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Autographa californica M nucleopolyhedrovirus open reading frame 109 affects infectious budded virus production and nucleocapsid envelopment in the nucleus of cells

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ABSTRACT

Autographa californica M nucleopolyhedrovirus (AcMNPV) open reading frame 109 (*ac109*) is conserved in all known baculovirus genomes, suggesting a crucial role in virus replication. Although viruses lacking *ac109* have been previously characterized, the phenotypes differ from production of non-infectious virions to lack of virion production. To re-examine *ac109* function, we constructed a recombinant AcMNPV bacmid, AcBAC109KO, with a deletion in *ac109*. We did not detect infectious budded virus after transfection of AcBAC109KO DNA into cells. In the nucleus, nucleocapsids had envelopment defects and polyhedra lacked virions. DNA synthesis and gene expression between AcBAC109KO and a control virus were similar. However, lower levels of non-infectious budded virus were detected from AcBAC109KO DNA-transfected cells compared to the parental virus using Q-PCR to detect viral DNA or by immunoblotting to detect a budded virus protein. Therefore, deletion of *ac109* affects envelopment of nucleocapsids in the nucleus and the production of infectious budded virus.

Introduction

Baculoviruses are a large family of enveloped, rod-shaped viruses containing double-stranded DNA genomes. These viruses infect arthropods and have been primarily isolated from members of the order Lepidoptera, although some infect insects from two other orders. The replication cycle of the prototypical baculovirus, *Autographa californica* M nucleopolyhedrovirus (AcMNPV) can be divided by the production of two distinct viruses, budded virus (BV) and occlusion-derived virus (ODV). BVs are necessary for cell-to-cell virus transmission and are produced after nucleocapsids egress from their site of assembly in the nucleus, traffic through the cytoplasm of the cell, and bud off the infected cell acquiring a cell-derived envelope. ODVs, which are responsible for host-to-host virus transmission, are formed when nucleocapsids remaining in the nucleus at very late times after infection are thought to obtain a nuclear-derived envelope. ODVs are then embedded in a protein matrix mainly composed of the protein polyhedrin and form an occlusion body.

Recently, two independent reports were published on the characterization of AcMNPV recombinants lacking open reading frame 109 (*ac109*). In both of these reports, regions of *ac109* were deleted by homologous recombination in *Escherichia coli*. Deletions of *ac109* did not affect either viral DNA replication or the production of the very late protein polyhedrin; however, there was no cell-to-cell virus transmission. The hypothesized cause of a defect in virus transmission differed in these reports. Fang et al. (2009) detected virions in the supernatant of cells transfected with DNA from their *ac109* deletion virus and concluded that the deletion of *ac109* resulted in the production of non-infectious BV. Lin et al. (2009) were unable to find any evidence of nucleocapsid formation in cells transfected with their *ac109*-defective virus and this resulted in no BV or ODV production. They concluded that *ac109* was essential for nucleocapsid assembly.

To resolve the differences between these two reports and further characterize *ac109* in cell culture, we constructed a recombinant virus, AcBAC109KO, which carried a 94% deletion within the *ac109* coding region. In our experiments, deletion of *ac109* resulted in a defect in cell-to-cell virus infection and we could only detect the production of non-infectious BV; however, DNA replication, late gene synthesis, and polyhedrin production were similar to that of a control virus encoding *ac109*. In addition, nucleocapsids localized within the virogenic stroma and along the ring zone of the nucleus. At later times, the nucelocapsids lacked membrane envelopment prior to occlusion although microvesicles were formed in the ring zone. These results suggest that Ac109 is essential for both the production of infectious BV and the formation of an occlusion-derived envelope prior to occlusion.

Results

Homologs of *ac109* have been predicted in all baculoviruses sequenced to date (Fig. 1). However, BLAST and Structural

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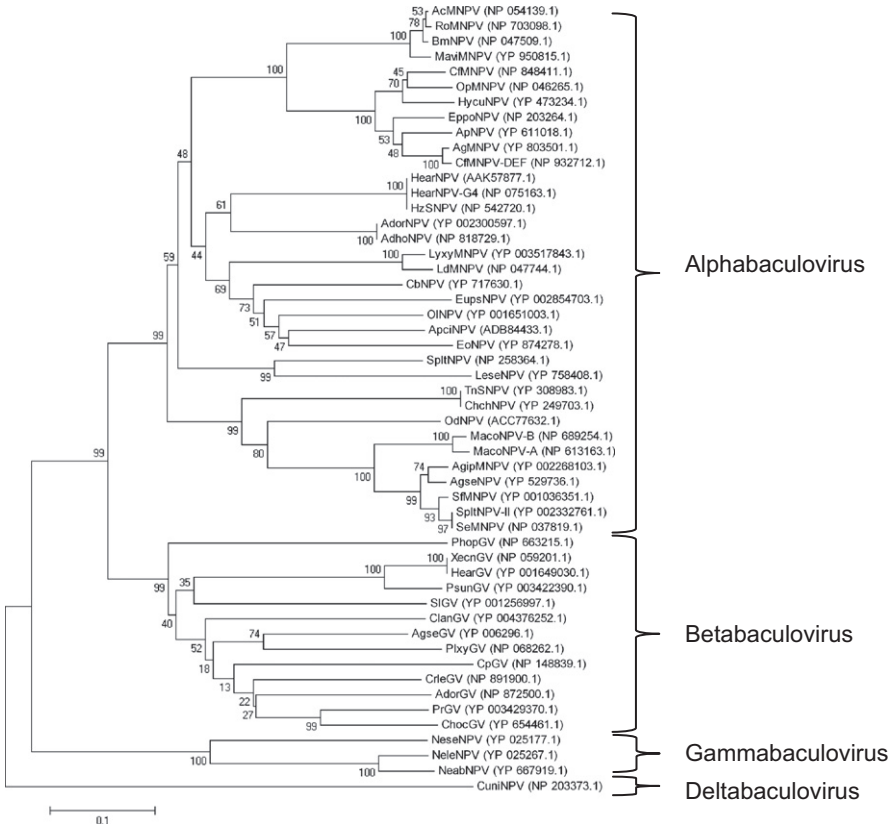

Fig. 1. Phylogenetic analysis of Ac109 homologs. Protein sequences with homology to Ac109 were aligned from 52 baculovirus genomes and a phylogenetic tree was obtained using MEGA 4.0.2 with 1000 bootstrap replicates. Numbers at nodes indicate bootstrap scores for the NJ analysis. Baculovirus genera are indicated to the right. Sequence similarity to Ac109 ranged from 98% (RoMNPV) to 47% (CuniMNPV). The numbers and letters in parenthesis refer to the National Center for Biotechnology Information RefSeq accession number. The virus abbreviations are: AcMNPV: *Autographa californica* M nucleopolyhedrovirus; AdhoNPV: *Adoxophyes honmai* nucleopolyhedrovirus; AdorGV: *Adoxophyes orana* granulovirus; AdorNPV: *Adoxophyes orana* nucleopolyhedrovirus; AgipMNPV: *Agrotis ipsilon* multiple nucleopolyhedrovirus; AgMNPV: *Anticarsia gemmatalis* nucleopolyhedrovirus; AgseGV: *Agrotis segetum* granulovirus; AgseNPV: *Agrotis segetum* nucleopolyhedrovirus; ApciNPV: *Apocheima cinerarium* nucleopolyhedrovirus; ApNPV: *Antheraea pernyi* nucleopolyhedrovirus; BmNPV: *Bombyx mori* nucleopolyhedrovirus; CbNPV: *Clanis bilineata* nucleopolyhedrosis virus; CfMNPV: *Choristoneura fumiferana* multiple nucleopolyhedrovirus; CfMNPV-DEF: *Choristoneura fumiferana* DEF multiple nucleopolyhedrovirus; ChchNPV: *Chrysodeixis chalcites* nucleopolyhedrovirus; ChocGV: *Choristoneura occidentalis* granulovirus; ClanGV: *Clostera anachoreta* granulovirus; CpGV: *Cydia pomonella* granulovirus; CrleGV: *Cryptophlebia leucotreta* granulovirus; CuniNPV: *Culex nigripalpus* nucleopolyhedrovirus; EoNPV: *Ecotropis obliqua* nucleopolyhedrovirus; EppoNPV: *Epiphyas postvittana* nucleopolyhedrovirus; EupsNPV: *Euproctis pseudoconspersa* nucleopolyhedrovirus; HearGV: *Helicoverpa armigera* granulovirus; HearNPV: *Helicoverpa armigera* nucleopolyhedrovirus; HearNPV-G4: *Helicoverpa armigera* nucleopolyhedrovirus G4; HycuNPV: *Hyphantria cunea* nucleopolyhedrovirus; HzSNPV: *Helicoverpa zea* single nucleopolyhedrovirus; LdMNPV: *Lymantria dispar* multiple nucleopolyhedrovirus; LeseNPV: *Leucania separata* nucleopolyhedrovirus; LyxyMNPV: *Lymantria xylina* multiple nucleopolyhedrovirus; MacoNPV-A: *Mamestra configurata* nucleopolyhedrovirus-A; MacoNPV-B: *Mamestra configurata* nucleopolyhedrovirus-B; MaviMNPV: *Maruca vitrata* multiple nucleopolyhedrovirus; NeabNPV: *Neodiprion abietis* nucleopolyhedrovirus; NeleNPV: *Neodiprion lecontii* nucleopolyhedrovirus; NeseNPV: *Neodiprion sertifer* nucleopolyhedrovirus; OdNPV: *Ophiusa disjungens* nucleopolyhedrovirus; OlNPV: *Orgyia leucostigma* nucleopolyhedrovirus; OpMNPV: *Orgyia pseudotsugata* multiple nucleopolyhedrovirus; PhopGV: *Phthorimaea operculella* granulovirus; PlxyGV: *Plutella xylostella* granulovirus; PrGV: *Pieris rapae* granulovirus; PsunGV: *Pseudaletia unipuncta* granulovirus; RoMNPV: *Rachiplusia ou* multiple nucleopolyhedrovirus; SeMNPV: *Spodoptera exigua* multiple nucleopolyhedrovirus; SfMNPV: *Spodoptera frugiperda* multiple nucleopolyhedrovirus; SlGV: *Spodoptera litura* granulovirus; SpltNPV: *Spodoptera litura* nucleopolyhedrovirus; SpltNPV-II: *Spodoptera litura* nucleopolyhedrovirus II; TnSNPV: *Trichoplusia ni* single nucleopolyhedrovirus; XecnGV: *Xestia c-nigrum* granulovirus.

