Deletion of AcMNPV AC16 and AC17 results in delayed viral gene expression in budded virus infected cells but not transfected cells

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A B S T R A C T

This study investigated the combined function of the Autographa californica multiple nucleopolyhedrovirus overlapping genes ac16 (BV/ODV-E26, DA26) and ac17. Ac17 is a late gene and the promoter is within the ac16 open reading frame. A double ac16–ac17 knockout virus was generated to assess the function of each gene independently or together. Loss of ac17 did not affect viral DNA synthesis but budded virus (BV) production was reduced. Deletion of both ac16–ac17 resulted in reduced viral DNA synthesis and a further reduction in BV production. In BV infected Sf9 cells, viral gene expression was delayed up to 12 h in the absence of both AC16 and AC17 but not if either gene was present. Cells infected by transfecting viral DNA, by-passing the BV particle, exhibited no delay in gene expression from the double knockout virus. AC16 and AC17 are therefore required for rapid viral gene expression in cells infected by BV.

Introduction

The baculoviridae consists of a large group of insect viruses with circular double stranded DNA genome in size of 80 kb to 180 kb (Miller, 1997). The viruses are divided phylogenetically into four genera: alpha-, beta-, gamma- and delta-baculovirus (Jehle et al., 2006). Alpha- and beta-baculoviruses infect larvae from lepidoptera; gamma- and delta-baculoviruses infect larvae from hymenoptera and diptera, respectively. The infection of baculoviruses is a sequential process that is divided into early and late phases, and baculoviral genes are categorized into early, late and very late genes (Miller, 1997). Early genes are transcribed before viral DNA replication using host RNA polymerase II (Friesen, 1997); whereas late genes are transcribed following or concurrently with the onset of viral DNA replication using a viral RNA polymerase complex (Fuchs et al., 1983). The appropriate expression and regulation on viral early genes is critical for the success of viral replication.

In this study we investigated the roles of the overlapping AcMNPV gene pair ac16 (BV/ODV-E26, DA26) and ac17 during viral replication. Ac16 is expressed early (O’Reilly et al., 1990) and is one of seventeen genes specific to Group I alpha-baculoviruses (Herniou et al., 2001). Ac16 is an envelope protein of both budded virus (BV) and occlusion derived virus (ODV) and is suggested to be involved in the trafficking of ODV-E66 along with FP25 (Beniya et al., 1998). Recently, we have shown that AC16 is found to interact with both AcMNPV IE0 and IE1, suggesting it might play a role in the viral transcription or replication. However the deletion of AC16 does not affect viral DNA synthesis or BV production but does result in increased levels of IE0 relative to IE1 (Nie et al., 2009). Bombyx mori NPV (BmNPV) BM8 is a homolog of AC16 and has been shown to co-localize in an hr-dependent manner with BmNPV IE1 in the nucleus of infected BmN cells (Kang et al., 2005).

Genes homologous to ac17 are common to all alpha-baculoviruses sequenced to date except for the closely related Trichoplusia ni single NPV (TnSNPV), Chrysosidexia clutches NPV (ChcNPV) (Rohrmann, 2008) and Ogyia leucostigma NPV (OrlNPV, accession number EU390941). Ac17 and its homolog BMNPV bm9 have been reported to be expressed as early genes (An et al., 2006; Yang et al., 2009); however, the homolog from Helicoverpa amigerata NPV (HearNPV), ha128, is reported to be a late gene (An et al., 2005). All three proteins AC17, HA128 and BM9 however have been shown to be cytoplasmic proteins (An et al., 2005, 2006; Yang et al., 2009). Deletion of bm9 was found to have no effect on viral DNA replication, but reduced the levels of BV produced (Yang et al., 2009).

To further clarify the functional roles of AcMNPV AC16 and AC17, we made a double knockout (KO) virus as ac16 and ac17 open reading frames (ORFs) overlap. To determine the impact of deleting both genes and each gene individually the double knockout virus was repaired with either ac16, ac17, or both ac16 and ac17. Deletion of AC17 was also shown to decrease BV production but did not affect viral DNA synthesis. Significantly, the deletion of both ac16 and ac17 was shown to cause a delay in the viral gene expression when cells
were infected by BV, but not when transfected by naked viral DNA. The delay was also not observed when either ac16 or ac17 was present suggesting a synergistic role of ac16 and ac17 at the immediately early stage of viral gene expression but only when cells are infected by virions. Transcriptional analysis showed that ac17 in contrast to previous reports is a late gene. Cellular localization showed that AC17 is located in both the cytoplasm and the nucleus at late times post-infection.

