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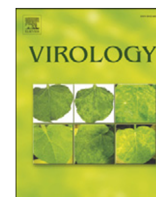

Deletion of AcMNPV AC16 and AC17 results in delayed viral gene expression in budded virus infected cells but not transfected cells

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ABSTRACT

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.
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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 phylogenically 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), *Chrysodeixis chalcites* NPV (ChchNPV) (Rohrmann, 2008) and *Ogyia leucostigma* NPV (OrleNPV, accession number EU309041). *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 amigera* 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

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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*

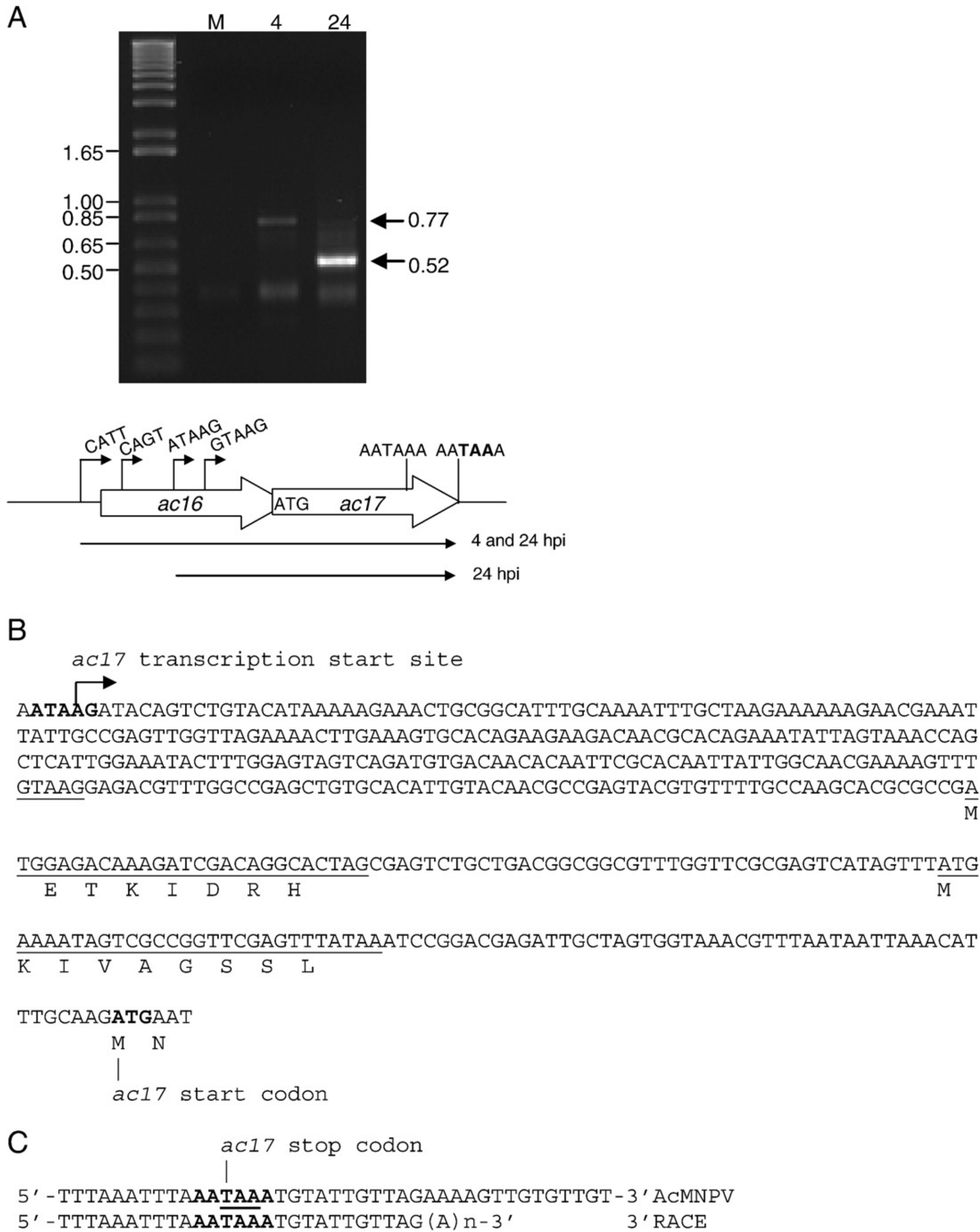

Fig. 1. 5' and 3' RACE analysis of *ac17* transcription. (A) Agarose gel analysis of *ac17* 5' RACE products at 4 and 24 hpi. Sizes of products are shown on the right in kbp. M, mock infected cells. Numbers on the left show sizes of markers in kbp. The schematic below the gel shows the *ac16* and *ac17* ORFs and the location of potential transcription start sites and 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 mini-cistrons 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 showed 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-cristrons 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