Classification of Proteins domain searches using Ac109 as the query did not show significant sequence similarity with non-baculovirus proteins. An *in silico* examination of the predicted *ac109* gene product, suggests a basic 44.8 kilodalton (kDa) protein with multiple post-translational modification sites according to protein sequence analysis.

The region upstream of *ac109* has a consensus late/very late gene initiator site GTAAG (Blissard et al., 1992; Kogan et al., 1995) at −14 nucleotides and a TATA-like element (Pullen and Friesen, 1995) at −8 nucleotides upstream of the translational start codon (Fig. 2B). Northern blot analysis using total RNA extracted from SF-21 cells infected with AcMNPV and hybridized to an *ac109* RNA-specific probe, identified a ~1.6 kb RNA first evident at 9 h p.i. The transcript was slightly reduced in infected cells treated with aphidicolin, a viral DNA synthesis inhibitor, but it was not affected by the presence of cycloheximide. It is not clear if an early transcript also hybridizes with the probe or if the inhibitor did not fully block gene transcription (Ross, Guarino, 1997). A slightly longer transcript was apparent at 24 and 48 h p.i. (Fig. 2A). These can potentially be bicistronic messages of *ac109* and *ac110* or *ac109* and *ac108*.

To monitor Ac109 synthesis during virus infection, we constructed a virus, AcBAC109glyHA, encoding Ac109 with a C-terminal HA tag and three glycines prior to the tag in the AcBAC109KO backbone. The modified Ac109, Ac109glyHA, was observed starting at 12 h p.i. and accumulated through the remainder of the time course. The protein was not produced in the presence of aphidicolin, suggesting that DNA replication was necessary for its synthesis (Fig. 2C). As expected, proteins were not produced when the protein synthesis inhibitor cycloheximide was used.

To determine the role of Ac109 during AcMNPV replication, a recombinant AcMNPV lacking *ac109*, AcBAC109KO, was generated (Fig. 3). Briefly, 94% of *ac109* was replaced with the coding sequence of the chloramphenicol resistance gene using homologous recombination and the commercially available AcMNPV-based bacmid

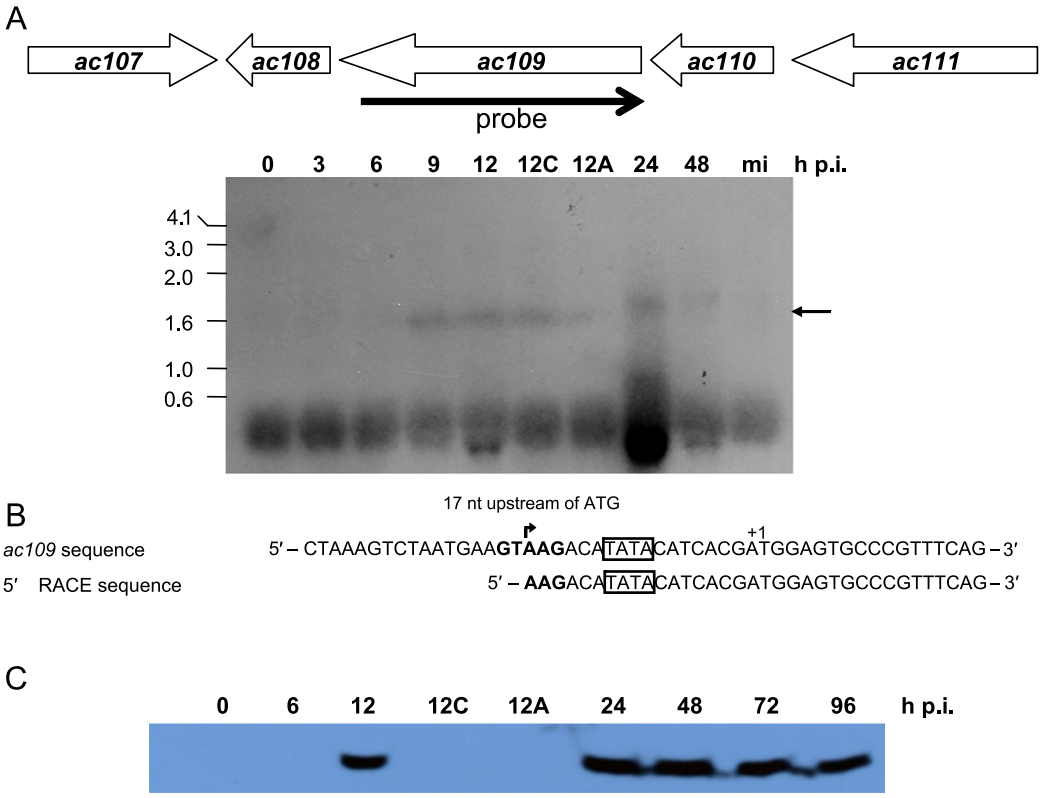

Fig. 2. *ac109* transcription and protein synthesis. (A) Region flanking *ac109* is shown in the top panel. Open arrows illustrate relative size and direction of transcription of open reading frames. RNA probe (black arrow) used for the Northern blot in the lower panel is indicated. Numbers at the top indicate h p.i. Total RNA collected at 12 h p.i from cells treated with cycloheximide (12C) or aphidicolin (12A) is also shown. Numbers to the left correspond to standards in kb. (B) *ac109* transcription start site (arrow) determined by 5′ RACE. The late promoter GTAAG is shown in bold-type, the TATA element is boxed, and the *ac109* translation start codon is indicated as +1. (C) SF-21 cells were infected with AcBAC109glyHA and treated with cycloheximide (12C) or aphidicolin (12A). At the times indicated at the top, the cells were collected, lysed and proteins fractionated on an SDS-polyacrylamide gel. After transfer to a PVDF membrane, the samples were immunoblotted using anti-HA antibody.

bMON14272. Since this virus lacks the polyhedrin (*polh*) gene, we used transposition to introduce two cassettes, *polh* and its promoter and the enhanced green fluorescent protein (*egfp)* gene under *Drosophila* heat shock protein (hsp) 70 promoter control (Detvisitsakun et al., 2006). To ensure that the effects of deleting *ac109* were specific and other mutations were not introduced during virus manipulation, we also constructed a repaired virus, AcBAC109Rep, in which *ac109* was reintroduced into the *ac109* deficient background. In this case, in addition to *egfp* and *polh*, *ac109* driven by a promoter element contained in the 49-bp region upstream of *ac109* was introduced into the bacmid genome at the *polh* locus. The 49-bp promoter region contains both the TATA element and a late promoter GTAAG motif. After construction of AcBAC109KO and AcBAC109Rep, PCR was used to confirm that recombination and translocation occurred as designed (Fig. 3B).

DNA from AcBAC109KO and AcBAC109Rep were individually transfected into SF-21 cells, and cells were observed for cytopathic effects and eGFP expression. At 24 h p.t., between 20 and 35% of AcBAC109KO DNA- and AcBAC109Rep DNA-transfected cells were expressing eGFP, corresponding to cells initially transfected with bacmid DNA (Fig. 4A and C, respectively). At 48 h p.t., the majority of the AcBAC109Rep DNA-transfected cells expressed eGFP (Fig. 4D), whereas in AcBAC109KO DNA-transfected cells, the number of fluorescent cells remained relatively constant (Fig. 4B). The increase in fluorescent cell number suggested that AcBAC109Rep DNA-transfected cells produced infectious BV, which led to the infection of untransfected cells. On the other hand, AcBAC109KO DNA-transfected cells did not appear to support secondary infections and only those cells initially transfected showed eGFP expression. However, by 96 h p.t., AcBAC109KO DNA-transfected cells formed occlusion bodies, suggesting that viral replication proceeded to very late times (Fig. 4M).

