Autographa californica Multiple Nucleopolyhedrovirus ac76 Is Involved in Intranuclear Microvesicle Formation

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In this study, we characterized Autographa californica multiple nucleopolyhedrovirus (AcMNPV) orf76 (ac76), which is a highly conserved gene of unknown function in lepidopteran baculoviruses. Transcriptional analysis of ac76 revealed that transcription of multiple overlapping multicistronic transcripts initiates from a canonical TAAG late-transcription start motif but terminates at different 3’ ends at 24 h postinfection in AcMNPV-infected S9 cells. To investigate the role of ac76 in the baculovirus life cycle, an ac76-knockout virus was constructed using an AcMNPV bacmid system. Microscopy, titration assays, and Western blot analysis demonstrated that the resulting ac76-knockout virus was unable to produce budded viruses. Quantitative real-time PCR analysis demonstrated that ac76 deletion did not affect viral DNA synthesis. Electron microscopy showed that virus-induced intranuclear microvesicles as well as occlusion-derived virions were never observed in cells transfected with the ac76-knockout virus. Confocal microscopy analysis revealed that Ac76 was predominantly localized to the ring zone of nuclei during the late phase of infection. This suggests that ac76 plays a role in intranuclear microvesicle formation. To the best of our knowledge, this is the first baculovirus gene identified to be involved in intranuclear microvesicle formation.

Baculoviruses are arthropod-specific, rod-shaped, enveloped viruses with circular, supercoiled double-stranded DNA genomes that replicate in the nuclei of host cells (38). The Baculoviridae are divided into four genera: Alphabaculovirus (lepidopteran-specific nucleopolyhedrovirus [NPV]), Betabaculovirus (lepidopteran-specific granuloviruses), Gammabaculovirus (hemipteran-specific NPV), and Deltabaculovirus (dipteran-specific NPV) (38). Autographa californica multiple nucleopolyhedrovirus (AcMNPV) is the archetype species of Alphabaculovirus (38). During the AcMNPV life cycle, two virion phenotypes, budded virus (BV) and occlusion-derived virus (ODV), are produced. Both types of virions have similar nucleocapsid structures and identical genetic information, but they differ in the origin and composition of their envelopes and in the roles they play in the baculovirus life cycle (32). During the early phase of infection, viral DNA replicates in the virogenic stroma, and newly synthesized viral genomes are condensed and packaged into rod-shaped capsids to form nucleocapsids. Nucleocapsids are transported into the cytoplasm and become enveloped by budding through the GP64-modified plasma membrane, thereby forming BVs. BVs are responsible for spreading infection between susceptible insect tissues and between cells in cell culture. During the late phase of infection, nucleocapsids remain localized within a peristomial ring zone, where they are enveloped in intranuclear microvesicles (whose formation is induced by viral infection) to form ODVs. ODVs are subsequently embedded into a paracrystalline protein matrix to form occlusion bodies (OBs). ODVs play a role in the horizontal transmission of infection among insect hosts (42).

Baculovirus gene expression follows a temporal cascade, which is primarily regulated at the transcriptional level (32). Viral gene transcription can be subdivided into three major phases: early, late, and very late. Early genes are transcribed by host RNA polymerase II. A common regulatory motif of many baculovirus early genes includes a TATA promoter sequence and the transcriptional initiation consensus sequence CAGT (10). Transcription of late and very late genes is dependent on viral DNA replication, is mediated by a virus-encoded RNA polymerase, and is generally initiated from the baculovirus late promoter motif TAAG (22).

AcMNPV has a genome of approximately 134 kbp that contains 154 predicted open reading frames (ORFs) (2). AcMNPV orf76 (ac76) encodes a putative 9.4-kDa protein (2). Homologs of ac76 have been identified in all sequenced lepidopteran baculovirus genomes (14, 40). Analysis with the InterProScan program shows that the ac76 homologs constitute a DUF843 baculovirus protein family (IPR008561) of unknown function. In this study, we generated an ac76-knockout mutant to investigate the role of ac76 in the AcMNPV life cycle. We found that ac76 is essential for both BV and ODV development but is not required for viral DNA synthesis. Electron microscopy showed that ac76 is not required for nucleocapsid assembly but that it is required for intranuclear microvesicle formation, ODV envelopment, and the subsequent embedding of virions into OBs.

MATERIALS AND METHODS

Viruses and cell lines. Bacmid bMON14272, containing an AcMNPV genome, was propagated in Escherichia coli DH10B as previously described (23). The S9 cell strain, the clonal isolate 9 of the parent cell line IPLB-S21-AE which is derived from the fall armyworm Spodoptera frugiperda (41), was cultured at 27°C.
in TMF-H medium (Invitrogen) supplemented with 10% fetal bovine serum, penicillin (100 μg/ml), and streptomycin (30 μg/ml). BV titers were determined with a TCD_{50} endpoint dilution assay using SF9 cells (29).