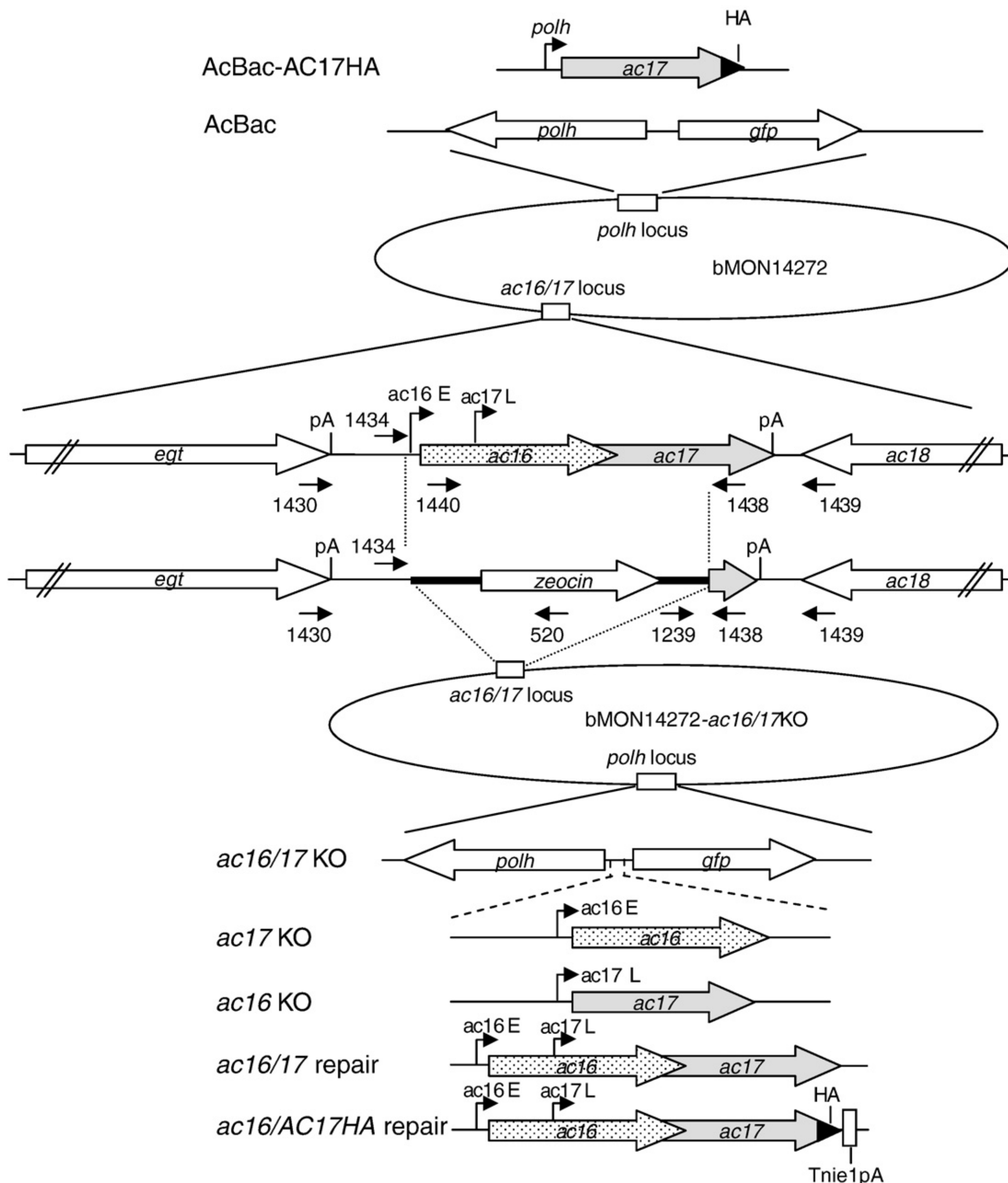

Fig. 2. Construction of *ac16*/*17*KO and repair bacmids. The sequence encoding both *ac16* and *ac17* were replaced by the *zeocin* resistance gene cassette (amplified with primers 1434 and 1438) via homologous recombination in *E. coli* to generate bMON14272-*ac16*/*17*KO. The correct deletion was 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*/*17*KO to generate the knockout and repair bacmids *ac16*/*17*KO, *ac17*KO, *ac16*KO, *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.

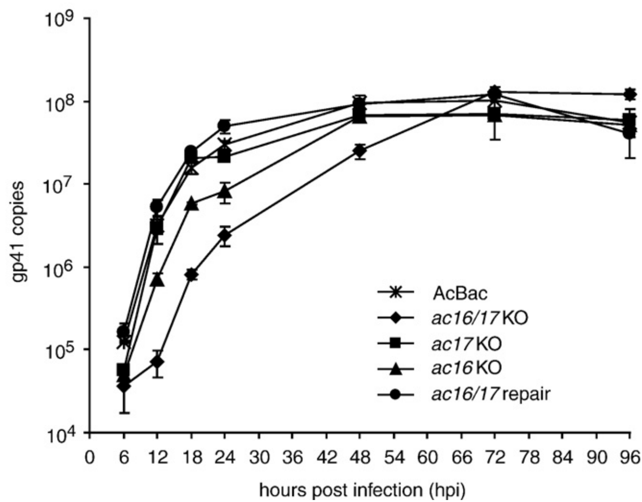

Fig. 3. Analysis of viral DNA replication by qPCR. Sf9 cells were infected by *ac16/17*KO and *ac17*KO, *ac16*KO, *ac16/17repair* and AcBac at a MOI of 5 and collected at various times post-infection. Viral DNA replication was analyzed using qPCR based on the amplification of a 100 bp fragment of the viral gene *gp41*. Each value represents the average of two samples from two independent infections. Error bars represent standard error.