Results

Transcriptional analysis of ac17

To design viruses that contain deletions of ac17 it was necessary to identify the transcriptional start site of ac17. A previous study using RT-PCR concluded that ac17 was an early gene however the design of that experiment could not distinguish between the overlapping ac16

Fig. 1. 5’ and 3’ RACE analysis of ac17 transcription. (A) Agarose gel analysis of ac17 5’ RACE products at 4 and 24 hpi. Sizes of products are shown on the right in kbp. M, mock infected cells. Numbers on the left show sizes of markers in kbp. The schematic below the gel shows the location of the transcribed ac16 and ac17 transcripts (arrows). (B) Location of the ac17 transcription start site as determined by 5’ RACE. The arrowhead shows the initiation site of ac17 transcription and the baculovirus late promoter motif (ATAAG) and ac17 translation start codon (ATG) are shown in bold. Another potential late motif (GTAAG) and two minicistrons within the UTR are underlined with the predicted translation products shown underneath. (C) 3’ RACE analysis of ac17 transcription. Both the sequencing result of the 3’ RACE products and the AcMNPV genome sequence are shown. The canonical polyadenylation (AATAAA) is shown in bold and the ac17 stop codon is in bold and underlined. Transcription of ac17 was found to terminate 11 bp downstream of the polyadenylation site.
transcript and the ac17 transcript (An et al., 2006), as it has been shown that the early gene ac16 transcription terminates downstream of the ac17 ORF (Guarino and Summers, 1988). The HearNPV homolog ha128 has also been transcriptionally analyzed and is reported to be a late gene (An et al., 2005).

Rapid amplification of cDNA ends (RACE) analysis was performed to identify the transcription start and termination sites of ac17. At 4 h post-infection (hpi), a single specific 5’ RACE product of approximately 0.77 kbp was produced which mapped to the reported CATT early transcriptional start site for ac16 (Burks et al., 2007) (Fig. 1). At 24 hpi the major 5’ RACE product using ac17 gene specific primers was approximately 0.52 kbp product that when sequenced mapped to the baculovirus late promoter ATAAG, 430 bp upstream of the start codon of ac17 (Fig. 1A). These results show that ac17 is a late gene and is transcribed with a leader sequence that incorporates two thirds of the ac16 ORF. Analysis of the leader sequence reveals the presence of two potential mini-cistrons of 27 and 30 nt (Fig. 1B). Previous studies have shown that mini-cistrons in baculovirus leader sequences can regulate the expression of downstream ORFs (Chang and Blissard, 1997; Theilmann et al., 2001). The late expression of ac17 is consistent with the HearNPV ha128 homolog (An et al., 2005). Interestingly there is a second late promoter motif (GTAAG) closer to the ac17 ORF but no RACE product was mapped to this location (Fig. 1A).

The 3’ RACE produced a major 0.65 kbp band specific to virus infected cells at both 4 and 24 hpi. Sequencing of this band showed that the transcription terminated at 11 bp downstream of a predicted polyadenylation signal AATAAA in which the TAA is also the stop codon of ac17 (Fig. 1C). As the 3’ RACE product is observed at both 4

![Fig. 2](image-url)

**Fig. 2.** Construction of ac16/17KO and repair bacmids. The sequence encoding both ac16 and ac17 were replaced by the zeocin resistance gene cassette (amplified with primers 1434 and 1438) via homologous recombination in E. coli to generate bMON14272-ac16/17KO. The correct deletion was confirmed by PCR using primer pairs 1430/520, 1239/1439 and 1440/1439. The relative positions of these primers are shown on the diagram. Shown in the lower part of the figure are the genes inserted into the polh locus of bMON14272-ac16/17KO to generate the knockout and repair bacmids ac16/17KO, ac17KO, ac16KO, ac16/17repair, ac16/17HArepair. The wild type bacmid (bMON14272) was repaired at the polyhedrin locus with polyhedrin (polh) and gfp to generate AcBac. For confocal microscopy bMON14272 was repaired with ac17-HA under the control of the polyhedrin promoter to generate the virus AcBac-AC17HA.
and 24 hpi it would indicate that the ac17 and ac16 transcripts utilize the same termination site producing a 1073 nt late transcript and a 1324 nt early transcript without considering the size of the poly(A) tail. This would agree with the approximate sizes of the early and late transcripts previously described by Northern blot for this gene region (O’Reilly et al., 1990).

**Generation of ac16/17KO and repair viruses**

As shown in Fig. 1, ac16 and ac17 are intimately associated ORFs that overlap. We have recently shown that ac16 could be deleted and viable virus could be recovered (Nie et al., 2009). This was in contrast to previous studies which suggested that this gene was essential (Burks et al., 2007; Imai et al., 2004; Nie et al., 2009). A possible explanation for these different results was that the knockout of ac16 in other studies also interrupted the expression of ac17, which initiates from within the ac16 ORF. Therefore to address the question of ac17 function, an ac16–ac17 double knockout virus (bMON14272-ac16/17KO) was constructed (Fig. 2). To enable the observation of virus propagation and occlusion body formation, gfp and polyhedrin were transposed into polh locus of bMON14272-ac16/17KO, generating ac16/17KO. To examine the function of ac17, the double KO virus bMON14272-ac16/17KO was repaired with ac16, ac17, ac16–ac17 or ac16–ac17HA, respectively via transposition along with gfp and polyhedrin. The viruses generated were named ac17KO, ac16KO, ac16/17repair and ac16/17HArepair (Fig. 2). The control virus AcBac (Fig. 2) was generated by repairing the native bacmid bMON14272 with gfp and polyhedrin (Dai et al., 2004).

