, J.J., Arif, B.M., Krell, P.J., 2011. Interaction of Autographa californica multiple nucleopolyhedrovirus cathepsin protease progenitor (proV-CATH) with insect Baculovirus chitinase as a mechanism for proV-CATH cellular retention. J. Virol. 85 (8), 3918–3929.
Huang, J., Hao, B., Deng, F., Sun, X., Wang, H., Hu, Z., 2008. Open reading frame Bm21 of Bombyx mori nucleopolyhedrovirus is not essential for virus replication in vitro, but its deletion extends the median survival time of infected larvae. J. Gen. Virol. 89 (4), 922–930.
Jehle, J.A., Blissard, G.W., Bonning, B.C., Cory, J.S., Herniou, E.A., Rohrmann, G.F., Theilmann, D.A., Thiem, S.M., Vlak, J.M., 2006. On the classification and nomenclature of baculoviruses: a proposal for revision. Arch. Virol. 151 (7), 1257–1266.
Lange, M., Wang, H., Zhihong, H., Jehle, J.A., 2004. Towards a molecular identification and classification system of lepidopteran-specific baculoviruses. Virology 325 (1), 36–47.
Li, A., Zhao, H., Lai, Q., Huang, Z., Yuan, M., Yang, K., Sandri-Goldin, R.M., 2015. Posttranslational modifications of Baculovirus protamine-like protein P6.9 and the significance of its hyperphosphorylation for viral very late gene hyperexpression. J. Virol. 89 (15), 7646–7659.
Liang, C., Lan, D., Zhao, S., Liu, L., Xue, Y., Zhang, Y., Wang, Y., Chen, X., 2014. The Ac124 protein is not essential for the propagation of Autographa californica multiple nucleopolyhedrovirus, but it is a viral pathogenicity factor. Arch. Virol. 160 (1), 275–284.
Lu, A., Krell, P.J., Vlak, J.M., Rohrmann, G.R., 1997. Baculovirus DNA replication. In: Miller, L.K. (Ed.), The Baculoviruses. Plenum Press, New York, N.Y, pp. 171–192.
Luckow, V.A., Lee, S.C., Barry, G.F., Olins, P.O., 1993. 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. 67 (8), 4566–4579.
Monsma, S.A., Oomens, A.G., Blissard, G.W., 1996. The GP64 envelope fusion protein is an essential baculovirus protein required for cell-to-cell transmission of infection. J. Virol. 70 (7), 4607–4616.
Nie, Y., Fang, M., Theilmann, D.A., 2009. AcMNPV AC16 (DA26, BV/ODV-E26) regulates the levels of IE0 and IE1 and binds to both proteins via a domain located within the acidic transcriptional activation domain. Virology 385 (2), 484–495.
O'Reilly, D.R., Miller, L., Luckow, V.A., 1992. Baculovirus Expression Vectors: A Laboratory Manual, Xiii. 347 p.. W.H. Freeman, New York.
Passarelli, A.L., Miller, L.K., 1993. Identification and characterization of *lef-1*, a baculovirus gene involved in late and very late gene expression. J. Virol. 67 (6), 3481–3488.
Pearson, M.N., Rohrmann, G.F., 2002. Transfer, incorporation, and substitution of envelope fusion proteins among members of the Baculoviridae, Orthomyxoviridae, and Metaviridae (insect retrovirus) families. J. Virol. 76 (11), 5301–5304.
Prikhod'ko, E.A., Miller, L.K., 1998. Role of baculovirus IE2 and its RING finger in cell cycle arrest. J. Virol. 72 (1), 684–692.
Roberts, T.E., Faulkner, P., 1989. Fatty acid acylation of the 67K envelope glycoprotein of a baculovirus: autographa californica nuclear polyhedrosis virus. Virology 172 (1), 377–381.
Rohrmann, G.F., 2013. Baculovirus Molecular Biology. U.S. National Library of Medicine, National Center for Biotechnology Information, Bethesda, MD.
Saville, G.P., 2004. Deletion of the Autographa californica nucleopolyhedrovirus chitinase KDEL motif and in vitro and in vivo analysis of the modified virus. J. Gen. Virol. 85 (4), 821–831.
Schmittgen, T.D., Livak, K.J., 2008. Analyzing real-time PCR data by the comparative C(T) method. Nat. Protoc. 3 (6), 1101–1108.
Takagi, T., Taylor, G.S., Kusakabe, T., Charbonneau, H., Buratowski, S., 1998. A protein tyrosine phosphatase-like protein from baculovirus has RNA 5'-triphosphatase and diphosphatase activities. Proc. Natl. Acad. Sci. U. S. A. 95 (17), 9808–9812.
Thomas, C.J., Brown, H.L., Hawes, C.R., Lee, B.Y., Min, M.K., King, L.A., Possee, R.D., 1998. Localization of a baculovirus-induced chitinase in the insect cell endoplasmic reticulum. J. Virol. 72 (12), 10207–10212.
van Oers, M.M., Vlak, J.M., 2007. Baculovirus genomics. Curr. Drug Targets 8 (10), 1051–1068.
Vaughn, J.L., Goodwin, R.H., Tompkins, G.J., McCawley, P., 1977. The establishment of two cell lines from the insect Spodoptera frugiperda (Lepidoptera; Noctuidae). In Vitro 13 (4), 213–217.
Volkman, L.E., 1986. The 64K envelope protein of budded Autographa californica nuclear polyhedrosis virus. Curr. Top. Microbiol. Immunol. 131, 103–118.
Wang, R., Deng, F., Hou, D., Zhao, Y., Guo, L., Wang, H., Hu, Z., 2010. Proteomics of the *Autographa californica* Nucleopolyhedrovirus budded virions. J. Virol. 84 (14), 7233–7242.
Wei, D., Wang, Y., Zhang, X., Hu, Z., Yuan, M., Yang, K., 2013. Autographa californica nucleopolyhedrovirus Ac76: a dimeric type II integral membrane protein that contains an inner nuclear membrane-sorting motif. J. Virol.
Whitford, M., Stewart, S., Kuzio, J., Faulkner, P., 1989. Identification and sequence analysis of a gene encoding gp67, an abundant envelope glycoprotein of the baculovirus Autographa californica nuclear polyhedrosis virus. J. Virol. 63 (3), 1393–1399.
Wu, W., Lin, T., Pan, L., Yu, M., Li, Z., Pang, Y., Yang, K., 2006. *Autographa californica* multiple nucleopolyhedrovirus nucleocapsid assembly is interrupted upon deletion of the *38K* gene. J. Virol. 80 (23), 11475–11485.
Yoo, S., Guarino, L.A., 1994. The Autographa californica nuclear polyhedrosis virus ie2 gene encodes a transcriptional regulator. Virology 202 (2), 746–753.
Yuan, M., Huang, Z., Wei, D., Hu, Z., Yang, K., Pang, Y., 2011. Identification of Autographa californica nucleopolyhedrovirus ac93 as a core gene and its requirement for intranuclear microvesicle formation and nuclear egress of nucleocapsids. J. Virol. 85 (22), 11664–11674.
Zeng, X., Nan, F., Liang, C., Song, J., Wang, Q., Vlak, J.M., Chen, X., 2009. Functional analysis of the Autographa californica nucleopolyhedrovirus IAP1 and IAP2. Sci. China C Life Sci. 52 (8), 761–770.