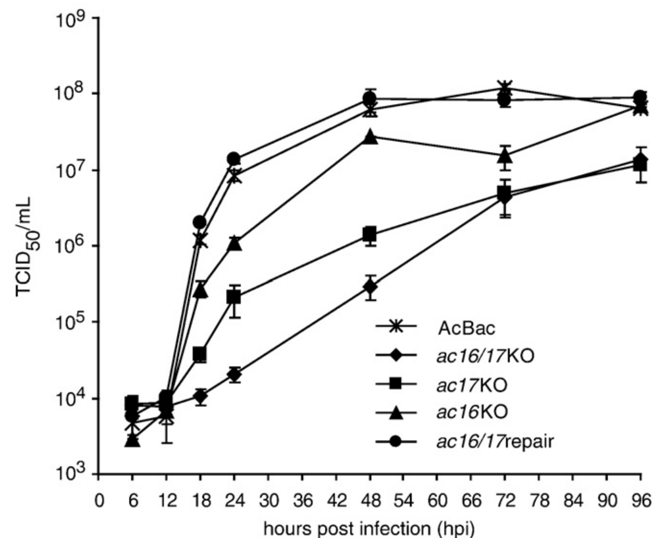

Fig. 4. Growth curve analysis of *ac16/17*KO and repair viruses. Sf9 cells were infected by *ac16/17*KO and repairs *ac17*KO, *ac16*KO, *ac16/17repair* and AcBac at a MOI of 5. BV supernatant was collected at various times post-infection and titred using qPCR based on the amplification of a 100 bp fragment of the viral *chintinase* gene as described in the Materials and Methods. Each value represents the average of two samples from two independent infections. Error bars represent standard error.

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-*ac16/17*KO) 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-*ac16/17*KO, generating *ac16/17*KO. To examine the function of *ac17*, the double KO virus bMON14272-*ac16/17*KO was repaired with *ac16*, *ac17*, *ac16–ac17* or *ac16–ac17HA*, respectively via transposition along with *gfp* and *polyhedrin*. The viruses generated were named *ac17*KO, *ac16*KO, *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 *ac16/17*KO, *ac17*KO, *ac16*KO, *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 (*ac16/17*KO) 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 *ac17*KO, 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 *ac16*KO 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 *ac16*KO-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 *ac17*KO, *ac16*KO, *ac16/17*repair 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 *ac16/17*KO BV levels were equal to *ac17*KO but remained nearly a log lower than AcBac. The reduced BV could be related to the lower viral DNA replication levels (Fig. 3).