**Analysis of viral DNA replication**

Transfection of each bacmid DNA showed that all virus constructs replicated and produced BV (Data not shown). BV stocks were prepared and used to infect SF9 cells to enable the comparison of replication properties for each virus. To determine the impact of deleting ac17 or both ac16 and ac17 on viral DNA replication, viruses ac16/17KO, ac17KO, ac16KO, ac16/17repair and AcBac control were used to infect SF9 cells. Cells were collected for the analysis of viral DNA replication at various times post-infection using real-time quantitative PCR (Fig. 3). The results showed that deleting both AC16 and AC17 (ac16/17KO) from the viral genome resulted in significantly lower levels of viral DNA replication between 6 and 48 hpi but levels similar to AcBac were obtained by 72 hpi. The double repair virus ac16/17repair had equivalent viral DNA replication levels as AcBac showing that the observed decrease in DNA replication was due to the loss of ac16 and ac17. The ac17 knockout virus ac17KO, showed similar levels of viral DNA replication as the wild type control AcBac or the double repair virus ac16/17repair. This indicates that deletion of ac17 does not have a detectable impact on viral DNA replication which is similar to the results reported for the homologous BmNPV bm9 gene (Yang et al., 2009). The ac16 knockout virus ac16KO had reduced levels of DNA replication from 6 to 24 hpi but reached normal levels relative to AcBac or the double repair virus ac16/17repair by 48 hpi. This is not exactly the same as what was previously observed with the single gene knockout virus ac16KO-GFP-PH (Nie et al., 2009) which showed no difference in viral DNA replication compared with wild type. One possible reason is that the expression of ac17 in the two different ac16 knockout viruses could be different. In the present study the ac17 gene is in the polyhedrin locus with different 5’ and 3’ sequence contexts as opposed to its native locus. Nevertheless, these results showed that deletion of AC16 in conjugation with AC17 results in a synergistic effect causing a greater reduction in viral DNA levels from 6 to 72 hpi.

**Virus growth curve analysis**

To investigate the impact of deleting ac17 and ac16 on BV production, virus growth curves of ac17KO, ac16KO, ac16/17repair and AcBac were analyzed. Both of the single gene knockout viruses had reduced BV production (Fig. 4). The deletion of ac17 had a greater impact with levels being reduced 1–2 logs. Loss of Ac16 also resulted in reduced BV levels up to 72 hpi but levels were equivalent to AcBac by 96 hpi. As seen with DNA replication there was a synergistic negative impact on BV production up to 48 hpi when both ac16 and ac17 were deleted. By 72 hpi ac16/17KO BV levels were equal to ac17KO but remained nearly a log lower than AcBac. The reduced BV could be related to the lower viral DNA replication levels (Fig. 3).
Viral gene expression

The results described above showed reduced levels of viral DNA replication in the ac16/17KO and ac16KO viruses which suggest that early viral gene expression may be affected by these deletions. To determine if viral early or late gene expression was affected in cells infected with ac16/17KO or repair viruses, the temporal expression of the early proteins IE0, IE1, GP64, P35, LEF3, P143, the late protein VP39 and the polyhedrin protein was measured.

Fig. 5. Western blot analysis of the temporal expression of the viral early and late genes IE0, IE1, GP64, LEF-3, P143, P35, VP39 and POLYHEDRIN. Sf9 cells were infected with AcBac (1), ac16/17KO (2), ac17KO (3), ac16KO (4), and ac16/17repair (5) at a MOI of 5. Infected cells were harvested at the times indicated in the presence of protease inhibitors and analyzed by SDS-PAGE and Western blot.
VP39, and the very late protein POLYHEDRIN were analyzed by Western blots from cells infected by ac16/17KO, ac17KO, ac16KO, ac16/17repair and AcBac viruses. The viruses AcBac, ac17KO, ac16KO and ac16/17repair showed no significant differences in the timing of expression of the early genes IE0/IE1, GP64, P143, or P35 (Fig. 5). However there was a significant delay in expression from the double knockout virus ac16/17KO. In cells infected by ac16/17KO, IE0 was only detectable at 12 hpi while IE1 levels were not detectable until 18 hpi (Fig. 5). Additionally, the relative levels of IE0 to IE1 were increased, which is similar to what was observed in the ac16 knockout virus, ac16KO and as previously reported (Nie et al., 2009). The other early proteins analyzed in the ac16/17KO infected cells including P143, LEF3, P35 and GP64 also exhibited the same significant delay of expression relative to the control virus AcBac. A similar delay in protein expression was observed with late and very late VP39 and POLYHEDRIN. These results therefore suggest that in cells infected with the ac16/17KO virus there is a global delay of viral gene expression. If the levels of replication proteins P143, LEF3 and P35 were also reduced in the ac16KO infected cells, it could result in the decreased levels of DNA replication that were observed (Fig. 3). However, a quantitative analysis of the protein expression levels is needed before this conclusion can be drawn.