To determine the production of infectious budded virus, supernatant from AcBAC109KO DNA- or AcBAC109Rep DNA-transfected cells was harvested at 108 h p.t. and used to treat uninfected SF-21 cells. By 24 h p.i., all the cells infected with AcBAC109Rep showed signs of infection as indicated by fluorescent cells (Fig. 4J) and contained occlusions by 48 h p.i. (data not shown), whereas none of the cells treated with AcBAC109KO supernatants appeared to be infected (Fig. 4I). In addition, supernatant from AcBAC DNA-, AcBAC109KO DNA- or AcBAC109Rep DNA-transfected cells was used to perform a single-step growth curve in SF-21 cells (Fig. 4N). Cells treated with AcBAC109KO supernatants failed to show signs of infection throughout the time course, while cells infected with the AcBAC109Rep and AcBAC viruses had similar kinetic profiles. The replication kinetics of AcBAC109glyHA were also tested, and it produces less infectious budded virus than AcBAC109Rep but not as low as that of AcBAC109HA (data not shown), confirming that addition of epitope tags affects function.

We confirmed that the phenotype in AcBAC109KO DNA-transfected cells was due to a disruption of *ac109* by transcomplementing with plasmids expressing Ac109 or a truncated Ac109. The plasmids p109 (encoding Ac109 and Ac110) and p109FS (containing a frame shift mutation within *ac109* that introduces a premature stop codon and truncates Ac109 by 40%) were cotransfected with AcBAC109KO DNA into SF-21 cells (Fig. 4E and G, respectively). After 48 h p.t., all the cells cotransfected with p109 expressed eGFP (Fig. 4F), while the percentage of those cotransfected with the frame-shift mutant of *ac109* remained constant

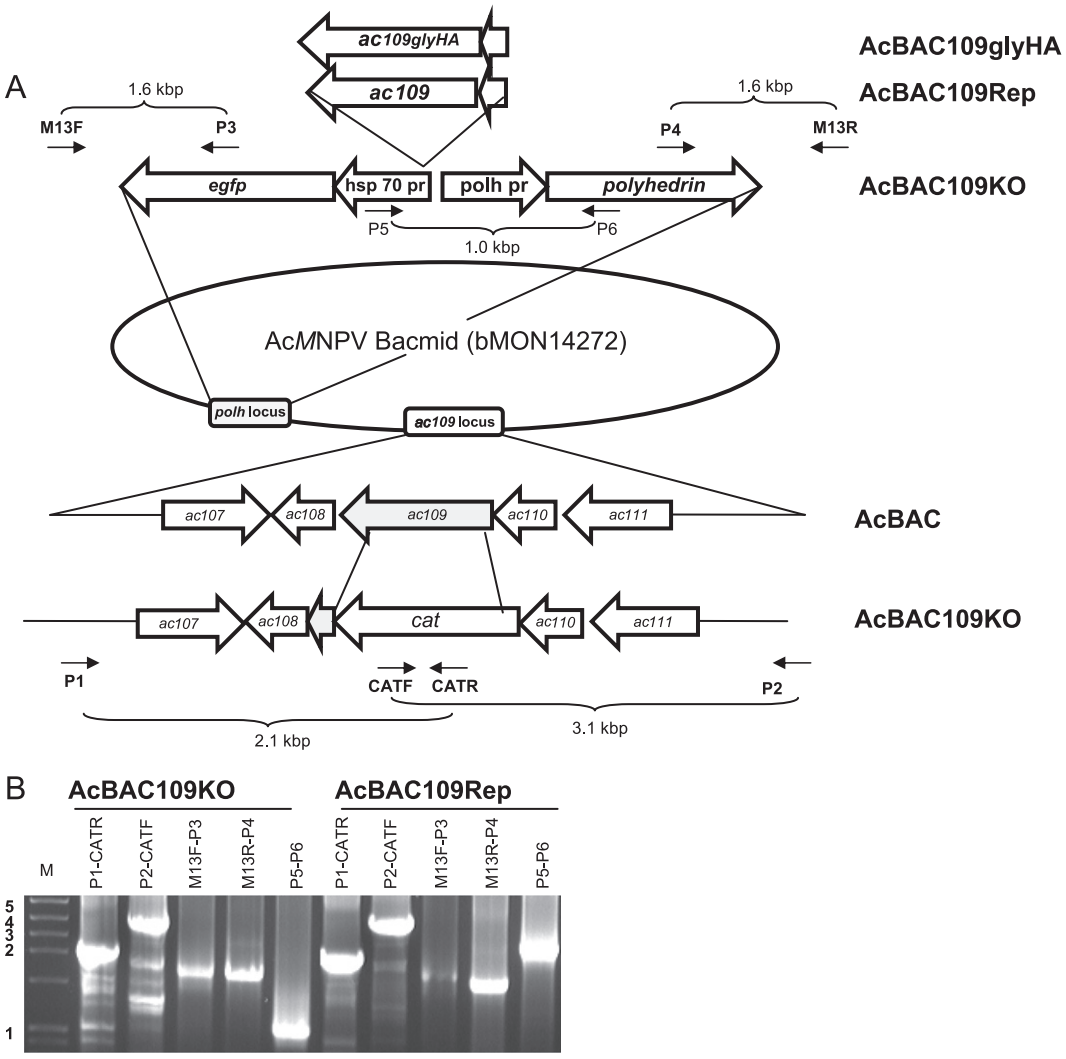

Fig. 3. Construction of AcBAC109KO, AcBAC109Rep and AcBAC109glyHA viruses. (A) Using homologous recombination, 94% of *ac109* was replaced with the chloramphenicol resistance gene, leaving a 69-nucleotide C-terminal fragment to serve as the promoter of *ac108*. After recombination, transposition was used to reintroduce the *polyhedrin* (*polh*) gene under its promoter (pr) control and the enhanced green fluorescent protein (*egfp*) gene under the *Drosophila* heat shock protein 70 (hsp70) promoter control. For AcBAC109Rep and AcBAC109glyHA, in addition to transposition of *polh* and *egfp*, *ac109* or the HA epitope tagged *ac109* was reintroduced into the AcBAC109KO backbone driven by a 49-nucleotide *ac109* promoter element. Primers (small arrows) used to characterize bacmids and expected PCR fragment sizes are indicated. (B) To determine correct recombination and transposition events, PCR using specific primer sets indicated at the top was used to characterize AcBAC109KO and AcBAC109Rep. Numbers to the left indicate the mass of DNA standards in kbp (lane M).

(~30% of cells) (Fig. 4H). Supernatant from these transfected cells was used to treat uninfected SF-21 cells, but only the supernatant from AcBAC109KO DNA- and p109-cotransfected cells produced infectious budded virus (Fig. 4, compare K to L).

AcBAC109KO-infected cells may not produce BVs or produce non-infectious BV. Lack of BV production may be due to defective viral DNA replication, late viral gene expression, packaging, or shuttling of nucleocapsids to the cell surface or their subsequent budding from cells. To explore some of these possibilities, we first evaluated viral genome replication and gene expression. SF-21 cells were transfected with DNA from AcBAC, AcBAC109KO, AcBAC109Rep, or vAc^{gp64-}, a bacmid with a mutation in the envelope fusion protein gene, *gp64*, that is unable to spread in cells but able to replicate viral DNA (Oomens and Blissard, 1999). At specific h p.t., total DNA from transfected cells was harvested and viral DNA quantified using quantitative real time polymerase chain reaction (Q-PCR). The viral genome copy number was determined for all the samples. Both, AcBAC109KO- and AcBAC109Rep DNA-transfected cells, showed increasing amounts of viral DNA with time, suggesting that both viruses had the ability for DNA biosynthesis (Fig. 5A). The lack of increase in viral genome copy number in AcBAC109KO and vAc^{gp64-} after 48 h p.i., compared to AcBAC and AcBAC109Rep, is likely due to defects in infectious virus spread.

To determine if AcBAC109KO could express late genes, we used reverse transcriptase (RT)-PCR and gene-specific primer sets to determine the expression of a representative early gene, *late expression factor-1* (*lef-1*), a late gene, *vp39*, and a very late gene, *polh*. SF-21 cells were transfected with either AcBAC109KO or vAc^{gp64-} DNA, and total RNA was harvested at 12, 24, and 48 h p.t. and transcription from each of the target genes was detected, suggesting that deletion of *ac109* did not adversely affect these genes and probably other genes in these stages (Fig. 5B).