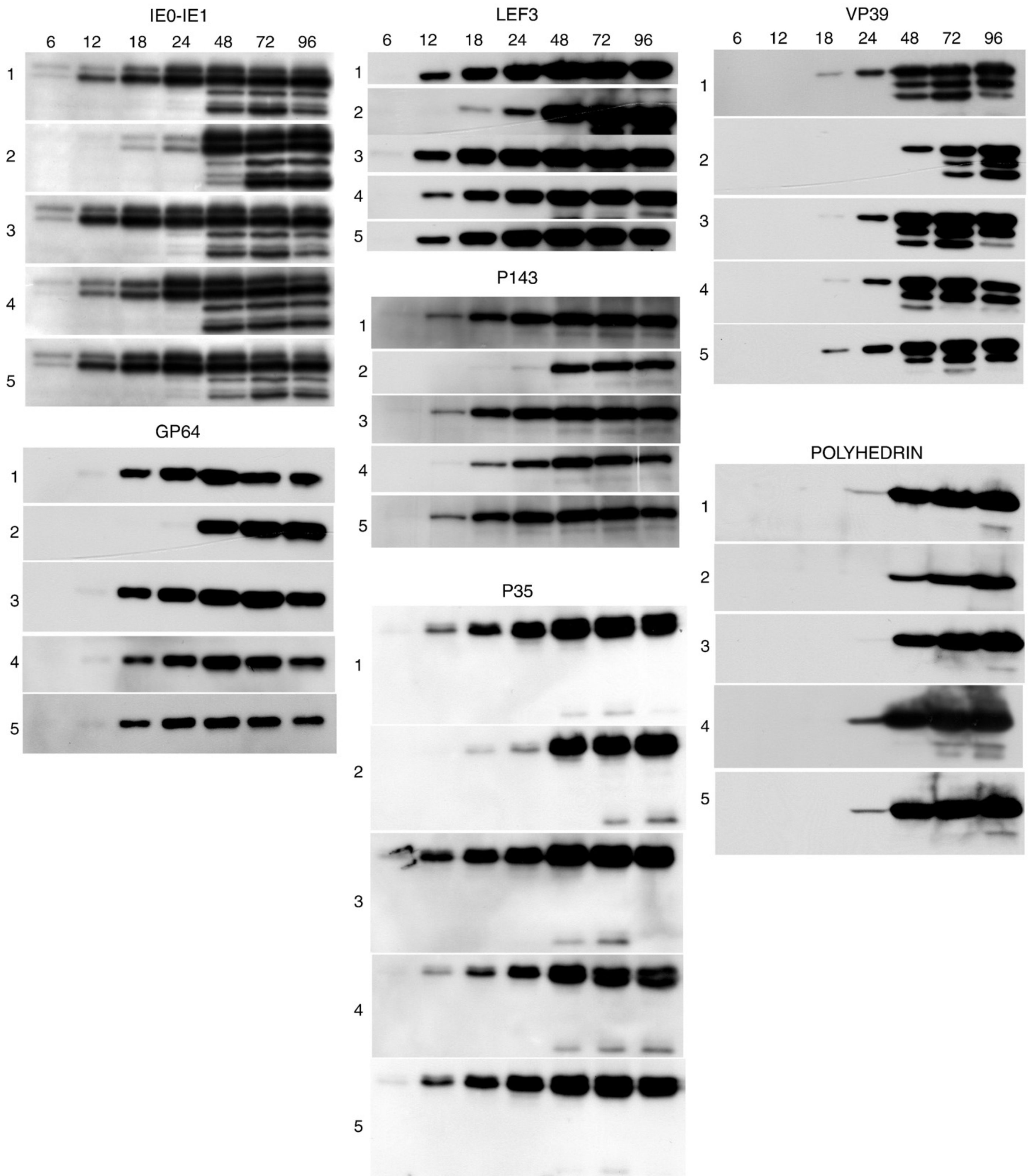

Fig. 5. Western blot analysis of the temporal expression of the viral early and late genes IE0, IE1, GP64, LEF-3, P143, P35, VP39 and POLYHEDRIN. Sf9 cells were infected with AcBac (1), *ac16/17*KO (2), *ac17*KO (3), *ac16*KO (4), and *ac16/17repair* (5) at a MOI of 5. Infected cells were harvested at the times indicated in the presence of protease inhibitors and analyzed by SDS-PAGE and Western blot.

Viral gene expression

The results described above showed reduced levels of viral DNA replication in the *ac16/17*KO and *ac16*KO 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 *ac16/17*KO or repair viruses, the temporal expression of the early proteins IE0, IE1, GP64, P35, LEF3, P143, the late protein