**Transcription analysis of gp64, ie0 and ie1**

The Western blot analyses show that there is a delay in viral gene expression at the level of translation in ac16/17KO BV infected cells. However this could be due to the delayed or reduced transcription of viral genes. Therefore to determine if the transcription of viral genes was altered, we analyzed expression of three early genes, gp64, ie0 and ie1 in cells infected by ac16/17KO, ac17KO, ac16KO, ac16/17repair and AcBac respectively. The temporal expression of gp64 was analyzed by Northern blot and the expression of ie0 and ie1 was determined by real-time quantitative reverse transcription PCR (qRT-PCR). The Northern blot results (Fig. 6A) showed that the gp64 mRNA was detected at 4 hpi with increasing levels up to 24 hpi from AcBac, ac17KO, ac16KO and ac16/17repair. However in ac16/17KO infected cells gp64 transcript was not detected until 12 hpi. In addition, the levels of the gp64 transcript were reduced at 16 and 24 hpi compared with AcBac, ac17KO, ac16KO and ac16/17repair. This data showed that the delayed expression of GP64 detected by
Western blot (Fig. 5) was due at least in part to the delayed and reduced transcription of gp64.

The levels of expression from the primary baculovirus transcription factors ie0 and ie1 were analyzed using qRT-PCR with two sets of primer pairs. The first pair are specific for ie0 and only detect ie0 transcripts (Fig. 6B). The second pair detect ie1 and in addition ie0, since the ie0 transcript also contains the entire ie1 transcript (Fig. 6C). At 4 hpi, ac17 KO, ac16 KO, ac16/17 repair had ie0 expression...
levels equivalent to the control AcBac, whereas ac16/17KO did not have any detectable expression above background (Fig. 6B). At 12 hpi ie0 expression from ac16/17KO is detected above background indicating an increase in transcript levels. This was in contrast to the other viruses which all showed a decline in ie0 expression between 4 and 12 hpi. Analysis of combined levels of both ie0 and ie1 transcripts (Fig. 6C) showed that ac17KO, ac16KO, ac16/17repair and AcBac. Total cell protein was collected at various times post-transfection and analyzed by SDS-PAGE and Western blot.

To determine if AC17 is a component of BV, virions were purified from supernatant of Sf9 cells infected by ac16/17HArepair and analyzed by SDS-PAGE and Western blot. BV purified from AcBac was used as HA negative control and antibodies against VP39 and GP64 were used as markers for BV purification (Fig. 7C). The results showed that AC17 was associated with BV and therefore may be a structural protein of BV.

Expression of viral proteins in bacmid transfected cells

Analysis of ie0, ie1 and gp64 expression revealed that deletion of ac16–ac17 results in delayed and reduced transcription that also leads to delayed expression of viral proteins (Figs. 5 and 6). As shown by Western blot analysis (Fig. 5) and in previous studies (Beniya et al., 1998) both AC16 and AC17 are found in the BV particle. Therefore as virion structural components it is possible that AC16 and AC17 accelerate gene expression after being transported into the cell upon infection with BV. If this is the case, in the absence of virion proteins no delay in viral gene expression between ac16/17KO and AcBac should be observed under conditions where the viral genome is delivered to the nucleus as naked DNA. To test this hypothesis, we transfected Sf9 cells with bacmid DNA of ac16/17KO, ac16KO, ac16/17repair and control AcBac. Cells were collected at 4, 7, 10, 13, 16 and 24 h post-transfection (hpt) and total cell protein was analyzed by Western blot for the expression of the early proteins IE0 and IE1, LEF-3, P143 and the late structural capsid protein P39 (Fig. 8). The results showed that there was no difference in the temporal expression of
the early proteins IE0 and IE1, LEF-3, P143 or the late protein P39 between AcBac and the double KO ac16/17/KO. The same result was also observed for ac16KO and ac16/17/repair. This result shows that AC16 and AC17 facilitate the rapid expression of viral genes only when cells are infected by BV.

Discussion

In this study, the analysis of an ac17 knockout virus has shown that AC17 is required for efficient high level production of BV. In the absence of AC17 BV yields are reduced by approximately 10 fold however viral DNA levels are unaffected (Figs. 3 and 4). This would suggest that AC17 affects post-replication events such as the efficiency of assembly of nucleocapsids or nucleocapsid egress and budding. A number of baculovirus genes have been reported to affect BV production but do not appear to impact viral DNA synthesis, including gp64, f-protein, gp41, exon0, pp31, ac66, vif-1, 38K, ac101(BV/ODV-C42), ac142, ac143 and me53 (Dai et al., 2004; de Jong et al., 2009; Fang et al., 2007; Ke et al., 2008; McCarthy et al., 2008; McCarthy and Theilmann, 2008; Monsma and Blissard, 1995; Monsma et al., 1996; Olszewski and Miller, 1997; Oomens and Blissard, 1999; Pearson et al., 2000; Vanardsall et al., 2004; 2007; Wu et al., 2006; Yamagishi et al., 2007). The deletion or mutation of ac142, ac143, gp41, vif-1, 38K, ac101, gp64, f-protein abolishes infectious BV production whereas the deletion of pp31, exon0 and me53 only results in a reduction of BV production by 100 to 1000 fold. Three genes vif-1, 38K, and ac101 have been shown to impair the assembly of nucleocapsids. Loss or reduced BV production in the absence of gp41, exon0, ac66 or ac142 however is suggested to be due to the compromised nucleocapsid transport from the nucleus to the cytoplasm (Ke et al., 2008; McCarthy et al., 2008; Olszewski and Miller, 1997). Loss of GP64 or F-protein affects the budding of nucleocapsids directly at the plasma membrane (Monsm et al., 1996; Oomens and Blissard, 1999; Westenberg and Vlak, 2008; Westenberg et al., 2002). Given the viability of the ac17KO BV, it is unlikely that the assembly of nucleocapsids are severely impaired in the absence of AC17. AC17 in comparison to the above proteins therefore appears to be more of an auxiliary factor for the BV production. The 10 fold higher levels of BV achieved in the presence of AC17 represent a large increase in the number of virions produced per cell and could have a potentially significant impact in vivo enabling rapid systemic infection in lepidopteran larvae. However, it is still possible that the loss of AC17, a BV associated protein, could result in structurally compromised nucleocapsids, which have reduced stability, inefficient transport or budding. The precise nature of the AC17 role in BV assembly and production however, remains to be determined.