To explore further if AcBAC109KO produced BV, Sf9 cells were transfected with AcBAC109KO or AcBAC109Rep DNA and supernatants were collected at 48 and 72 h p.t. and lysates immunoblotted with anti-GP64, an antibody against the envelope fusion protein. GP64 was detected in all samples which suggests that Ac109KO produced non-infectious BV (Fig. 6A); however, we detected less GP64 in the AcBAC109KO lysates, suggesting a defect

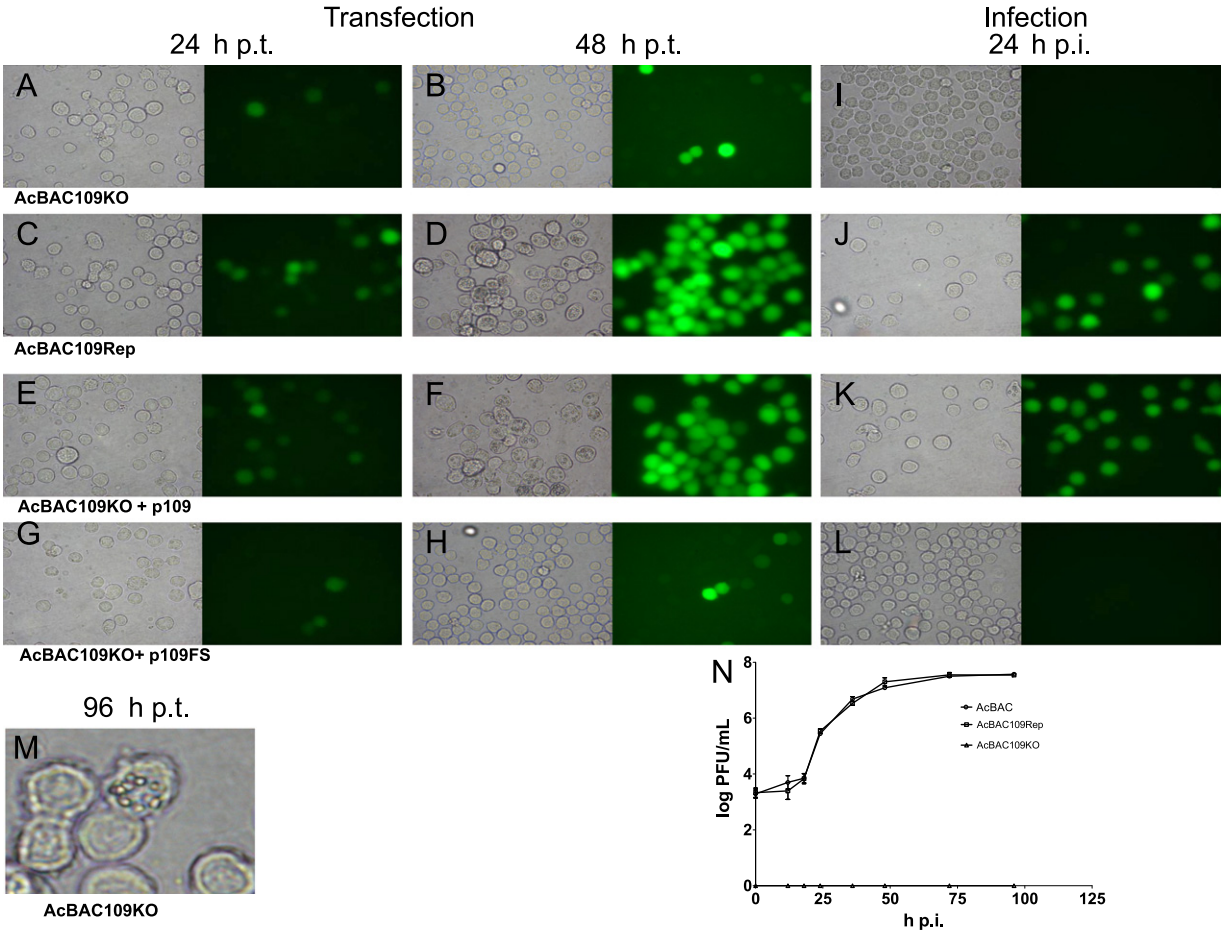

Fig. 4. Infectivity and complementation of AcBAC109KO. AcBAC109KO (A, B) or AcBAC109Rep (C, D) DNA was transfected into SF-21 cells and cells examined for eGFP expression at the times post transfection (p.t.) indicated. At 48 h p.t., supernatant was removed from the transfected cells and used to treat naïve SF-21 cells. eGFP expression was visualized at 24 h p.i. (I and J). To transcomplement the deletion phenotype, a plasmid containing *ac109* under its promoter control (p109) or a frame shift mutant (p109FS) was cotransfected with AcBAC109KO DNA into SF-21 cells and eGFP expression was visualized (E to H). The supernatant from each transfection was collected at 48 h p.t. and used to treat naïve SF-21 cells and visualized at 24 h p.i. (K and L). Occlusion bodies present in SF-21 cells transfected with AcBAC109KO DNA were observed at 96 h p.t. (M). Single-step growth curves from the supernatants from AcBAC-, AcBAC109KO- and AcBAC109Rep DNA-transfected cells are shown (N).

in producing budded virions. We obtained similar results using SF-21 cells (data not shown). To confirm this result, we determined if concentrated supernatants from bacmid-transfected cells contained viral DNA using Q-PCR . AcBAC109KO and AcBAC109Rep samples had viral DNA, although it was lower in the AcBAC109KO samples (Fig. 6B). To confirm that we did not have DNA contamination from lysed cells, we treated or untreated DNA samples with DNase I prior to DNA purification. Similar results were obtained with mock-infected samples spiked with bacmid DNA suggesting that our DNA purification was not contaminated with bacmid transfected DNA or DNA from nucleocapsids in the cells (Fig. 6B, mi lanes).

Since AcBAC109KO DNA-transfected cells were able to express representative viral genes in the early, late and very late gene expression phases and viral DNA replication was unaffected, we considered that the inability of AcBAC109KO DNA-transfected cells to produce infectious BV may be related to events occurring during nucleocapsid assembly. To examine nucleocapsid structures in cells, SF-21 cells were transfected with AcBAC109KO or AcBAC109Rep DNA and, at set intervals, cells were prepared for electron microscopy. In four independent transfections, cells transfected with AcBAC109Rep DNA produced nucleocapsids visualized in the cytoplasm and nucleus (Fig. 7, top and middle right panels). In cells transfected with AcBAC109KO DNA, nucleocapsids were detected in the nucleus but not in the cytoplasm of at least fifty transfected cells that were individually visualized (Fig. 7, top left panel and data not shown). Our inability to detect nucleocapsids in the cytoplasm may be related to the reduced levels of budded virus production in AcBAC109KO DNA-transfected cells.

At 72 h p.t., nucleocapsids aligned in the nucleus in both AcBAC109Rep- or Ac-BAC109KO DNA-transfected cells (Fig. 7, middle panels). However, many of the nucleocapsids in the AcBAC109KO DNA-transfected cells were devoid of the characteristic ODV envelope. ODVs embedded in the occlusion bodies were not apparent in AcBAC109KO DNA-transfected cells compared to AcBAC109Rep DNA-transfected cells (Fig. 7, lower panels).

In a recent study with *Helicoverpa armigera* nucleopolyhedrovirus (HearNPV), the interactions between ODV proteins using a yeast two-hybrid screening method were reported. Ha-EC43, a homolog of Ac109, interacted with HA9, the homolog of Ac142 (Peng et al., 2010). To confirm these results with AcMNPV proteins, we performed interaction studies using plasmids expressing an HA- or FLAG-epitope tagged Ac109 and Ac142. Ac142 immunoprecipitated Ac109 (Fig. 8, third lane). Interestingly, the deletion of Ac142, another baculovirus core gene of unknown function, from the genome of AcMNPV results in a phenotype similar to the one observed when deleting Ac109; deletion of Ac142 results in the loss of BV production and lack of nucleocapsid envelopement in the nucleus and their occlusion (Vanarsdall et al., 2007; McCarthy et al., 2008). Although this interaction could only be verified in one direction, Ac142 pulling down Ac109, and we could not detect self-interactions, Ac109 or