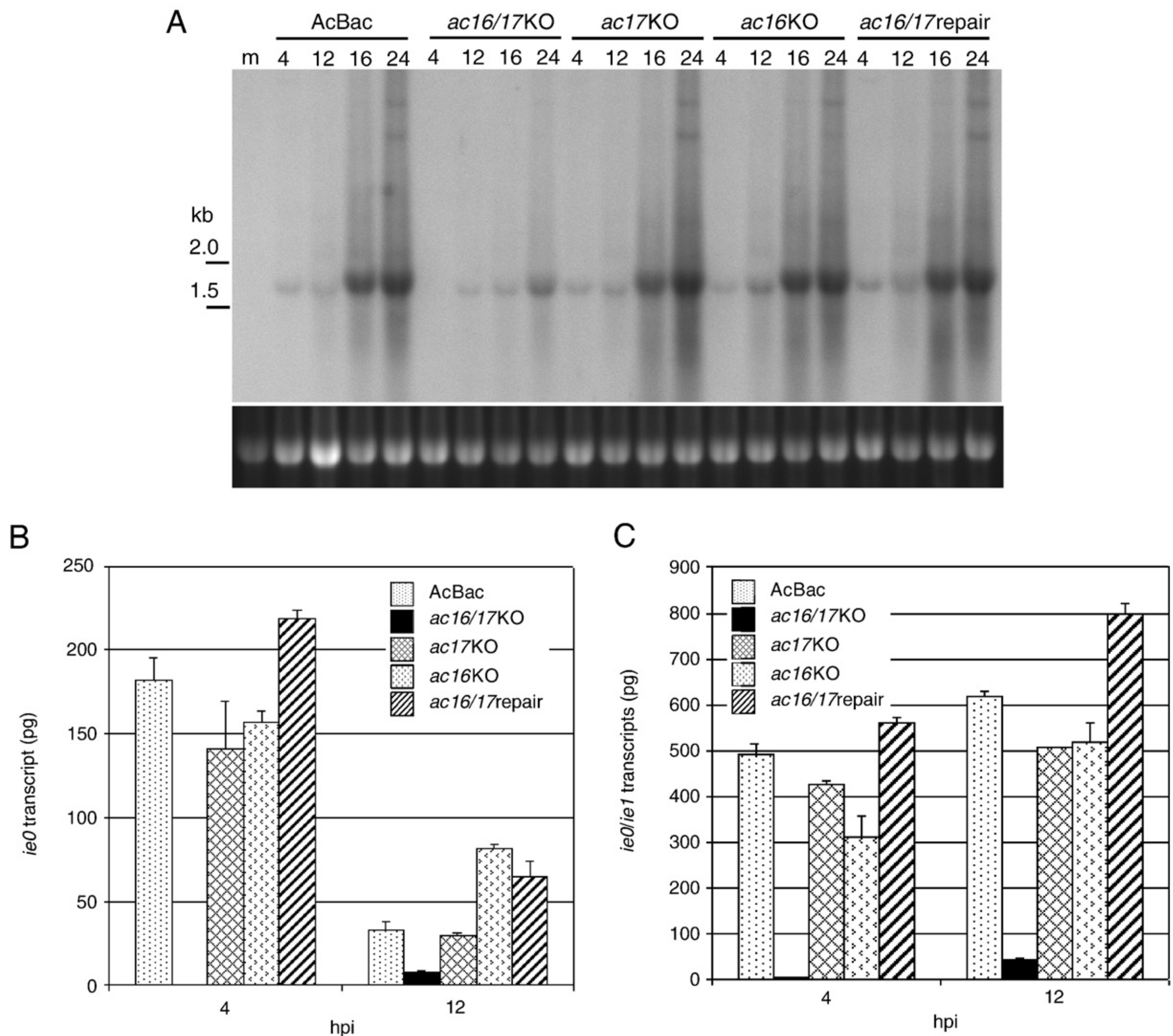

Fig. 6. Transcriptional analysis of *gp64, ie0* and *ie1*. (A) Northern blot analysis of *gp64* expression at 4, 12, 16, and 24 hpi in Sf9 cells infected at a MOI of 5 with AcBac, *ac16/17*KO, *ac17*KO, *ac16*KO, *ac16/17repair*. Total RNA (10 μg) of each sample were separated on a 1% formaldehyde agarose gel, blotted and probed with a *gp64* strand specific RNA. Below the Northern blot is a picture of the ethidium bromide stained ribosomal bands of the agarose gel. (B, C) Analysis of *ie0* and combined *ie0* and *ie1* transcripts using qRT-PCR. Total RNA (5 μg) was analyzed from Sf9 cells infected with AcBac, *ac16/17*KO, *ac17*KO, *ac16*KO, *ac16/17repair* at 4 and 12 hpi. Background was obtained by omitting reverse transcriptase in the cDNA synthesis reactions and was subtracted from each sample values. A pair of primers specific to *ie0* was used for qPCR measurement of *ie0* transcript (B), and a pair of primers that amplifies both *ie0* and *ie1*(C) were used for the measurement of combined levels of the *ie0* and *ie1* transcripts. Each value represents the average of two qPCR reactions. Error bars represent standard error.

VP39, and the very late protein POLYHEDRIN were analyzed by Western blots from cells infected by *ac16/17*KO, *ac17*KO, *ac16*KO, *ac16/17repair* and AcBac viruses. The viruses AcBac, *ac17*KO, *ac16*KO 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 *ac16/17*KO. In cells infected by *ac16/17*KO, 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, *ac16*KO 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 also observed with late and very late VP39 and POLYHEDRIN. These results therefore suggest that in cells infected with the *ac16/17*KO 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 *ac16*KO 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 *ac16/17*KO 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 *ac16/17*KO, *ac17*KO, *ac16*KO, *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, *ac17*KO, *ac16*KO and *ac16/17repair*. However in *ac16/17*KO 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, *ac17*KO, *ac16*KO and *ac16/17repair*. This data showed that the delayed expression of GP64 detected by