In addition to the impact on BV production the deletion of ac17 in combination with ac16 had a dramatic impact on the virus life cycle compared to the deletion of either gene independently. This includes significantly delayed early and late viral gene expression, a further reduction in BV production and decreased levels of viral DNA replication. The most intriguing result however was the difference between infection and transfection on viral gene expression. There was an approximately 12 h delay in early and late gene expression when cells were infected with ac16/17KO BV compared AcBac or the other deletion viruses. However, when cells were infected by transfecting viral DNA no difference in viral gene expression was observed between ac16/17KO and AcBac or the repair viruses. This shows that the delay is specific to the BV infection process and that AC16 and AC17 are required for the rapid start of viral gene expression. Interestingly no delay is observed with the single gene deletions indicating a synergistic impact on the virus life cycle when both genes are absent. AC17 is expressed late and as a result de novo ac17 expression could not affect the early events that occur upon BV infection. However, both AC16 and AC17 are found in the BV particles (Fig. 7; Beniya et al., 1998) and therefore could influence the early events of the infection process by being introduced into the cell by the virion. The delay of viral transcription in cells infected but not transfected with ac16/17KO would suggest a defect in the BV entry, transport to the nucleus, uncoating viral genome from the nucleocapsid or potentially recruitment of RNA Pol II to early viral promoters. Entry may be affected but previous studies have clearly shown that GP64 is required for BV binding to target cells and low-pH-dependent membrane fusion (Blissard and Wenz, 1992; Monsma and Blissard, 1995; Oomens and Blissard, 1999). After fusion of the BV envelope with membranes of a late endosome, nucleocapsids are released and traverse the cytoplasm. AC16 is included in the sorting of ODV envelope proteins to the inner nuclear membrane and viral envelope (Braunagel et al., 2009) and therefore could potentially facilitate the endosome-virion envelope fusion and release of nucleocapsids into the cytoplasm. Additionally, AC16 is reported to share homology to tropomyosin (Beniya et al., 1998) and therefore could also be an auxiliary factor in the formation of F-actin bundles (Goley et al., 2006; Lanier and Volkman, 1998) enabling the transport of nucleocapsids to the nucleus. Possible functions of AC17 could be attachment of nucleocapsids at the nuclear pore complex, uncoating and release of the viral genome into the nucleus. Once in the nucleoplasm, AC16 could also be involved in enabling access to the viral early promoters by the host RNA Pol II system as we and others have shown that AC16 binds the major viral transcriptional transactivators IE0 and IE1 and therefore could be directly affecting transcription (Kang et al., 2005; Nie et al., 2009).

Comparison of all baculovirus genomes sequenced to date has shown that AC17 is conserved in all the alpha-baculoviruses reported with the exception of TnSNPV, ChchNPV and OrleNPV. Ac16 is suggested to be one of seventeen genes specific to Group I alpha-baculoviruses, but there is an ORF, non-homologous to ac16 present at the same locus adjacent to the ac17 homologs in the group II alpha-baculoviruses. It is possible that the Group II protein could be a functional homolog of AC16 as analyses show that the predicted protein products of the group II ORFs contain a similar predicted coiled-coil structure (Data not shown). The AC16 coiled-coil domain has been shown to be essential for the binding of AC16 to IE0 and IE1 (Kang et al., 2005; Nie et al., 2009). Therefore the ac16–ac17 gene pair and their homologs could be playing a critical role in accelerating the early events of most alpha-baculovirus infections. The acquisition of this gene cluster compared to beta-, gamma- and deltabaculoviruses may have contributed to the diversification of alphabaculoviruses.

Materials and methods

Viruses and cells

Spodoptera frugiperda clone 9 (Sf9) cells were maintained at 27 °C in TC100 medium supplemented with 10% fetal bovine serum. AcMNPV recombinants were derived from bacmid bMON14272 (Invitrogen Life Technologies) in Escherichia coli DH10B cells as described previously (Datsenko and Wanner, 2000; Luckow et al., 1993).