**RNA preparation and Northern blot analysis.** SF9 cells were infected with AcMNPV at a multiplicity of infection (MOI) of 10 to 50% tissue culture infective doses (TCID_{50}) per cell. Cells were collected at various time points. Total cellular RNA was isolated using an RNeasy Mini-Kit (Qiagen) according to the manufacturer's instructions. The RNA samples were quantified by optical density measurements at 260 nm.

Total RNA (10 μg) was denatured by glyoxal for 1 h at 55°C and electro-phoresed on 1.5% agarose gel in 1× BPTE buffer (10 mM piperrazine-N,N'-bis(2-ethanesulfonic acid) (PIPES), 30 mM Bis-Tris, 10 mM EDTA, pH 8.0). RNA was transferred to a positively charged nylon membrane (Hybond-N+; Amersham) and fixed to the membrane by UV cross-linking. The probe was a 59-nucleotide (nt) single-stranded DNA sequence (5'-TTAGAATCGCATCAAGCGCTTG-3'). The PCR products were analyzed by agarose gel electrophoresis, and the bands of the product that was amplified from the RNA isolated at 24 h post-infection (p.i.) were purified and cloned into pMD18-T vector (TaKaRa) for sequencing.

**Analysis by 3' and 5' RACE.** 3' Rapid amplification of cDNA ends (RACE) was performed using a 5'-3' RACE Kit, 2nd Generation (Roche), with 1 μg of total RNA. Following the manufacturer's instructions, first-strand cDNA synthesis was performed with the Superscript III reverse transcriptase (Invitrogen) and an oligo(dT) anchor primer. The cDNA mixtures were amplified via PCR with a PCR oligo(dT) anchor primer and the ac76-specific forward primer (5'-ATAT TTGTGTGGTGGCAGCAGTTG-3'). The PCR products were analyzed by agarose gel electrophoresis, and the bands of the product that was amplified from the RNA isolated at 24 h post-infection (p.i.) were purified and cloned into pMD18-T vector (TaKaRa) for sequencing.

Generation of the ac76 knockout AcMNPV bacmid. An ac76 knockout AcMNPV bacmid was generated via homologous recombination in E. coli employing the bacmid BMON14227 as previously described (4, 43). A vector in which the ac76 locus was replaced with a chloramphenicol resistance (Cm) gene for antibiotic selection in E. coli was constructed as follows. The 1.089-kb Cm gene cassette was excised by PstI/BamHI from the pUC18-US-Cm gene cassette, ligated, and inserted into pUC18 to generate the recombinant plasmid pUC-Cm. The Cm cassette was replaced by high purity PCR Purification Kit (Promega), and a poly(A) tail was added to the 5' end using terminal transferase and dATP. The tailed cDNAs were amplified by PCR using an oligo(dT) anchor primer and the nested GSP2 primer (5'-AGAATTCGCTACGTGCTTTCG-3'). The PCR products were gel purified and cloned into the pMD18-T vector (TaKaRa) for sequencing.

**Construction of knockout, repair, and wt AcMNPV bacmids.** In order to facilitate the detection of recombinant virus infection and to examine whether the ac76 knockout has any effect on OB morphogenesis, the polyhedrin gene (polh; designated “PH” in constructs) of AcMNPV and the green fluorescent protein gene (gfp) was inserted into the polh locus of AcMNPV by site-specific homologous recombination, as previously described (43). A 950-bp fragment containing polyh and its own promoter and a poly(A) signal was amplified via PCR using the primers ac76-US2 and ac76-DS1. The PCR product was digested with EcoRI and cloned into pFB1-PH-GFP (43) to generate pFB1-ac76-PH-GFP. Electroporlent DH10B cells containing the pMON1724 helper plasmid and AcMV ac76-KO were transformed with either the pFB1-PH-GFP or pFB1-ac76-PH-GFP donor plasmid to generate the ac76 knockout bacmid AcMV ac76-KO-PH-GFP or the ac76 repair bacmid AcMV ac76-REP-PH-GFP, respectively. Electroporlent DH10B cells containing the helper plasmid pMON1724 and the bMON14227 bacmid were transformed with pFB1-PH-GFP to generate the wild-type (wt) control bacmid, which we named AcVPH-GFP. Successful transposition was confirmed by PCR. The correct recombinant bacmids were electroporlated into E. coli DH10B cells and were screened for tetacycline sensitivity to ensure that the isolated bacmids were free of helper plasmids. Bacmid DNA was then extracted and purified using a Quiagen Large-Construct Kit and was quantified by optical density.

**Analysis of viral growth.** SF9 cells (1.0 × 10^6) cells/35-mm diameter dish) were transfected in triplicate with 1.0 μg of each bacmid construct (AcMV ac76-KO-PH-GFP, AcMV ac76-REP-PH-GFP, or AcVPH-GFP) using Cellfectin liposome reagent (Invitrogen), or cells were infected in triplicate with BV at an MOI of 5. The cell debris was removed by centrifugation for 10 min at 4°C while the pelleted cells were resuspended in double-distilled H_2O (ddH_2O) for Western blot analysis. The supernatants containing BVs were collected at different time points, and titers were determined by a TCD_{50} endpoint dilution assay using SF9 cells (29).