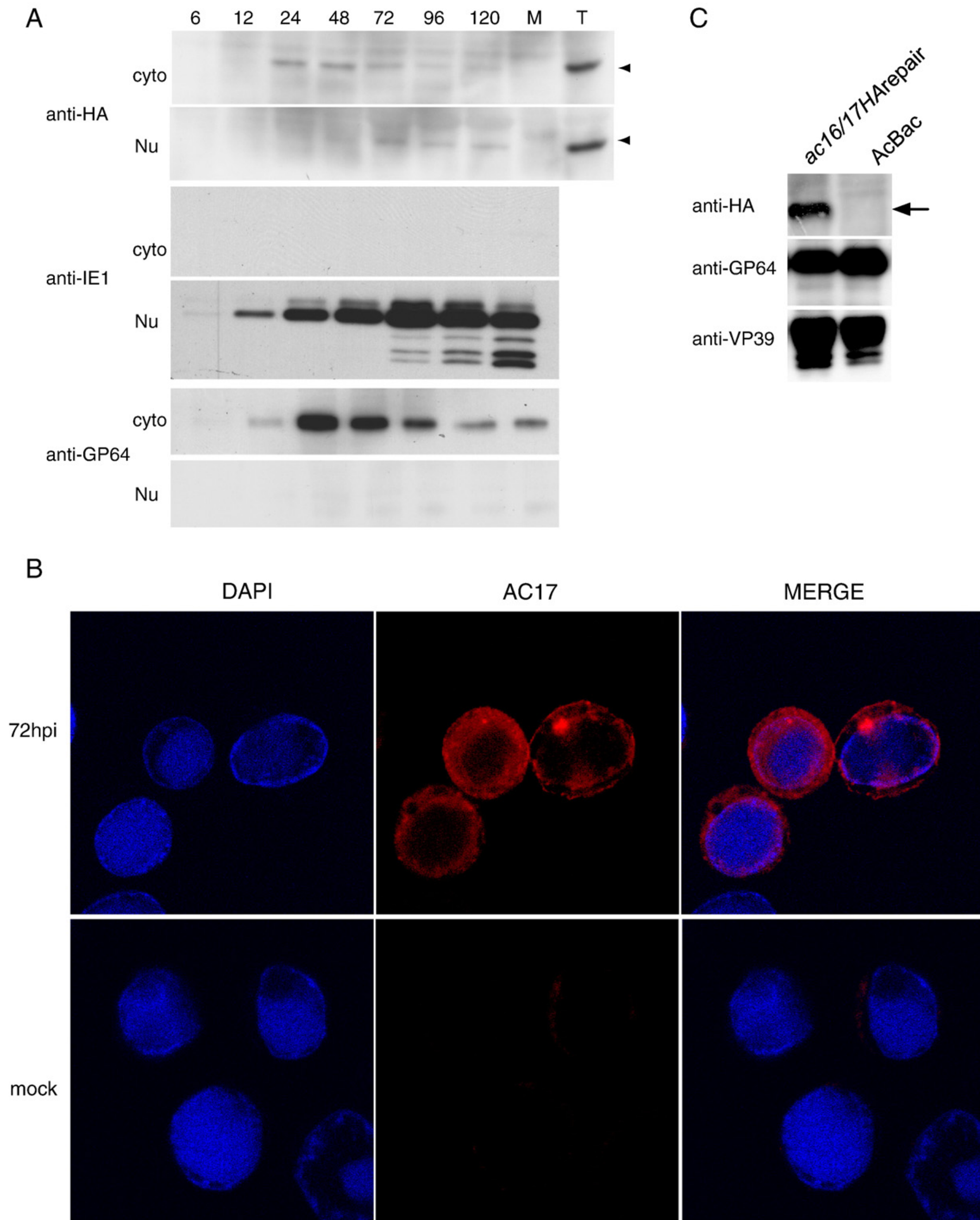

Fig. 7. Detection of AC17 in infected cells and budded virus. (A) Sf9 cells were infected with *ac16/17HArepair* (MOI = 5) and cytoplasmic (cyto) and nuclear (nu) fractions were prepared at the indicated times post-infection. Fractions were analyzed by Western blot probed with anti-HA antibody to detect AC17-HA. To confirm the correct fractionation the samples were also probed with anti-IE1 and anti-GP64. M, mock infected Sf9 cells. T, total cell protein from non-fractionated Sf9 cells transfected with *ac16/17HArepair* bacmid DNA used as a positive control and size marker. (B) Immunofluorescence confocal microscopy analysis of AC17 distribution in Sf9 cells. Cells were infected with AcBac-AC17HA (MOI = 10) which expresses *ac17* under control of the polyhedrin promoter. To detect AC17 cells were fixed at 72 hpi and incubated with anti-HA and Alexa 635 conjugated goat anti-mouse IgG. Nuclei were visualized by staining with DAPI. (C) Western blot analysis of purified BV for the presence of AC17. BV was isolated from both the HA labelled AC17 virus *ac16/17HArepair* and AcBac which does not express the HA epitope.

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, *ac16/17repair* had *ie0* expression

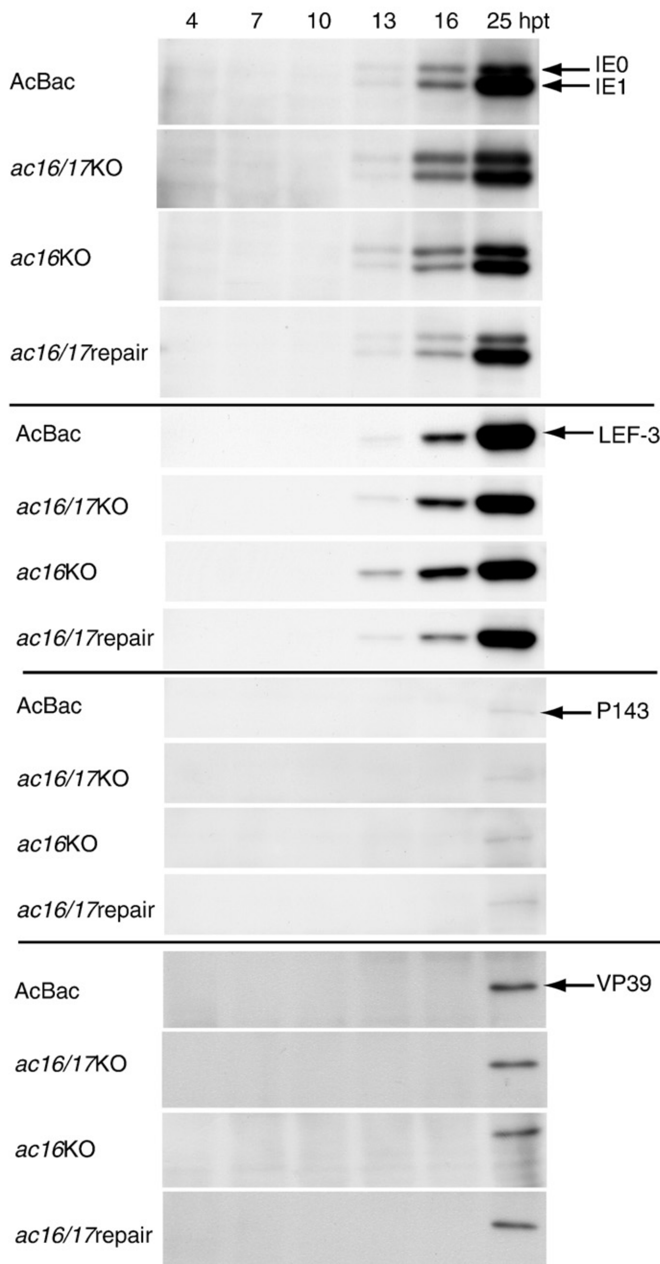

Fig. 8. Temporal analysis of early and late proteins IE0, IE1, LEF-3, P143 and VP39 in transfected Sf9 cells. Sf9 cells were transfected with bacmid DNA (1 µg) of *ac16/17*KO, *ac16*KO, *ac16/17repair* and AcBac. Total cell protein was collected at various times post-transfection and analyzed by SDS-PAGE and Western blot.