Plasmid construction

The fragment containing ac17 promoter, ORF and polyadenylation signal was amplified with 1558 (′-GGGTTGAGGACTGTTATTTTGG-3′) and 1559 (′-GGGTTGAGGACTGTTATTTTGG-3′) using AcMNPV genomic DNA as the template and cloned into pAct-GFP at XbaI/Sacl sites, generating pAct-GFP–AC17. To tag ac17 with the HA epitope, primer pair 1558/1781 (′-GAGGGCTCCTGGTTTGAAGTTTGG-3′) were used to amplify ac17 from the

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AcMNPV genomic DNA and the fragment containing the ac17 promoter and ORF was cloned into pFACT-gfp-Tnie1pa at Xhol and NotI sites, generating pFACT-GFP-AC17-HA. The ac17-HA cassette was further subcloned into the Xhol/Sacl sites of pFACT to produce pFACT-AC17-HA. To drive ac17-HA with polyhedrin promoter, ac17 was also amplified with primers 1818 (5'-GCCTCAATGGATACCTGAACTGTAATTAAAGCG-3') and 1783 (5'-GGCAAGCTTATAGGGCTATGCGAACGCGTGAGATTTATTTTAAATAAAAATAGT-3') using AcMNPV genomic DNA as the template and subsequently cloned into pFastbacI using the BamHI/SrI sites, generating pFastbac-AC17HA. The fragment containing both ac16 and ac17 was amplified with primer pair 1430 (5'-GCGCTCGAGCTACCTACAAAAAACCATGG-3') and 1438 (5'-ATTTTTATTATTATATTTTATCTATTTTATAATTTTACTATGCGTGACG-TACCCCTG-3') using AcMNPV genomic DNA as the template, and the PCR product was cloned into pFACT-GFP at the Xhol/Sacl sites, generating pFACT-GFP-AC16-AC17. The ac16/17 fragment was also amplified with primers 1430/1781 and cloned into pFACT-GFP-Tnie1pa generating pFACT-GFP-AC16-AC17HA.

Virus construction

Construction of ac16/17 double KO AcMNPV bacmid

AcMNPV bacmid (bMON14272) was used to generate an ac16/17 double knockout virus by recombination in E. coli as previously described (Matsumoto and Wanner, 2000; Hou et al., 2002). A zeocin resistance cassette with ac16/17 flanking regions was amplified using primers 1434 (5'-TGTGCGACGCTACCCATCAAGCTTATTTAAACACATCAG-3') and 1437 (5'-ATTTTTTTTATATTTTTATATTTTTTTATATTTTTCTATGCGTGACG-TACCCCTG-3') with p2ZeoKS as the template. These primers contain 50 bp homologous sequences to the 5' flanking region of ac16 and 50 bp homologous region to the 3' of ac17. The PCR fragment of zeocin resistance cassette was purified and electroporated into E. coli BW25113-pKD46 cells which contained the AcMNPV bacmid bMON14272. The electroporated cells were incubated at 37 °C for 4 h in 1 ml of LB medium and plated onto agar medium containing 25 µg/ml of zeocin and 50 µg/ml of kanamycin. Plates were incubated at 37 °C overnight and colonies resistant to both zeocin and kanamycin were selected and further confirmed by PCR. Three different pairs of primers were used to confirm the correct knockout of ac16/17 had been produced. Primers 1500/520 (5'-CAGGAAACGGCACCAGTTACAAATAATTTTTTATTTTTACTATGCGTGACG-TACCCCTG-3') and primers 1239 (5'-CTGACGGAGCGGCCAACAA-3') and 1439 (5'-ATAGCTATACCTGCATCGTTTGT-3') were used to confirm the correct insertion of the zeocin cassette and primer pair 1440 (5'-CTGACGGAGCGGCATGCATACATT-3') and 1439 was used to confirm the deletion of the desired sequence. One recombinant bacmid confirmed by PCR was selected and named bMON14272-ac16/17KO.

Construction of ac16/17KO and repair bacmids containing polyhedrin and gfp

To generate ac16/17KO and repair viruses with POLYHEDRIN and GFP, pFACT-GFP was transposed into bMON14272-ac16/17KO as previously described (Luckow et al., 1993) to make ac16/17KO; pFACT-GFP-AC16, pFACT-GFP-AC17, pFACT-GFP-AC16/17 and pFACT-GFP-AC16-AC17-HA vectors were used to transpose bMON14272-ac16/17KO to generate ac17KO, ac16KO, ac16/17repair and ac16/17HArepair respectively. To enable the immunofluorescence confocal analysis of AC17, a Fastbac-AC17HA was used to transpose bMON14272, producing AcBac-AC17HA.