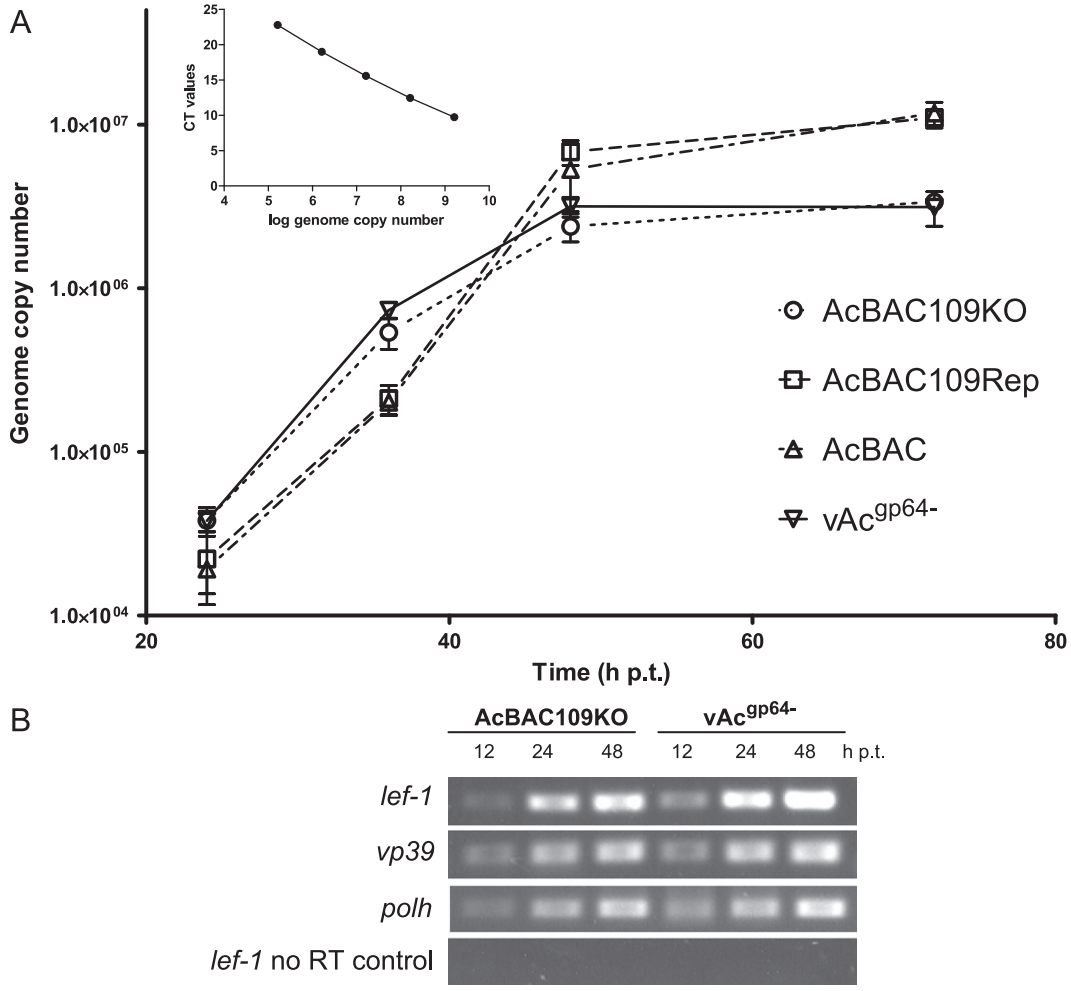

Fig. 5. Viral DNA synthesis and expression of selected viral genes. SF-21 cells were transfected with 1 μg of AcBAC, AcBAC109KO, AcBAC109Rep, or vAc^{gp64-}. At specific time points post transfection (p.t.), total DNA and RNA were harvested from cells. (A) Total DNA of each sample (2 μg total) was used to amplify a segment of *gp41*. The number of viral genome copies present in the transfected cells was obtained using the standard curve (inset). (B) Total RNA (60 ng total) from the vAc^{gp64-} DNA- and AcBAC109KO DNA-transfected cells was used with reverse transcriptase (RT) and specific primer sets to assess expression from the viral genes indicated to the left. The last row contains no RT.

Ac142, the similarity in phenotypes of the deletion mutants, makes it tempting to speculate that Ac109 and Ac142 form a functional complex during the course of infection necessary for virus replication.

Discussion

There are 31 core genes present in all baculovirus genomes sequenced to date, including seven with unknown function (Rohrmann, 2011). Phylogeny studies using Ac109 show a clear relationship between the baculoviruses and their host, indicating that Ac109 may be useful to define virus evolution. ac*109* has been only partially characterized. To examine the function of *ac109* during virus replication, we constructed AcBAC109KO, a virus lacking *ac109*, and two *ac109* repaired viruses, AcBAC109Rep and AcBAC109glyHA, in which *ac109* or *ac109* with an HA tag was introduced into the genome of the AcBAC109KO. Since we and others (Fang et al., 2009) had difficulty repairing AcBAC109KO with a tagged *ac109*, we introduced glycines prior to the tag to alleviate possible defects in protein conformation. The repaired viruses, AcBAC109Rep and AcBAC109glyHA, either fully or partially repaired AcBAC109KO, respectively (Fig. 3 and data not shown). Following transfection with AcBAC109KO DNA, there was no production of infectious budded virus. This phenotype was rescued by either reintroducing *ac109* into the AcBAC109KO backbone or by complementing *ac109* function by cotransfecting a plasmid expressing *ac109* with AcBAC109KO DNA. Deletion of *ac109* from the viral genome does not appear to affect viral DNA replication or expression of baculovirus genes expressed at different transcriptional stages. Transmission electron microscopy of cells transfected with AcBAC109KO DNA showed nucleocapsids in the nucleus of cells similar to those produced after transfection with AcBAC109Rep DNA. At late times, nucleocapsids present in the nucleus stacked but failed to acquire virus envelopes or become incorporated into occlusions.

Our results agree with previous findings where deletion of *ac109* resulted in the production of non-infectious BV (Fang et al., 2009). Fang et al. also detected reduced levels of the structural proteins in *ac109*-deficient BV, suggesting that *ac109* was necessary for efficient BV production. This is consistent with our Q-PCR and similar immunobloting analyses.

The phenotype of a virus lacking *ac109* reported by Lin et al. (2009) is different to that observed by Fang et al. (2009) and to that described here. Although they describe a deficiency in cell-to-cell transmission, they were unable to detect any nucleocapsids in transfected cells. Transmission electron microscopy images of cells transfected with DNA from an *ac109* deleted virus, showed enlarged

nuclei and electron dense material similar to virogenic stroma but no nucleocapsids. We show detailed transmission electron micrographs showing the specific stage at which nucleocapsids lacking Ac109 are defective (no envelopment of nucleocapsids and no incorporation into occlusion bodies). Lin et al., deleted a 278-nucleotide internal fragment (514–791 nt) from *ac109* and replaced it with a *zeocin*-expressing selection marker cassette. This method resulted in an N-terminal a*c109* fragment that can potentially encode an important function by itself or interact with another protein. Whether this affected the phenotype is not clear.

A similar phenotype to that observed here for *ac109* (no ODV formation but formation and stacking of nucleocapsids) was also reported for *ac103*- (Yuan et al., 2008) and *ac142*-deficient (McCarthy et al., 2008) viruses. Ac109, Ac142, and Ac103 are the only genes reported to be involved in envelopment of nucleocapsids

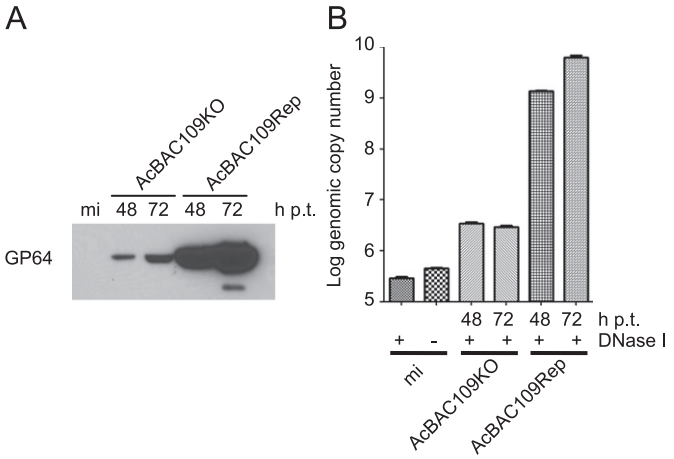

Fig. 6. Detection of BV from AcBAC109KO-transfected Sf9 cells. Sf9 cells were transfected with AcBAC109KO or AcBAC109Rep DNA or mock-transfected. At the indicated times (h post transfection, h p.t.), supernatants were collected, passed through a 0.8 μm filter, and centrifuged at 20,000*g* to pellet any virus particles. Virus particles were used for immunoblotting or Q-PCR. (A) Proteins derived from purified virus particles were separated on a SDS-12% polyacrylamide gel. After transfer to a PVDF membrane, proteins were immunoprobed with anti-GP64 V5 antibody and goat-anti-mouse-HRP secondary antibody. (B) The pelleted particles were resuspended in H_2O and treated with DNase I (+) and then lysed with virus disruption buffer, DNA was extracted by phenol/chloroform and precipitated with ethanol. Purified DNA was resuspended in 50 μl H_2O and 5 μl were used as template for Q-PCR.