levels equivalent to the control AcBac, whereas *ac16/17*KO did not have any detectable expression above background (Fig. 6B). At 12 hpi *ie0* expression from *ac16/17*KO 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 *ac17*KO, *ac16*KO, *ac16/17repair* had significant levels of expression at 4 hpi which increased by 12 hpi similar to AcBac. However, for *ac16/17*KO no detectable expression above background was observed at 4 hpi. At 12 hpi *ie0–ie1* expression from *ac16/17*KO was detected but at levels significantly lower than any of the other viruses. The levels detected with the *ie1* primers were approximately 5.5 fold higher than *ie0* alone, which indicates both *ie1* and *ie0* are being expressed by 12 hpi for *ac16/17*KO. These results are similar to the *gp64* Northern blot results and show that deletion of both *ac16* and *ac17* results in a significant delay in the transcription of the essential immediate early genes *ie0* and *ie1*.

Localization of AC17

To determine the subcellular localization of AC17, Sf9 cells infected by *ac16/17HArepair* were collected at 6, 12, 24, 48, 72, 120 hpi. Cytoplasmic and nuclear fractions of infected cells were prepared and subjected to SDS-PAGE followed by Western blot analyses. AC17-HA was detected with HA antibody and was found to be expressed as a late protein and locates mainly in the cytoplasm at 24 and 48 hpi. However, from 72 to 120 hpi AC17-HA was observed at approximately equal levels in both the nucleus and the cytoplasm (Fig. 7A). This differs from previous studies which reported that AC17, a C-terminal truncated AC17-GFP fusion protein or the homologue HA128 were present mainly in the cytoplasm or at the nuclear envelope (An et al., 2005, 2006). Therefore to further analyze the cellular location of AC17 we used confocal microscopy. A virus expressing AC17 (full length) fused with YFP was constructed and used to detect AC17 by direct fluorescence. In addition, immunofluorescence was attempted using the virus expressing AC17-HA. However, the fluorescent signal for AC17-YFP or AC17-HA was too low and reliable localization could not be determined. The low level of expression from each virus may be due to the native *ac17* late promoter used to drive expression of AC17-YFP or AC17-HA, which contains two mini-cistrons. Therefore to increase the expression level of AC17 a third virus was made, AcBac-AC17HA, which expresses AC17-HA under the control of *polyhedrin* promoter. Under these conditions detectable levels of AC17-HA were obtained (Fig. 7B). At 72 hpi AC17-HA was detected primarily in the cytoplasm but was also in the nucleus similar to the Western blot results. The nuclear AC17 was localized on the periphery of the virogenic stroma and potentially had enhanced signal at the nuclear envelope. It is possible that higher expression obtained from the *polyhedrin* promoter could affect the relative ratio of cytoplasmic and nuclear AC17.

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 *ac16/17*KO 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 *ac16/17*KO, *ac16*KO, *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 *ac16*KO and *ac16/17repair*. 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 therefore 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*, *vlf-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; Vanarsdall et al., 2004, 2007; Wu et al., 2006; Yamagishi et al., 2007). The deletion or mutation of *ac142*, *ac143*, *gp41*, *vlf-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 *vlf-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 (Monsma et al., 1996; Oomens and Blissard, 1999; Westenberg and Vlak, 2008; Westenberg et al., 2002). Given the viability of the *ac17*KO 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 *ac16/17*KO 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 *ac16/17*KO 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 *ac16/17*KO 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 involved 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 alphabacululoviruses.

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 *Escherichi 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 (5'-GCGTCTAGACGCACTTGAATTT-CAATAAG-3') and 1559 (5'-GCGGAGCTCCTAACAATACATTTATT-TAAATTT-3') using AcMNPV genomic DNA as the template and cloned into pFAcT-GFP at *Xba*I/*Sac*I sites, generating pFAcT-GFP-AC17. To tag *ac17* with the HA epitope, primer pair 1558/1781 (5'-GCGGCGGCCGCTTAGGCGTAGTCGGGCACGTCGTAGGGGTATTTAAATT-TAAAAATAAATAAAATAGT-3') were used to amplify *ac17* from the