Time course analysis of viral DNA replication and BV production

Sf9 cells (2.0 × 10^6 cells/35 mm diameter well of a six-well plate) were infected by AcBac, ac16/17KO, ac17KO, ac16KO, ac16/17repair, respectively at a MOI of 5 in duplicate. At various hours post-infection, supernatant containing BV was collected and stored at 4 °C until analysis after the cell debris was removed by centrifugation at 8000 × g for 5 min. For viral DNA replication analysis, infected cells were washed once with phosphate-buffered saline (PBS, 137 mM NaCl, 10 mM phosphate, 2.7 mM KCl, pH 7.4), scraped off with rubber policeman, pelleted by centrifugation at 2000 × g for 5 min and cell pellets were stored at −80 °C until analysis. BV titer was determined by real-time quantitative PCR (qPCR) (Lo and Chao, 2004). Briefly, 100 µl of BV supernatant collected during the course of the same time were aliquoted and incubated at 50 °C overnight in the lysis buffer (10 mM Tris-Cl Ph8.0, 100 mM EDTA, 0.5% SDS, 80 µg/ml Proteinase K). Viral DNA was extracted with phenol-chloroform-isooamyl alcohol followed by extraction with chloroform. 2 µl viral DNA extracts was used directly for the qPCR with primers 850 (5'-TTTTTGGAGGAAGATTCTTGTC-3') and 851 (5'-CAAAACTGCAGGAGAAGAG-3') as well as 2× DyNAmo HS Master Mix (DyNAmo HS SYBR Green qPCR Kit, New England Biolabs) in a 20 µl reaction to amplify a 100-bp fragment of ac126 (chitinase). To create the standard curve qPCR is performed on a serial dilution of an AcMNPV E2 stock that had been titered using the 50% tissue culture infective dose (TCID50). The thermal profile used was based on McCarthy et al. (2008). Analysis of viral DNA replication using qPCR has also been described previously (Nie et al., 2009). The method was adapted from Vanarsdall et al. (2004) which is based on the amplification of 100 bp fragment of gfp41 gene. The results were analyzed by the MX4000 software (Stratagene).

Northern blot

S9 cells were infected by ac16/17KO, ac17KO, ac16KO, ac16/17repair and AcBac at MOI = 5 and cells were collected at 6, 12, 16, 24 hpi. Total RNA was extracted from S9 cells using Trizol (Invitrogen). 10 µg of total RNA from each sample were separated on a 1% formaldehyde gel and blotted and hybridized with α-32P-labelled single stranded RNA probe of gp64 (Fournier et al., 1988). The blot was visualized by exposure to Perkin Elmer Multisensitive Phosphorscreens, which was scanned using a Cyclone Phosphor Imager (Perkin Elmer) and analyzed with Optiquant Acquisition and Analysis Software V5.0 (Perkin Elmer). For the synthesis of a strand specific probe, a gp64 fragment of 300 bp was amplified using primer 1867 (5'-TCAATAACGACTCATATAGGGCTACGTTCTTGTGAA-TATGCA-3') containing the T7 promoter sequence (underlined) and gp64 homologous sequence and primer 1868 (5'-GTACATGCT-CAAAAAAGCTCTACG-3') was used to probe labelled with α-32P-UTP and synthesized using GeneScribeTM T7 RNA probe kit (USB).

qRT-PCR

Total RNA was extracted using Trizol (Invitrogen) from S9 cells infected by ac16/17KO, ac17KO, ac16KO, ac16/17repair and control AcBac collected at 4 hpi and 12 hpi. To synthesize cDNA, 5 µg of total RNA was used for the reverse transcription using Superscript III (Invitrogen) following the manufacturer's protocol. Control reactions were performed by omitting the Superscript III reverse transcriptase from the cDNA synthesis reactions with total RNA from 4 hpi. Background qRT-PCR values obtained from the control cDNA reactions were subtracted from each sample. For qPCR, the synthesized cDNA was diluted five times with sterilized distilled water before using as templates for the qPCR. Series of amounts of an iel expressing plasmid pAcie1delta (Huijser et al., 2004) were used as the standard for the qPCR. The qPCR reaction was set up using the SYBER green qPCR kit (DyNAmo HS SYBR Green qPCR Kit, New England Biolabs) with primers 1446 (5'-CATATTGCTGCCAGGCAACG-3') and 1414 (5'-GAGTACACAGCGGTTAATAAT-3') for iel and 1505 (5'-GACAAAGCTATTCAGAT-3') and 1523 (5'-GAGGTTGCAGTGGC-CAAAA-3') for ie1, amplifying a fragment of 126 bp and 144 bp,
respectively. The program setting for the qPCR was one cycle of 95 °C for 15 min, 40 cycles of 95 °C for 30 s, 52 °C for 24 s, 72 °C for 30 s.

**RACE**

To map the transcription start site for ac17, total RNA was extracted using RNeasy kit (Qiagen) from mock Sf9 cells or cells infected with AcMNPV-E2 virus that were collected at 4 hpi and 24 hpi. 5 µg of total RNA was used to generate RACE ready cDNA using the GeneRacer Kit (Invitrogen) following the manufacturer's protocol. For RNA ligase-mediated and oligo-capping RACE methods, which eliminate the truncated mRNA from the amplification process and allow only the amplification of full-length transcript ends. For 5' RACE PCR, gene specific primer 1 (GSP-1) 1731 (5'-AGCCATCTCAATAATCA-3') was paired with GeneRacer 5' primer (5'-CGACTGGACCCAGGACACGTGA-3') for the initial amplification. GSP-2 1732 (5'-GGGAGATCGGCATATTTG-TTACC-3') was paired with GeneRacer 5' nested primer (5'-GGACACTGACATGGACTGAAGGAGTA-3') to specifically amplify the ac17 transcript. Another gene specific primer 1432 (5'-GGCCTCAGTGTGAAGGTGGAGAAG-3') was paired with GeneRacer 3' primer (5'-GCCCTCAGATGCTAGAGGACTAGC-3') for the 3' RACE analysis. The PCR RACE products were cloned and sequenced with M13 forward and reverse primers.