**Western blot analysis.** SF9 cells (1.0 × 10^6) cells were transfected with 1.0 μg of each bacmid (AcVPH-GFP-PH-GFP, AcVPH-GFP-PH-GFP, or AcVPH-GFP) BVs were pu- rified as previously described (24, 44). At 12 h posttransfection (p.t.), the cultures were harvested and centrifuged at 2,000 × g for 20 min at room temperature to pellet the cells. The cell pellets were resuspended in double-distilled H_2O (ddH_2O) for Western blot analysis. The supernatants (3 ml) were loaded onto a 25% sucrose cushion and were centrifuged at 80,000 × g for 90 min at 4°C in a SW41 Ti rotor. The pellets of AcMV ac76-KO-PH-GFP, transfected and mock-transfected cells were resuspended in 9.9 ml of 250 mM Tris-Cl (pH 7.8) and 0.1 μl of protease inhibitor cocktail (Protease Inhibitor Cocktail Set III; Calbiochem), while the pellets of AcMV ac76-REP-PH-GFP, transfected and AcVPH-GFP transfected cells were resuspended in four times the volume of the same solutions due to the abundance of BVs present. For Western blot analysis, one-quarter of the AcVPH-GFP-PH-GFP, AcVPH-GFP-PH-GFP, and AcVPH-GFP-PH-GFP was loaded onto a 10% SDS-PAGE gel and were electrophoretically transferred to a nitrocellulose transfer membrane (Schleicher and Schuell) according to the manufacturer's instructions (Gene Images AlkPhos Direct Labeling and Detection System; Amersham).

Construction of knockout, repair, and wt AcMNPV bacmids. In order to facilitate the detection of recombinant virus infection and to examine whether the ac76 knockout has any effect on OB morphogenesis, the polyhedrin gene (polh; designated “PH” in constructs) of AcMNPV and the green fluorescent protein gene (gfp) was inserted into the polh locus of AcMNPV by site-specific homologous recombination, as previously described (43). A 950-bp fragment containing polyh and its own promoter and a poly(A) signal was amplified via PCR using the primers ac76-US2 and ac76-DS1. The PCR product was digested with EcoRI and cloned into pFB1-PH-GFP (43) to generate pFB1-ac76-PH-GFP. Electroporlent DH10B cells containing the pMON1724 helper plasmid and AcMV ac76-KO were transformed with either the pFB1-PH-GFP or pFB1-ac76-PH-GFP donor plasmid to generate the ac76 knockout bacmid AcMV ac76-KO-PH-GFP or the ac76 repair bacmid AcMV ac76-REP-PH-GFP, respectively. Electroporlent DH10B cells containing the helper plasmid pMON1724 and the bMON14227 bacmid were transformed with pFB1-PH-GFP to generate the wild-type (wt) control bacmid, which we named AcVPH-GFP. Successful transposition was confirmed by PCR. The correct recombinant bacmids were electroporlated into E. coli DH10B cells and were screened for tetacycline sensitivity to ensure that the isolated bacmids were free of helper plasmids. Bacmid DNA was then extracted and purified using a Quigen Large-Construct Kit and was quantified by optical density.

**Analysis of viral growth.** SF9 cells (1.0 × 10^6) cells/35-mm diameter dish) were transfected in triplicate with 1.0 μg of each bacmid construct (AcVPH-GFP-PH-GFP, AcVPH-GFP-PH-GFP, or AcVPH-GFP) BVs were pu- rified as previously described (24, 44). At 12 h posttransfection (p.t.), the cultures were harvested and centrifuged at 2,000 × g for 20 min at room temperature to pellet the cells. The cell pellets were resuspended in double-distilled H_2O (ddH_2O) for Western blot analysis. The supernatants (3 ml) were loaded onto a 25% sucrose cushion and were centrifuged at 80,000 × g for 90 min at 4°C in a SW41 Ti rotor. The pellets of AcVPH-GFP-PH-GFP, transfected and mock-transfected cells were resuspended in 9.9 ml of 250 mM Tris-Cl (pH 7.8) and 0.1 μl of protease inhibitor cocktail (Protease Inhibitor Cocktail Set III; Calbiochem), while the pellets of AcVPH-GFP-PH-GFP, transfected and AcVPH-GFP transfected cells were resuspended in four times the volume of the same solutions due to the abundance of BVs present. For Western blot analysis, one-quarter of the AcVPH-GFP-PH-GFP, AcVPH-GFP-PH-GFP, and AcVPH-GFP-PH-GFP was loaded onto a 10% SDS-PAGE gel and were electrophoretically transferred to a nitrocellulose transfer membrane (Schleicher and Schuell) according to the manufacturer's instructions (Gene Images AlkPhos Direct Labeling and Detection System; Amersham).
tion of 1:5,000. Blots were detected using an enhanced chemiluminescence system (ECL; Amersham) according to the manufacturer's instruction.