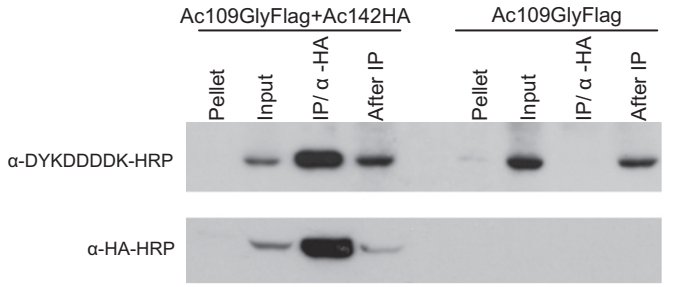

Fig. 8. Coimmunoprecipitation of Ac109 and Ac142. SF-21 cells were cotransfected with plasmids pHS70PL-orf109GlyFlag-polyA and pHS70-Ac142-HA or transfected with pHS70PL-orf109GlyFlag-polyA. At 24 h p.t., cells were lysed and proteins immunoprecipitated (IP) with α-HA antibody and separated on a SDS-12% polyacrylamide gel, transferred to a PVDF membrane, and probed with α-DYKDDDDK-HRP conjugated antibody (similar to anti-FLAG-HRP from Sigma). The same membrane was antibody stripped and probed again with α-HA-HRP conjugated antibody.

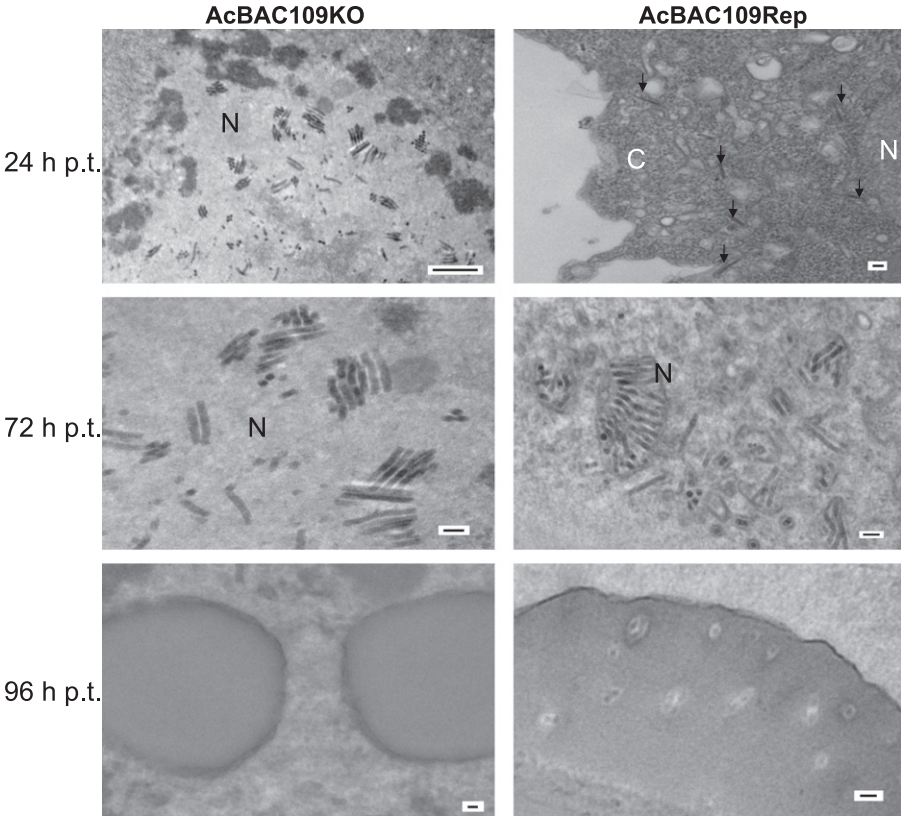

Fig. 7. Transmission electron microscopy of AcBAC109KO DNA- and AcBAC109Rep DNA- transfected cells. SF-21 cells were transfected with AcBAC109KO (left column) or AcBAC109Rep (right column) DNA, and cells were fixed at the times shown to the left. N, Nucleus; C, cytoplasm; arrows nucleocapsids. Occlusion bodies from viral DNA-transfected cells are shown in the lower panels. Scale bars: Left top panel, 500 nm; others, 100 nm.

destined to become ODV. More in depth characterization and interrelationships will be important to define this process.

In this study, we contributed new information to the function of *ac109* by providing *ac109* expression and Ac109 production profiles (Fig. 2), the effects of *ac109* on early, late and very late gene transcription (Fig. 5), detailed ultrastructural visualization of nucleocapsids in AcBAC109KO DNA-transfected cells (Fig. 7), and interactions between Ac142 and Ac109, two proteins that appear to have similar functions (Fig. 8). This information along with additional studies presented here and previously reported (Fang et al., 2009 and Lin et al., 2009) will allow determining the specific role of *ac109*.

Materials and methods

Cells and viruses

The cell line IPLB-SF-21 (SF-21) (Vaughn et al., 1997), and the Sf9, clonal isolate 9 from IPLB-SF-21-AE, were derived from the fall armyworm, *Spodoptera frugiperda*, and were maintained in TC-100 medium (Invitrogen) supplemented with 10% fetal bovine serum (Atlanta Biologicals) and 0.26% tryptose broth.

Transfection of cells

Bacmid DNA (1 μg) was mixed with 4 μl of a liposome preparation and added to 1×10^6 cells as previously described (Crouch and Passarelli, 2005). Cells were maintained at 27 °C for 4 h in the liposome–DNA mixture, then the media was removed from the cells and the cells were washed once with TC-100 media. After washing, TC-100 media containing 10% fetal bovine serum was added to the transfected cells and incubated at 27 °C until they were harvested.

Imaging cells expressing GFP

Cells were imaged on a Nikon Eclipse TE200 microscope with a 40× lens. Pictures were captured with a Nikon Coolpix 955 camera set at maximum resolution. The images shown are representative of four independent experiments.

Generation of AcBAC-Δorf109, a bacmid lacking ac109

AcMNPV DNA was digested with *Eco* RI and the 4035 bp fragment corresponding to nucleotides 93,589 to 97,623 (Ayers et al., 1994) was ligated to pBlueScript (Stratagene), creating pB1E4-3 . pB1E4-3 was digested with *Mlu* I (AcMNPV site 94,788–94,793) and *Nde* I (AcMNPV site 96,379–96,384) to remove 1592 bp, including orfs *ac111, ac110*, and 94% of *ac109*. Using pB1E4-3 as a template and oligonucleotides 109MluI (5′-GAACGCGTCGTGATGTATATGTCTTACTTCA-3′) and 111NdeI (5′-GGAATTCCATATGCATCAGTGACATTGCCT-3′), a PCR fragment containing full-length *ac111* and *ac110* was obtained (corresponding to 491 bp of AcMNPV from nt 95,894 to 96,384). This fragment was ligated to pCR II (Invitrogen) for sequencing and then digested with *Mlu* I and *Nde* I and ligated to the digested pB1E4-3 to generate pB1E4-3-Δ109. The chloramphenicol resistance gene (*cat*) and promoter was amplified from pUC18CMR (ATCC # 37,718) using the primer set CAT1 (F 5′-GAACGCGTTCCGTCGATCATATCGTCAA-3′) and CAT2 (R 5′-GAACGCGTCGCCACATAGCAGAACTTTA-3′). This 1579 bp fragment flanked by *Mlu* I sites was digested with *Mlu* I and ligated to pB1E4-3-Δ109 digested with the same enzyme to make the recombination vector pB1E4-3-Δ109CAT. This vector was digested with *Ava* I and a 4.5-kpb fragment containing the *cat* cassette and *ac109* flanking regions was purified. Following the method of Bideshi and Federici (2000), 1.5 ug of this fragment were used to transform BJ5183 cells containing bMON14272, the commercially available AcMNPV bacmid (Invitrogen). After incubation (4 h) in Super Optimal broth with catabolite repression (SOC) media at 37 °C, the transformed cells were plated on Luria Bertani (LB) broth agar plates supplemented with kanamycin (50 μg/ml) and chloramphenicol (25 μg/ml). Colonies growing after 24 h were screened using PCR for the presence of *cat* within the *ac109* locus, using the primer sets P1 and CAT-R (5′-TGTGCTGCTTACTGTGCCTGTAT-3′ and 5′-AACGTTTTCATCGCTCTGGAGT-3′, respectively), and CAT-F and P2 (5′-CCAGGTTTTCACCGTAACACG-3′ and 5′-CGACATTAACAAAGAGCCATTGA-3′, respectively). Both P1 (nt 93,336–93,358 of AcMNPV) and P2 (nt 97,918–97940 of AcMNPV) were outside the area of recombination in order to confirm the correct insertion site. The resulting *ac109*-knockout bacmid, AcBAC-Δorf109, was electroporated into MAX Efficiency DH10B competent *E. coli* cells (Invitrogen) in the presence of the T7 translocation helper plasmid pMON7124 and cells were plated on LB plates supplemented with kanamycin (50 μg/ml), chloramphenicol (25 μg/ml) and tetracycline (10 μg/ml).