**Western blot analysis**

Total cell protein was collected during time course analyses or purified BV were mixed with 4 × SDS protein sample buffer (4 × PBS: 0.25 M Tris–Cl, pH6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.02% bromophenol blue) and were heated at 100 °C for 10 min. Protein samples were separated by 10% or 7.5% SDS-PAGE (Laemmlli, 1970) using a Bio-Rad Mini-Protein II and transferred to Immobilon-P membrane (Millipore) using a liquid transfer apparatus (Bio-Rad). Western blot hybridizations were performed following the standard protocol (Harlow and Lane, 1988). Membranes were probed with one of the following antibodies: mouse monoclonal HA antibody 1:1000 (Covance, HA11); mouse monoclonal IE1 antibody 1: 8000 (Ortho, 1:1000; Ross and Friesen for the P35 antiserum. Enhanced Chemiluminescence System (ECL, Perkin Elmer) was used to visualize bound antibodies.

**Immunofluorescence**

Sf9 cells infected by AcBac-Ac17HA at 72 hpi were washed once in PBS and fixed with 3% paraformaldehyde in PBS for 15 min. The fixed cells were washed three times in PBS for 5 min each time, followed by permeabilization in 0.15% Triton X-100 in PBS for 20 min. Cells were then blocked for 60 min in 2% bovine serum albumin in PBS and incubated with anti-HA antibody (1:100, HA11, Covance). After three washes in PBS, cells were incubated with Alexa 635 conjugated goat anti-mouse IgG (Molecular Probes) for 60 min followed by staining with 200 ng/ml 4′,6-diamidino-2-phenylindole (DAPI) (Sigma) for 2 min, and examined with a Leica confocal microscope after three washes in PBS.

**BV purification**

Sf9 cells were infected at a MOI of 0.1 at a cell density of 2 × 10^6/ml with ac16/17HArepair or AcBac in two spinner flasks for each virus. At 4 days post-infection, the BV supernatants (80 ml) were harvested. The purification of BV was performed as previously described (O'Reilly et al., 1992). The supernatant was cleared of cell debris by centrifugation at 8000 × g (Beckman F50C rotor) for 10 min, followed by centrifugation for 60 min at about 100,000 × g (28,000 rpm) in Beckman SW28 rotor. Pelleted BV were resuspended in 1 ml PBS with 1% protease inhibitor cocktail (Sigma) and loaded onto a 25–65% sucrose (w/w) gradient and centrifuged for 90 min in a SW41 (Beckman) at about 80,000 × g (28,000 rpm). The BV band was collected, diluted with PBS and pelleted by centrifugation for 60 min at about 100,000 × g (28,000 rpm) in Beckman SW28 rotor to clear of sucrose. Purified BV was resuspended in 120 µl of PBS with 1% protease inhibitor cocktail, and 15 µl was used for separation on SDS-PAGE followed by Western blot.

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Chang, M.-J., Pearson et al., 1988; mouse monoclonal GP64 antibody 1:250 (Hohnmann and Faulkner, 1983); mouse monoclonal OPMNPV POLH antibody 1:10000 (Quant et al., 1984); rabbit polyclonal anti-AcMNPV LEF3 antibody 1:2000 (Chang et al., 2004); rabbit polyclonal anti-AcMNPV P143 antibody 1:200 (Ito et al., 2004); rabbit polyclonal AcMNPV P35 antibody 1:1000 (Hershberger et al., 1983). To detect bound primary antibodies membranes were incubated with 1:10000 goat anti-mouse or goat anti-rabbit peroxidase-conjugated secondary antibodies. Enhanced Chemiluminescence System (ECL, Perkin Elmer) was used to visualize bound antibodies.

**Immunofluorescence**

Sf9 cells infected by AcBac-Ac17HA at 72 hpi were washed once in PBS and fixed with 3.5% paraformaldehyde in PBS for 15 min. The fixed cells were washed three times in PBS 5 min each time, followed by permeabilization in 0.1% Triton X-100 in PBS for 20 min. Cells were then blocked for 60 min in 2% bovine serum albumin in PBS and incubated with anti-HA antibody (1:100, HA11, Covance). After three washes in PBS, cells were incubated with Alexa 635 conjugated goat anti-mouse IgG (Molecular Probes) for 60 min followed by staining with 200 ng/ml 4′,6-diamidino-2-phenylindole (DAPI) (Sigma) for 2 min, and examined with a Leica confocal microscope after three washes in PBS.


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