**DNA synthesis analysis by real-time PCR.** To assess viral DNA synthesis, quantitative real-time PCR was performed as previously described with several modifications (39). A recombinant virus, Ac-GP64-KO which contains a deletion of the gp64 envelope fusion protein gene, was used as a noninfectious control as the deletion of gp64 results in a virus unable to propagate infection from cell to cell and could provide a more accurate comparison (25, 39). Briefly, 1 × 10⁶ Sf9 cells were transfected in triplicate with 1 μg of Ac-GP64-KO or VAcac76-KO-PH-GFP bacmid DNA, and cells were collected at selected time points. Total DNA from each sample was prepared with a Universal Genomic DNA Extraction Kit (TaKaRa) according to the manufacturer's protocol. The total DNA was resuspended in 150 μl of sterile water. Prior to PCR, 5 μl of total DNA from each time point was digested with 20 units of DpnI restriction enzyme (NEB) overnight in a 50-μl reaction volume to eliminate input bacmid DNA. Quantitative PCR (qPCR) was performed with 10 μl of the digested DNA and the Hot Start PCR Master Mix III (Chaoshi-Bio) according to the manufacturer's instructions using the primers targeting a 100-bp region of the gp64 gene and conditions described previously (39).

**Electron microscopy.** A total of 1 × 10⁶ Sf9 cells (per 35-mm-diameter dish) were transfected with 1.0 μg of VAc-PH-GFP, VAcac76-KO-PH-GFP, or VAcac76-KO-PH-PH-GFP. At 72 h p.i., cells were dislodged with a rubber policeman and pelleted at 3,000 g for 5 min. Then the cells were fixed, dehydrated, embedded, sectioned, and stained as described previously (19). Samples were examined with a JEM-100CXII transmission electron microscope at an accelerating voltage of 80 kV.

**Construction of GFP fusion recombinant bacmids and confocal microscopy.** To monitor the localization of Ac76 in AcMNPV-infected Sf9 cells, Ac76 was expressed in frame with GFP to create an Ac76-GFP fusion protein. A recombinant fusion bacmid VAc76-KO-PH-Ac76-GFP, and a control bacmid VAcPH-PH-GFP were constructed as previously described (43, 44). To generate a recombinant bacmid containing the polh gene cassette, a donor plasmid called pFB1-PbPh was first constructed as follows. The polh cassette was excised from pFB1-PH-GFP (43) with EcoRI and SmaI. The resulting fragment was cloned into pFastBac1 (Invitrogen), which was digested with EcoRI and SmaI, yielding pFB1-PbPh. The gfp ORF was excised from pUC19egfp (43) with XbaI and PstI, and the resulting fragment was cloned into pFB1-PbPh to generate the pFB1-PbPh-gfp. The ac76 gene (without its stop codon TGA) with its native promoter was amplified from the AcMNPV bacmid using the primers ac76CF-D2 (5′-TCTAGATATGTTTCTATTAAGCCGTTATTTTGTACAG-3′; XbaI site is underlined) and ac76CF-U (5′-GAATTCCGCGCTTCAAGATTCAAG-3′; EcoRI site is underlined). The PCR product was digested with EcoRI and XbaI and was cloned into pFB1-Pb-gfp to create the donor plasmid pFB1-Pb-ac76-gfp. Competent cells containing the VAc76-KO bacmid and the helper plasmid pMON7124 were transformed with pFB1-Pb-ac76-gfp, and the ac76-gfp chimera and polh were site-specifically transposed into the VAc76-KO bacmid polh locus. The resulting virus was named VAcac76-KO-PH-GFP, and this virus expressed Ac76 with a GFP tag under control of the native ac76 promoter. The ac76 promoter was amplified via PCR using the primers ac76CF-U and ac76CF-D1 (5′-TCTAGATATGTTTCTATTAAGCCGTTATTTTGTACAG-3′; XbaI site is underlined). The EcoRI/XbaI-digested PCR product was then inserted into pFB1-Pb-gfp to generate the donor plasmid pFB1-Pb-ph76-gfp. The control bacmid VAcPH-PH-GFP, in which only GFP was expressed (under the control of the ac76 promoter), was generated using a procedure similar to that used for the creation of VAc76-KO-PH-Ac76-GFP.

Sf9 cells (1 × 10⁶) were transfected with 1 μg of VAcPH-PH-GFP or VAcac76-KO-PH-GFP DNA. At 96 h p.i., supernatants were collected, and BV titers were determined by a TCID₅₀ endpoint dilution assay using Sf9 cells. For confocal microscopy analysis, Sf9 cells (1 × 10⁶) were seeded onto glass coverslips. Cells were infected with VAcPH-PH-GFP or VAcac76-KO-PH-PH-GFP at an MOI of 10. At 12, 24, 48, and 72 h p.i., cells were visualized with a Leica TCS SP2 confocal laser scanning microscope to search for fluorescence using a wavelength of 488 nm for GFP. All images were digitally recorded and were merged using the Leica software.