Generation of AcBAC109KO

To construct AcBAC109KO, *E. coli* DH10B carrying AcBAC-Δ*orf109* and the T7 helper plasmid were transformed with the T7 transplacement vector pFastBac–*polh*+*gfp*+ (Detvisitsakun et al., 2006). After transformation, bacteria were plated on LB plates supplemented with kanamycin (50 μg/ml), chloramphenicol (25 μg/ml), gentamicin (7 μg/ml), tetracycline (10 μg/ml), 100 μg/ml 5-bromo-4-chloro-3-indoxyl-beta-D-galactopyranoside, and 40 μg/ml isopropyl-1-thio-β-D-galactoside as directed in the Bac-to-Bac Expression System Manual (Invitrogen). White colonies were selected and screened for the presence of *egfp* and *polh* genes using PCR amplification. The primer sets used were M13F (F 5′-GTTTTCCCAGTCACGAC-3′) with P3 (R 5′-CATGGACGAGCTGTACAAGTAAAGC-3′), P5 (F 5′-CTGCAACTACTGAAATCAAC-3′) with P6 (R 5′-CAAGGAAAACATCCATCACTTCTTG-3′) and P4 (F 5′-CGAACATGAGATCGAAGAGGCTAC-3′) with M13R (R 5′-CAGGAAACAGCTATGAC-3′).

Construction of AcBAC109Rep and AcBAC109glyHA

We constructed a transfer vector to generate a recombinant of AcMNPV bacmid expressing *ac109* under its native promoter control. First, oligonucleotides (Sac I-F 5′-CGGTACGAGCTCACAAATTAAAATAATCTAAAG-3′ and Sac I-R 5′-GACGTGAGCTCCTACAAATAATAGTTGTACTTGA-3′) were used to amplify a 1222-bp fragment from pB1E4-3 consisting of a 49 bp *ac109* leader sequence and the complete *ac109* coding sequence. The PCR product was cloned into the transplacement vector pFastBac−*polh*+*gfp*+ (Detvisitsakun et al., 2006) at *Sac* I site to generate pFastBac−*polh*+-orf109-*gfp*+. The correct sequence was verified by nucleotide sequencing analysis. DH10B cells containing the AcBAC-Δorf109 backbone and T7 helper plasmid were transformed with pFastBac−*polh*+-orf109-*gfp*+. Transformed cells were grown on kanamycin (50 μg/ml), chloramphenicol (25 μg/ml), gentamicin (7 μg/ml), tetracycline (10 μg/ml), 5-bromo-4-chloro-3-indoxyl-beta-D-galactopyranoside (100 μg/ml), and isopropyl-1-thio-β-D-galactoside (40 μg/ml) media according to the Bac-to-Bac Expression System Manual (Invitrogen). White colonies were selected and the transposition event and flanking regions were verified by PCR analysis. AcBAC109glyHA was constructed similarly to AcBAC109Rep. Two primers, Native orf 109 promo (5′-ACAAATTAAAATAATCTAAAGTCTAATGAAG-3′) and HA-tag-Gly-orf109 (5′-CTAGGCGTAATCTGGGACGTCGTATGGGTATCCTCCTCCCAAATAATAGTTGTACTTGACG-3′) were used to amplify the 49 bp *ac109* promoter element and the complete *ac109* coding sequence with a three-glycine linker prior the C-terminal HA tag in pBIE4-3 . After amplification, the PCR product was ligated into the pCR II vector and the clone sequenced. The plasmid was digested with *Sac* I

and *Xba* I to release the a*c109* tagged orf and ligated to pFastBac-PH-GFP (Wu et al., 2006) which had been digested with the same enzymes to generate plasmid pFB-PG-native orf109glyHA. DH10B cells containing the AcBAC-Δorf109 backbone and T7 helper plasmid were transformed with pFB-PG-native orf109glyHA and the resulting virus, AcBAC109glyHA, was verified by PCR analysis. Budded virus was produced by transfection of AcBAC109Rep or AcBAC109glyHA DNA into SF-21 cells by liposome-mediated transfection as previously described (Crouch and Passarelli, 2005).

Complementation assay

SF-21 cells were transfected with 1 μg AcBAC109KO or AcBAC109KO DNA and 1 μg of plasmid p109 or p109FS obtained from Lois K. Miller. p109 contains *ac109* and *ac110* in a 1498 bp *Sal* I to *Sca* I fragment cloned into pBluescript KS+ (Invitrogen) at *Sal* I and *Eco* RV sites while p109FS contains a frameshift mutation at the *Nco* I site, created by blunt ending the *Nco* I digested plasmid with T4 DNA ligase and then re-ligating it to generate a premature stop codon in *ac109*. As a control, SF-21 cells were also transfected with AcBAC109Rep DNA and 1 μg pBluescript vector (Invitrogen). At 24 and 48 h p.t., transfected cells were examined for *egfp* expression. Budded virus production was determined by performing $TCID_{50}$ assays using the supernatant from the 48 h p.t sample (O′Reilly et al., 1994).

Virus growth curves

SF-21 cells were infected with AcBAC109Rep or AcBAC at a multiplicity of infection (MOI) of 5 plaque forming units (PFU)/cell. Budded virus was collected at different times post infection (p.i.) and titers were determined by $TCID_{50}$. Since AcBAC109KO is not infectious, the supernatant from AcBAC109KO DNA (1 μg)-transfected cells was collected at 48 h post transfection (p.t.) and a 1 ml aliquot was used to infect SF-21 cells. At specified time points p.i., the supernatant was collected and assayed for the presence of infectious BV using the $TCID_{50}$ method. Time 0 h p.i. is defined as the time after the 1 h virus adsorption period.

Immunoblotting

Protein samples were mixed with equal volumes of 2× Protein loading buffer (PLB, 0.25 M Tris-Cl, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol and 0.02% bromophenol blue) and incubated at 100 °C for 5 min. Samples were resolved by SDS-12% PAGE, transferred onto a PVDF membrane (MILLIPORE) and probed with one of the following primary antibodies: (i) mouse monoclonal anti-HA antibody (Covance) or; (ii) mouse monoclonal anti-GP64 antibody (eBioscience), followed by incubation with the HRP (horse radish peroxidase)-conjugated secondary antibodies (Sigma). Alternatively, immobilized proteins were probed with mouse monoclonal HA Tag HRP-conjugated antibody (Cell Signaling) or rabbit polyclonal DYKDDDDK Tag HRP-conjugated antibody (Cell Signaling). Blots were developed using the Super-Signal West Pico Chemiluminescent substrate (Pierce) and exposed to X-ray films.

RT-PCR

SF-21 cells were transfected with 1 μg AcBAC109KO DNA or vAc^{gp64-} DNA and lysed at specific times p.t. with Trizol reagent (Invitrogen). Samples were stored at −80 °C until the entire time course was completed. Total RNA from infected cells was extracted and quantified. Aliquots (1 μg) from each time point were treated with 2 units of DNase I (BioLabs) for 10 min and EDTA (Ethylenediaminetetraacetic acid) was added to a final concentration of 5 mM before heat inactivating DNase I at 75 °C for 15 min. After DNAse I treatment, the Access RT-PCR system (Promega) was used to amplify viral specific transcripts using 60 ng of DNase I-treated RNA as a template and the following oligonucleotides: *lef-1* primer set: AcLef1-F: 5′-GAGAACGTGTCAAGAGTCATGTATAC-3′ and AcLef1-R: 5′-AGTCGTCTGCATTGAACCG-3′; *vp39* primer set: Ac-vp39-F: 5′-AACTTTTTGCGCAACGACTTTAT-3′ and Ac-vp39-R: 5′-AGACGGCTATTCCTCCACCTG-3′; and *polyhedrin* primer set: Ac-ph-F 5′-ACCCGGCAAGAACCAAAAACTCACT-3′ and Ac-ph-R 5′-TCCAAGTTTCCCTGTAGAACTCTTTTCCTT-3. Parallel samples lacking AMV RT were subjected to amplification with the *lef-1* primers to monitor DNA contamination. PCR conditions were carried out at 42 °C for 1 h, followed by 25 cycles at 95 °C for 2 min, 50 °C for 30 s, and 72 °C for 30 s.

Rapid amplification of 5′ cDNA ends

To map the transcription start sites for *ac109*, RNA was extracted from AcBAC-infected SF-21 cells and collected at 12 and 48 h p.i. using the TRIzol reagent (Sigma), according to the manufacturer's protocol. Rapid amplification of 5′ cDNA ends (5′ RACE) was performed by using a 5′ RACE kit, version 2.0, and a gene specific primer (5′-GGAGCACGCTAAAAACAC-3′), according to the handbook provided by the manufacturer (Invitrogen). PCR products were gel-purified with the Gel Extraction kit (Qiagen) and cloned into pCR II (Invitrogen) prior to deriving the nucleotide sequence.