AcMNPV genomic DNA and the fragment containing the *ac17* promoter and ORF was cloned into pFAcT-gfp-Tnie1pA at *Xba*I and *Not*I sites, generating pFAcT-GFP-AC17-HA. The *ac17-HA* cassette was further subcloned into the *Xba*I/*Sac*I sites of pFAcT to produce pFAcT-AC17-HA. To drive *ac17-HA* with *polyhedrin* promoter, *ac17* was also amplified with primers 1818 (5'-GCGGGATCCATGAATCTCAAAGTGA-TATTAACGC-3') and 1783 (5'-GCGGAGCTCTTAGGCGTAGTCG GGCACGTCGTAGGGGTATTTAAATTTAAAAATAAATAAAATAGT-3') using AcMNPV genomic DNA as the template and subsequently cloned into pFastbacI using the *Bam*HI/*Sst*I sites, generating pFastbac-AC17HA. The fragment containing both *ac16* and *ac17* was amplified with primer pair 1430 (5'-GCGCTCGAGCTACCTACAAAAAACACATGG-3') and 1477 (5'-GCGGAGCTCAAGCGGTTTATGTCATGTAT-3') using AcMNPV genomic DNA as the template, and the PCR product was cloned into pFAcT-GFP at the *Xho*I/*Sac*I sites, generating pFAcT-GFP-AC16/17. 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 (Datsenko and Wanner, 2000; Hou et al., 2002). A *zeocin* resistance cassette with *ac16/17* flanking regions was amplified using primers 1434 (5'-TTGTGCGACTGCGCACTTCCAGCCTTTAT AAACGCTCAC-CAACCAAAGCATTCGGATCTCTGCAGCAC-3') and 1438 (5'-ATTTTTTT-TATTAATATTATAATTTTTATCTACCTTTATAAATTTTACTACATCGAGGTC-GACCCCCCTG-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 1430/520 (5'-CCGGAACGGCACTGGTCAACTT-3') and primers 1239 (5'-CTGACC-GACGCCGACCAA-3') and 1439 (5'-ATAGTTAATAGCTGTCTACCCGTA-3') were used to detect the correct insertion of the *zeocin* cassette and primer pair 1440 (5'-CTCGAGGTGCCAGTAGCAATCAATTT-3') and 1439 was used to confirm the deletion of the desired sequence. One recombinant bacmid confirmed by PCR was selected and named bMON14272-*ac16/17*KO.

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

To generate the *ac16/17*KO and repair viruses with POLYHEDRIN and GFP, pFAcT-GFP was transposed into bMON14272-*ac16/17*KO as previously described (Luckow et al., 1993) to make *ac16/17*KO; pFAcT-GFP-AC16, pFAcT-GFP-AC17, pFAcT-GFP-AC16/17 and pFAcT-GFP-AC16-AC17-HA vectors were used to transpose bMON14272-*ac16/17*KO to generate *ac17*KO, *ac16*KO, *ac16/17repair* and *ac16/17HArepair* respectively. To enable the immunofluorescence confocal analysis of AC17, pFastbac-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, *ac16/17*KO, *ac17*KO, *ac16*KO, *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 x *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 policemen, 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 time course 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-isoamyl alcohol followed by extraction with chloroform. 2 µl viral DNA extracts was used directly for the qPCR with primers 850 (5'-TTTGGCAAGGGAACTTTGTC-3') and 851 (5'-ACAAACCTGGCAGGA-GAGAG-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 ($TCID_{50}$). 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 *gp41* gene. The results were analyzed by the MX4000 software (Stratagene).

Northern blot

Sf9 cells were infected by *ac16/17*KO, *ac17*KO, *ac16*KO, *ac16/17repair* and AcBac at MOI = 5 and cells were collected at 6, 12, 16, 24 hpi. Total RNA was extracted from Sf9 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-^{32}P labelled single stranded RNA probe of *gp64* (Fourney 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'-TCATAATACGACTCACTATAGGGTCAGCTCCTCTTGAA-TATGCA-3') containing the T7 promoter sequence (underlined) and *gp64* homologous sequence and primer 1868 (5'-GTATGATTCT-CAAACAAAAGTCTACG-3'). The probe was labelled with α-^{32}P-UTP and synthesized using GeneScribe™ T7 RNA probe kit (USB).

qRT-PCR

Total RNA was extracted using Trizol (Invitrogen) from Sf9 cells infected by *ac16/17*KO, *ac17*KO, *ac16*KO, *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 manufacture'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 *ie0* expressing plasmid pAcie0delta (Huijskens et al., 2004) was 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'-CCATATTCGTGCGAGGCAACG-3') and 1414 (5'-GGTGTACGACGCGTTAAAAT-3') for *ie0* and 1505 (5'-GACAACAGCTATTCAGAGT-3') and 1523 (5'-CGAGTTGACGCTTGC-CAAAAA-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 manufacture's protocol that is based on 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'-AGCCATCTACAATAATCA-3') was paired with GeneRacer 5'primer (5′-CGACTGGAGCACGAGGACACTGA-3′) for the initial amplification. GSP-2 1732 (5'-GCGGGATCCTGGCATTATGG-TAATGCG-3') was paired with GeneRacer 5' nested primer (5′-GGACACTGACATGGACTGAAGGAGTA-3′) to specifically amplify the *ac17* transcript. Another gene specific primer 1432 (5'-GCGCTGCAGTTTGAAAGGTGAGGAAGA-3') was paired with GeneRacer 3' primer (5′-GCTGTCAACGATACGCTACGTAACG-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×PSB: 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 (Laemmli, 1970) using a Bio-Rad Mini-Protean 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 (Ross and Guarino, 1997); mouse monoclonal OpMNPV VP39 antibody (1:3000) (Pearson et al., 1988); mouse monoclonal GP64 antibody 1:250 (Hohmann and Faulkner, 1983); mouse monoclonal OpMNPV POLH antibody 1:10000 (Quant et al., 1984); rabbit polyclonal anti-AcMNPV LEF3 antibody 1:2000 (Chen et al., 2004); rabbit polyclonal anti-AcMNPV P143 antibody 1:2000 (Ito et al., 2004); rabbit polyclonal AcMNPV P35 antibody 1:1000 (Hershberger et al., 1994). 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 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 (Molelular 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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