**RESULTS**

**Comparison of the amino acid sequences of Ac76 homologs.** Homologs of Ac76 are found in all sequenced lepidopteran baculovirus genomes stored in the GenBank at the National Center for Biotechnology Information. A comparison of the predicted amino acid sequences showed that three regions are highly conserved: (i) a classical putative nuclear localization signal [(K/R)X₉KKK] located near the C terminus (Fig. 1, above-lined NLS); (ii) a highly conserved motif of unknown function, DAILNKNTSSID, located at the C terminus; and (iii) a positively charged amino acid (K/R) (Fig. 1, arrow) close to a highly hydrophobic transmembrane domain (Fig. 1, above-lined TM) (predicted by TMHMM [http://genome.cbs.dtu.dk/services/TMHMM/]). This sequence pattern is similar to the inner nuclear membrane-sorting motif (INM-SM) of the viral envelope protein ODV-E66 of AcMNPV (5, 8) which has been demonstrated to function as an N-terminal signal anchor and targets INM-SM-containing proteins to ODV envelope (6, 9).

**Transcriptional mapping analysis of ac76 transcripts.** The temporal expression of ac76 was examined with total RNA extracted from AcMNPV-infected Sf9 cells at different time points by using Northern blot analysis as well as 5′ and 3′ RACE analysis. Northern blot analysis demonstrated that a 1.8-kb and a 0.7-kb transcript were present with increasing abundance between 12 h and 24 h p.i. and remained present until 72 h p.i. (Fig. 2A). The transcriptional initiation site of the ac76 transcripts was determined by 5′ RACE using total RNA collected at 24 h p.i. Only one 5′ RACE product was obtained (Fig. 2B). Five clones derived from the product were sequenced, and the results revealed that all of the transcripts were initiated at the first A of the canonical baculovirus late promoter motif TAAG, which is located 12 nt upstream from the ac76 translation initiation codon ATG (Fig. 2D). The termination sites of ac76 were examined using 3′ RACE. A transcriptional pattern similar to that observed using Northern blot analysis was revealed by 3′ RACE. Two 3′ RACE products that were 1.8 kb and 0.7 kb in length were first detected at 12 h p.i. and continued to be detected until 72 h p.i. (Fig. 2C). Five clones derived from the 1.8 kb PCR product that was present at 24 h p.i. were sequenced. The results showed that this 1.8-kb mRNA terminated at the T of the putative ac73 stop codon TAA and that the transcript could potentially encode ac76, ac75, ac74, and ac73 of the AcMNPV genome (Fig. 2D). Ten PCR clones from the 0.7-kb product isolated at 24 h p.i. were sequenced and revealed two different transcript stop points located 13 and 40 nt downstream from the ac75 stop codon (Fig. 2D). These two transcripts could potentially encode ac76 and ac75.

**Generation of the ac76-knockout, repair, and wt AcMNPV bacmids.** The ac76-knockout AcMNPV bacmid, VAcac76-KO, was constructed using λ Red homologous recombination as previously described (43). To avoid affecting the transcription of either ac75 or vlf1, the resulting deletion left 145 bp at the 3′ end and 46 bp at the 5′ end of the ac76 ORF, respectively (Fig. 3A). The remaining 64-bp coding sequence was replaced with the Cm cassette (Fig. 3A).

PCR confirmed the correct insertion of the Cm gene into the ac76 locus of the bMON14272 bacmid (Fig. 3B and C). Southern blot analysis further indicated that the 64-bp fragment of the ac76 gene was successfully replaced by the Cm gene and confirmed the absence of an intact ac76 gene in the VAcac76-KO genome (Fig. 3D).

To examine if the ac76 deletion had any effect on OB morphogenesis and to facilitate observation of viral infection, the polh gene of AcMNPV and the gfp gene were inserted into the polh locus of VAcac76-KO via transposition. The resulting bacmid was named VAcac76-KO-PH-PH-GFP (Fig. 3E). As a positive
control, vAcPH-GFP was constructed by transposing polh and gfp into the polh locus of the bMON14272 bacmid (Fig. 3E). To ensure that the phenotype resulting from the ac76 knockout was due only to the deletion of ac76 and not to disruption of flanking genes, a repair bacmid was generated. A repair bacmid, vAcac76-REP-PH-GFP, was generated in which the ac76 gene with its own promoter and poly(A) signal, as well as polh and gfp, were inserted into the polh locus of vAc ac76-KO by transposition (Fig. 3E). All constructs were confirmed by PCR analysis (data not shown).

Analysis of vAcac76-KO-PH-GFP, vAcac76-REP-PH-GFP and vAcPH-GFP replication in transfected Sf9 cells. To examine the effect of ac76 deletion on viral propagation, Sf9 cells were transfected with vAc ac76-KO-PH-GFP, vAc ac76-REP-PH-GFP, or vAcPH-GFP and monitored by fluorescence microscopy. No difference was observed among the three viruses at 24 h posttransfection (p.t.), and the transfection efficiencies were comparable (approximately 10%) (Fig. 4A). There was almost no increase in the number of fluorescent cells in the vAc ac76-KO-PH-GFP-transfected cells up to 72 h p.t., indicating that there was no spread of the virus from the initially transfected cells. In contrast, fluorescence was observed in almost all cells transfected with vAcac76-REP-PH-GFP or vAcPH-GFP by 72 h p.t., indicating that both viruses were able to generate infectious BVs from the initial transfection (Fig. 4A).