Transmission electron microscopy

SF-21 cells (2×10^8) were transfected with 1 μg of AcBAC109Rep, AcBAC109KO, or AcBAC (Detvisitsakun et al., 2006) DNA. At 24, 72, and 96 h p.t., cells were collected, fixed with 2% paraformaldehyde/0.2% glutaraldehyde overnight and embedded in resin prior to sectioning. Sections were placed on Formvar carbon 500 mesh grids and visualized with a Philips MC-100 transmission electron microscope.

Genomic copy number

Purified AcMNPV DNA from budded virus was serially diluted from 230 ng/μl to 0.023 ng/μl to generate a standard curve. Using the protocol outlined by Vanarsdall et al., (2005), template DNA along with a *gp41* primer set (5′-CGTAGTGGTAGTAATCGCCGC-3′ and 5′-AGTCGAGTCGCGTCGCTTT-3′) was used to amplify viral DNA with the iQ Sybr Green Supermix® (BioRad), the BioRad iCycler system and Optical system software version 3.1. To calculate copy number from DNA concentration, 1 copy of the AcMNPV genome was equivalent to 1.34×10^{-4} pg of DNA (Carstens and Wu, 2007). The cycle threshold values were plotted against $\log_{10}$ genome copy number to generate a line and line slope equations. Sample DNA was obtained from the SF-21 cells transfected with 1 μg of AcBAC, AcBAC109KO, AcBAC109Rep, or vAc^{gp64-}(a recombinant virus lacking *gp64*) DNA (Oomens and Blissard, 1999). At specified times, cells were harvested and washed 3 times with phosphate buffered saline prior to addition of TRIzol reagent. Following the protocol of the manufacturer, genomic DNA was isolated and quantified using spectrophotometry. DNA (2 μg) was then digested with *Dpn* I for 4 h to remove any input bacmid DNA introduced during transfection. Cycle threshold values and melt curves were generated for each sample.

BV concentration and Q-PCR

Sf9 cells were transfected with AcBAC109KO or AcBAC109Rep DNA as described above. BVs from supernatants collected at 48 and 72 h p.t. were purified as described previously

(O'Reilly et al., 1994) with modifications. A total of 3 ml of supernatant with BV were centrifuged at 20,000*g* for 20 min at 4 °C. The pellet was resuspended in 50 μl of H_2O and mixed with an equal volume of 2 × PLB prior to immunoblotting.

To purify BV DNA, the cell pellet was resuspended in 100 μl of H_2O and treated with DNase I (New England Biolabs), according to the protocol provided. After heat inactivating DNase I, an equal volume of 2 × virus disruption buffer (20 mM Tris-HCl, pH 7.6; 20 mM EDTA, 0.5% SDS) was added, followed by proteinase K to a final concentration of 500 μg/ml. The mixture was incubated at 37 °C for 30 min with gentle mixing. BV DNA was purified using phenol/chloroform as described (O'Reilly et al., 1994), ethanol precipitated, and resuspended in 50 μl H_2O. In a control experiment to determine contamination from transfected bacmid DNA, 1 μg of AcBAC109KO or AcBAC109Rep DNA was added to the medium of uninfected or transfected Sf9 cells and incubated for 10 min. The supernatant was collected and treated with DNase I, followed by the BV DNA purification procedure described above. Q-PCR was performed as described above using 5 μl of purified BV DNA as template.

Northern blot

SF-21 cells (2×10^6) were infected with AcMNPV at an MOI of 20 PFU/cell for 1 h. At specified time points p.i., cells were harvested with TRIzol reagent and RNA extracted following the protocol of the manufacturer. Samples were treated with cycloheximide 30 min prior to infection at a final concentration of 100 μg/ml and maintained throughout the course of infection. Cells were treated with the DNA replication inhibitor aphidicolin at a final concentration of 5 μg/ml immediately after virus infection. Samples of total RNA (20 μg per lane) were electrophoresed on a formaldehyde–1 % agarose gel, transferred to a nylon membrane, and hybridized to α-^{32}P-radiolabeled strand-specific riboprobes. The probe was generated by first PCR-amplifying the sequence corresponding to the *ac109* translational start codon to the translational stop codon using pBIE4-3 . PCR products were cloned into pCR II (Invitrogen) and cRNA probes were synthesized by in vitro transcription using T7 RNA polymerase in the presence of α-^{32}P [UTP].

Ac109GlyFlag, Ac109GlyHA, Ac142HA and Ac142Flag plasmid constructions

Flag-tagged Ac109 plasmid was made using two primers, BSU-Flag-gly-orf109 [5′-CCTAAGGCTACTTATCGTCGTCATCCTTGTAATCTCCTCCCAAATAATAGTTGTACTTGACG-3′] and BSU36I-Orf 109 [5′-CCTAAGGATGGAGTGCCCGTTTCAGATTCAAGTTTGTATT-3′], to amplify the *ac109* from p109. This sequence has a two-glycine linker between the *ac109* and the C-terminal FLAG tag. Flanking the PCR product are *Bsu* 36I restriction sites. After PCR, the product was ligated into pCR II (Invitrogen) and clones were sequenced. The orf109GlyFlag fragment was released by *Bsu* 36I digestion and the fragment was ligated into pHS70PL-polyA (obtained from pHS70PL-GFP-polyA (Crouch, Passarelli, 2005)) cut with *Bsu* 36I to release the GFP, to obtain the plasmid pHS70PL-orf109GlyFlag-polyA . To make HA-tagged Ac109 plasmid, two primers, BSU-HA-gly-orf109 [5′-CCTAAGGCTAGGCGTAATCTGGGACGTCGTATGGGTATCCTCCCAAATATAGTTGTACTTGACG-3′] and BSU36I-Orf 109 [5′-CCTAAGGATGGAGTGCCCGTTTCAGATTCAAGTTTGTATT-3′], were used to amplify the *ac109* from the p109. This sequence has a two-glycine linker between the *ac109* and the C-terminal HA tag. Flanking the PCR product are *Bsu* 36I restriction sites. After PCR amplification, the product was ligated into pCR II (Invitrogen) and clones were subsequently sequenced. The orf109GlyHA fragment was released by *Bsu* 36I and ligated into pHS70PL-polyA to obtain the plasmid pHS70PL-orf109GlyHA-polyA . To make Flag-tagged Ac142 plasmid, BSU-Ac142-Forward [5′-CCTAAGGATGAGTGGTGGCGGCAACTTGTTGACTCTGGAAAG-3′] and BSU-Ac142-Flag [5′-CCTAAGGCTACTTATCGTCGTCATCCTTGTAATCTTGTACCGAGTCGGGGATTAATAAT-3′] were used to amplify *ac142* from a AcMNPV L1 strain DNA template. The resulting product has a C terminus FLAG tag and is flanked by *Bsu* 36I sites. The PCR product was ligated into the pCR II vector (Invitrogen) and subsequently sequenced. The orf142Flag fragment was released by *Bsu* 36I and ligated into pHS70PL-polyA to obtain the plasmid pHS70PL-orf142Flag-polyA . To make HA-tagged Ac142 plasmid, primers Ac142-F-Bsu36I [5′-GGCCCTAAGGATGAGTGGTGGCGGCAACTT-3′] and Ac142-R-HA-Bsu36I [5′-GGCCCTAAGGTTAGGCGTAATCTGGGACGTCGTATGGGTATTGTACCGAGTCGGGGATT-3′] were used to amplify *ac142* with an HA-tag at the C terminus. PCR products were digested with *Bsu* 36I and ligated into pHS70PL-polyA to generate pHS70-Ac142-HA . Clones were verified by sequencing DNA.

Coimmunoprecipitation assays

SF-21 cells (2×10^6) in 60-mm-diameter culture dishes were transfected as described above with 5 μg of plasmids expressing FLAG- or HA-tagged genes under *Drosophila melanogaster hsp70* promoter control. At 20 h p.t., cells were heat shocked for 30 min at 42 °C. Cells were harvested 3 h after heat shock, pelleted at 2000*g* for 3 min and lysed with NP-40 IP/Lysis buffer (50 mM Tris-HCl, pH 8.0; 150 mM NaCl; 1 mM EDTA; 1% NP-40) and two cycles of freeze–thawing . The lysate was clarified by centrifugation at 10,000*g* for 5 min and precleared by adding 50 μl of a 50% slurry of protein G beads (Sigma), followed by incubation at 4 °C for 1 h with rolling. The supernatant was then transferred to a fresh tube and mixed with anti-DYKDDDDK Tag or anti-HA antibody. After incubation at 4 °C for 3 h with rolling, 50 μl of a 50% slurry of protein G beads was added to the mixture and incubated for another 1 h at 4 °C with rolling. The beads were collected by centrifugation and washed five times with IP/Lysis buffer for 15 min each time and boiled in 25 μl of 2 × PLB for 5 min.

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