Light microscopy showed that OBs with a normal appearance formed in cells transfected individually with the three viruses and that the number of cells containing OBs did not differ among the three constructs at 48 h p.t. (Fig. 4B). However, by 96 h p.t., most of the cells transfected with vAcac76-REP-PH-GFP or vAcPH-GFP contained OBs, but the number of vAc ac76-KO-PH-GFP-transfected cells containing OBs did not increase (Fig. 4B).

These results suggested that the deletion of ac76 leads to a defect in infectious BV production and no secondary infection in Sf9 cells. To better assess the effect that ac76 deletion had on virus replication and to compare the replication kinetics of the virus constructs, virus growth was analyzed. Sf9 cells were transfected with vAc ac76-KO-PH-GFP, vAc ac76-REP-PH-GFP, or vAcPH-GFP individually. At selected time points, the BV titers
were determined using a TCID_{50} endpoint dilution assay. The virus titer from the supernatant of vAc^{ac76-KO-PH-GFP} transfected cells could not be determined at any time point up to 120 h p.t., indicating that no infectious progeny viruses were produced. In contrast, the Sf9 cells transfected with vAc^{ac76-REP-PH-GFP} and vAc^{PH-GFP} showed a normal increase in BV titers (Fig. 4C). To further determine whether or not the ac76-repair virus was able to rescue the defect in infectious BV production observed in vAc^{ac76-KO-PH-GFP}-transfected cells, a second growth analysis was performed with the BVs produced from cells transfected with vAc^{ac76-REP-PH-GFP} or vAc^{PH-GFP} at an MOI of 5. The ac76-repair virus showed similar replication kinetics to the wt virus, indicating that the defective phenotype could be rescued by inserting ac76 into the polyh locus of the vAc^{ac76-KO-PH-GFP} and that the defect in BV production in cells transfected with the ac76-knockout virus was due only to the deletion of ac76 (Fig. 4C).

To further determine if any noninfectious BVs budded from the vAc^{ac76-KO-PH-GFP}-transfected cells and if the expression of the primary capsid protein, VP39, was inhibited in vAc^{ac76-KO-PH-GFP}-transfected cells, Western blot analysis was performed to compare the levels of VP39 in the supernatants and the cell extracts of bacmid-transfected cells (Fig. 4D). VP39 was detected in the extracts of the cells transfected with vAc^{ac76-KO-PH-GFP}, vAc^{ac76-REP-PH-GFP}, or vAc^{PH-GFP}, but not in the mock-transfected cell extracts, indicating that VP39 expression was not affected in the vAc^{ac76-KO-PH-GFP}-transfected cells. However, VP39 was detected only in the supernatants of vAc^{ac76-REP-PH-GFP} and vAc^{PH-GFP}-transfected cells. In contrast, no VP39 protein was detected, even when a longer exposure time was used, in the supernatants of cells transfected with vAc^{ac76-KO-PH-GFP} or the supernatants of mock-transfected cells, indicating that ac76 deletion results in a defect in BV production (Fig. 4D).

**Quantitative analysis of viral DNA synthesis.** To determine whether or not ac76 deletion affected viral DNA synthesis, the level of viral DNA synthesis in vAc^{ac76-KO-PH-GFP}-transfected cells was assayed by quantitative real-time PCR (qPCR) analysis. A gp64-knockout bacmid was used as a noninfectious control virus (25, 39). The results of this analysis showed that the ac76 deletion virus was able to synthesize viral DNA at levels similar to those of the bacmid lacking gp64 during a 96-h period, indicating that the deletion of ac76 does not impact viral DNA synthesis (Fig. 5).

**Electron microscopy analysis of vAc^{ac76-KO-PH-GFP}, vAc^{ac76-REP-PH-GFP}, and vAc^{PH-GFP}-transfected cells.** The results described above showed that ac76 was required for BV production but that deletion of ac76 did not affect viral DNA synthesis or the progression of the viral infection to the very late phase, as evidenced by the presence of OBs in the nuclei of transfected cells. To further determine if the lack of ac76 had any effect on virion morphogenesis, electron microscopy analysis was performed with thin sections generated from vAc^{ac76-KO-PH-GFP}, vAc^{ac76-REP-PH-GFP}, and vAc^{PH-GFP}-transfected cells.

At 72 h p.t., cells transfected with vAc^{ac76-REP-PH-GFP} showed the typical characteristics of a baculovirus infection, such as the presence of a well-defined virogenic stroma inundated with electron-dense rod-shaped nucleocapsids (Fig. 6A), nucleocapsids accumulating and aligning with nuclear envelopes that had been synthesized de novo (Fig. 6B), and nucleocapsids acquiring their envelopes and embedding into the developing OBs (Fig. 6C). In cells transfected with vAc^{ac76-KO-PH-GFP}, the development of the virogenic stroma and abundant normal-appearing nucleocapsids could also be observed (Fig. 6D). Accumulation of nucleocapsids was observed in the ring zone; however, no virus-induced intranuclear microvesicles could be observed within the ring zone, and nucleocapsids were never enveloped to form ODVs (Fig. 6E). The OBs were purified from Sf9 cells transfected with vAc^{ac76-KO-PH-GFP} or vAc^{PH-GFP} to be subjected to Western blot analysis. Polyhedrin was detected in the OBs from cells transfected with either wt or knockout virus. However, VP39 was detected in the OBs only from cells transfected with wt but not the knockout
virus (data not shown). This result showed that the ac76-knockout virus OBs did not contain any ODVs although the shape and the size of OBs were similar to those observed in wt virus-transfected (data not shown) or repair virus-transfected cells (Fig. 6F). The electron microscopy indicated that the deletion of ac76 had no effect on nucleocapsid assembly but that it did disrupt intranuclear microvesicle formation as well as ODV envelopment and OB formation.

**Localization of Ac76 in AcMNPV-infected Sf9 cells.** To monitor the subcellular localization of Ac76, two recombinant viruses, vAcPH-GFP and vAcac76-KO-PH-GFP, were constructed (Fig. 7A). Ac76 was expressed in frame with GFP to
produce an Ac76-GFP chimera that was under the control of the ac76 promoter in vAcac76-KO-PH-Ac76GFP. The C-terminal GFP tag of the chimera facilitated visualization of the trafficking of Ac76 in virus-infected cells. As a control, GFP alone was expressed under the control of ac76 promoter in vAcPH-p76GFP (Fig. 7A).

It was observed that fluorescence could spread from the cells initially transfected with vAcPH-p76GFP or vAcac76-KO-PH-Ac76GFP (data not shown), indicating that both recombinant viruses were able to produce infectious budded virions in Sf9 cells. Sf9 cells infected with vAcPH-p76GFP or vAcac76-KO-PH-Ac76GFP were examined for GFP-specific fluorescence with a confocal laser scanning microscope. Fluorescence was found to be distributed along the outer periphery of the nucleus at 12 h p.i. and subsequently entered the nucleus and
localized in the intranuclear ring zone between 24 and 72 h p.i. in the vAcac76-KO-PH-Ac76GFP-infected cells (Fig. 7B). However, in the vAcPH-p76GFP-infected cells, fluorescence was observed throughout the whole cell at all time points selected between 12 h and 72 h p.i. (Fig. 7B).

DISCUSSION

AcMNPV ac76 is a highly conserved gene, homologs of which have been found in all sequenced lepidopteran baculoviruses, but the function of the gene is unknown. In this study, the transcription and function of ac76 were investigated.

Transcriptional analysis of ac76 in AcMNPV-infected cells revealed the presence of at least three overlapping RNA transcripts transcribed from the ac76 region. The three transcripts were first detected at 12 h p.i., and their transcription was initiated from a viral late gene promoter motif, TAAG. Analysis by 3′ RACE indicated that these transcripts possessed different transcription termination sites. Clusters of compactly arranged genes with the same transcriptional orientation are commonly found in baculovirus genomes. Each gene in such a cluster is transcribed from its respective promoter, resulting in a series of overlapping coterminal transcripts. In most cases, the overlapping transcripts generally share either a common 5′ end (7, 31) or a coterminal 3′ end (1, 12, 13, 21, 27, 28). Transcription of the distal upstream gene may interfere with the transcriptional initiation of the downstream gene via a regulatory mechanism called promoter occlusion (1, 11, 12, 31).

We investigated the role of AcMNPV ac76 in viral replication using an ac76-knockout bacmid and found that ac76 plays an essential role in the viral life cycle. Infection can be initiated without ac76, as evidenced by the presence of OBs in the ac76-knockout bacmid-transfected Sf9 cells, but the infection was restricted to the initially transfected cells, and the virus was unable to spread. The viral growth curve and Western blot assays confirmed that the ac76-deletion virus was unable to produce BVs.

As mentioned above, three overlapping multicistronic transcripts were transcribed from ac76. All of the transcripts have the same 5′ end but different 3′ ends. So the defect in BV production induced by deletion of ac76 from the AcMNPV
bacmid might have resulted from an interruption in the expression of the gene downstream of ac76. In our study, the deletion phenotype could be rescued by reinsertion of ac76 into the polh locus of the vAcac76-KO bacmid to generate vAcac76KO-PH-Ac76GFP. The control virus vAcPH-p76GFP was constructed by transposing gfp (under the control of the ac76 promoter) and polh into the AcMNPV bacmid. (B) Confocal images of Sf9 cells infected with recombinant virus. Cells were infected with vAcac76-KO-PH-Ac76GFP at an MOI of 10 and were observed for fluorescence by confocal laser scanning microscopy at 12, 24, 48, and 72 h p.i. As a control, cells were infected with vAcPH-p76GFP and examined with the confocal microscope. For each time point, GFP-specific fluorescence micrographs are shown to the left of the merged micrographs. Scale bar, 10 μm.

FIG. 7. Subcellular localization of the Ac76-GFP fusion protein in Sf9 cells infected with GFP-tagged virus. (A) Schematic diagram of the generation of GFP-tagged recombinant viruses. An ac76-gfp chimera, under the control of the native ac76 promoter, and polh were inserted into the polh locus of the vAcac76-KO bacmid to generate vAcac76KO-PH-Ac76GFP. The control virus vAcPH-p76GFP was constructed by transposing gfp (under the control of the ac76 promoter) and polh into the AcMNPV bacmid. (B) Confocal images of Sf9 cells infected with recombinant virus. Cells were infected with vAcac76-KO-PH-Ac76GFP at an MOI of 10 and were observed for fluorescence by confocal laser scanning microscopy at 12, 24, 48, and 72 h p.i. As a control, cells were infected with vAcPH-p76GFP and examined with the confocal microscope. For each time point, GFP-specific fluorescence micrographs are shown to the left of the merged micrographs. Scale bar, 10 μm.

Electron microscopy showed that abundant nucleocapsids with a normal appearance were both present in the intranuclear space of vAcac76-KO-PH-GFP-transfected cells and bundled in the ring zone, indicating that the deletion of ac76 had no effect on nucleocapsid assembly or on trafficking of the nucleocapsids out of the virogenic stroma. The normal, abundant nucleocapsids suggest that new viral DNAs have been embedded into capsids. This is consistent with the qPCR analysis which showed that ac76 is not involved in viral DNA synthesis. In vAcac76-REP-PH-GFP and vAcPH-GFP-transfected cells, the nucleocapsids approached the intranuclear microvesicles and acquired envelopes to form ODVs. However, membrane vesicles were not observed at the ring zone of vAcac76-KO-PH-GFP-transfected cells, and nucleocapsids were never enveloped to form ODVs. Intranuclear microvesicle formation is induced by baculovirus infection, and
these microvesicles ultimately become the envelope of the ODVs (8). There are two hypotheses about the origin of the nuclear microvesicles; some investigators believe that de novo synthesis occurs, while others believe that ODV envelopes are derived from the inner nuclear membrane (15, 42). Our results suggest that Ac76 plays a role in intranuclear microvesicle formation. Similar to viruses with knockouts of the 38K (43), ac142 (24), p48 (44), and ac53 (20) genes, no ODVs were present in the ac76-knockout virus occlusion bodies; interestingly, these genes have also been shown to affect the production of BV. Many ODV envelope proteins, including ODV-E56 (6), ODV-E66 (15), ODV-E25 (34), BV/ODV-E26 (3), and ODV-E18/ODV-E35 (7), are enriched in microvesicles, suggesting that virus-induced intranuclear microvesicles play an important role, either as a direct precursor or as an assembly focus for the ODV envelope (8). However, previous studies have indicated that disruption of ODV-E56 and ODV-E66 does not affect the formation of intranuclear microvesicles (6, 16), indicating that these proteins execute their functions after intranuclear microvesicle formation. It is possible that Ac76 interacts with other proteins and induces the formation of intranuclear microvesicles and that, after formation, the intranuclear microvesicles could be modified with specific ODV envelope proteins and could ultimately become the envelope of the ODV.

Using a recombinant virus expressing an Ac76-GFP chimera as a visual marker, we found that Ac76 predominantly localized to the ring zone during the late phase of infection. The intranuclear ring zone is the site of several morphogenic processes that are closely associated with ODV development (42). Some ODV-associated proteins, e.g., P74 (37), BV/ODV-E26 (3), and P91 (17, 33), also have similar localization patterns in virus-infected cells. In accordance with this localization pattern, Ac76 contains a highly hydrophobic transmembrane domain and a putative nuclear localization signal. The primary ODV envelope proteins have prominent hydrophobic transmembrane motifs that are involved in membrane insertion, anchoring, and localization in the ODV envelope (8, 36). The N-terminal hydrophobic transmembrane regions of ODV-E66 and ODV-E25 are sufficient to traffic fusion proteins to intranuclear membranes and microvesicles (16). In a study in which the hydrophobic C-terminal transmembrane region of P74 was fused to GFP, the resulting chimera localized to the intranuclear ring zone in a similar pattern to that of full-length P74-GFP (37). The Ac76 transmembrane region is similar to the INM-SM, which contains two features: a hydrophobic sequence of 18 to 20 amino acids that constitutes a transmembrane domain and a positively charged residue within 4 to 8 amino acids of the end of the hydrophobic sequence that is exposed to the cytoplasm/nucleoplasm (5, 8, 9). In this study, the hydrophobic transmembrane region of Ac76 was disrupted by the replacement of ac76 by the Cm gene, which resulted in the blockage of intranuclear microvesicle formation. Ac76 may therefore be an integral membrane protein that is associated with intranuclear microvesicle formation. Further studies need to be performed in order to determine whether Ac76 is a virion structural protein. Additionally, it is important to examine whether Ac76 localizes to the nuclear envelope and intranuclear microvesicles.

In conclusion, this study suggests that ac76 is essential for BV and ODV production and intranuclear microvesicle formation. Although the exact role of ac76 is unclear, we are hopeful that the results of our study will lead to a better understanding of the molecular mechanism of microvesicle formation by providing a foundation for further research on this topic.

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REFERENCES


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