Analysis of the *Autographa californica* Multiple Nucleopolyhedrovirus Overlapping Gene Pair *lef3* and *ac68* Reveals that AC68 Is a *Per Os* Infectivity Factor and that LEF3 Is Critical, but Not Essential, for Virus Replication

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Autographa californica multiple nucleopolyhedrovirus *ac68* is a core gene that overlaps *lef3* which encodes the single-stranded DNA binding protein. A knockout (KO) virus lacking both *lef3* and *ac68* was generated (*lef3-ac68* 2×KO) to enable the functional study of *ac68*. To produce an *ac68*KO virus that did not impact *lef3* expression, the *lef3-ac68* 2×KO virus was repaired with a DNA fragment containing *lef3* and *ac68*, in which *ac68* contained point mutations so that only LEF3 was expressed. Repair of *lef3-ac68* 2×KO with just *ac68* generated an *lef3*KO virus. Analysis of the *ac68*KO virus showed that viral DNA replication and budded virus (BV) levels were unaffected compared to levels in the double-repair or wild-type (WT) control virus. Bioassay analyses of *Trichoplusia ni* larvae injected with BV directly into the hemolymph, bypassing the gut, showed no difference in mortality rates between the *ac68*KO and the WT viruses. However, in oral bioassays the *ac68*KO occlusion bodies failed to kill larvae. These results show that the core gene *ac68* encodes a *per os* infectivity factor (*pif6*). The *lef3*KO virus was also analyzed, and virus replication was drastically reduced compared to WT virus, but very low levels of *lef3*KO virus DNA replication and BV production could be detected. In addition, in transfected cells P143 was transported to the nucleus in the absence of LEF3. This study therefore shows for the first time that even though the loss of LEF3 severely impairs virus replication, it is not absolutely essential for P143 nuclear import or viral replication.

Baculoviruses are large double-stranded DNA viruses, which specifically infect insects mainly from the orders Lepidoptera, Hymenoptera, and Diptera. *Baculoviridae* consists of alpha-, beta-, gamma-, and deltabaculoviruses (20, 35). Of the baculoviruses completely sequenced to date, 32 genes have been shown to be present in all genomes, and these have been categorized as core genes (17, 31, 46). Many of these core genes have been analyzed in *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV), the *Alphabaculovirus* type species, and most appear to be required for an essential viral function such as replication or transmission (25, 39, 40). One exception, however, is the core gene *ac68* for which no clear function has been identified, and the published results have been inconsistent (24, 25).

The AcMNPV *ac68* open reading frame (ORF) encodes a predicted protein of 192 amino acids (aa) that contains a C-terminal transmembrane domain (1, 24, 25). Using reverse transcription-PCR (RT-PCR) and 5' rapid amplification of cDNA ends (RACE), *ac68* has been reported to be an early gene; however, Western blot analysis demonstrated that it is a late gene and that the observed size of AC68 is smaller than predicted (24, 25). Functional analysis of *ac68* by the construction of a knockout (KO) virus (designated *ac68*KO) did not reveal any impact on viral replication kinetics in tissue culture. *In vivo* bioassays indicated no difference in 50% lethal dose (LD₅₀) values, but the mean time to death (LT₅₀) values were higher than for the wild type (WT). Occlusion bodies (OBs) produced by the *ac68* deletion mutant did not appear to be different from those of the WT and contained a similar amount of occlusion-derived virus (ODV) (25). The closely related *ac68* homologue from *Bombyx mori* NPV (BmNPV), *bm56*, has also been studied and in contrast has been reported to be a late gene. The

bm56 ORF encodes a smaller predicted protein (134 aa) and, unlike *ac68*, this gene product migrates at the predicted molecular mass as determined by Western blot analysis. Bioassays of BmNPV *bm56*KO virus via budded virus (BV) injection showed no difference in LD₅₀ values, but similar to the AcMNPV *ac68*KO, there was an extended LT₅₀. The AcMNPV *ac68*KO produced normal OBs, but the *bm56*KO virus produced OB-like structures that did not contain any embedded ODV (52). The functional analyses of *ac68* and *bm56*, closely related homologues from group I alphabaculoviruses, are therefore contradictory in terms of when the genes are expressed, the protein sizes, and the effects on OB development. Adjacent to *ac68* and *bm56* is the gene encoding LEF3, the single-stranded DNA binding (SSB) protein. The deletions of *ac68* and *bm56* in the previous studies involved the *lef3* or *ac69* promoter, and therefore expression of these genes could have been impacted and affected the observed phenotype; however, this was not thoroughly investigated (24, 25, 52).

lef3 encodes a late-gene expression factor found in all the

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TABLE 1 List of primers used for constructs and analyses

Primer no.	Sequence (5'–3')
1014	CCGATATACTATGCCGATGATT
1239	CTGACCGACGCCGACCAA
1271	CCCATTGTCATTTCTTCCAATTCGCTTTTAAAC ACATTACATTGTACGATCGAGGTCGA CCCCCTG
1273	GCCGTCTCTCGACAGTTAC
1564	TCTCTCGTTGTACATTGTTAAAGTTTTTGT TTAAATTGTACACTTCGGATCTCTG CAGCAC
1565	TTGTCGATCTCTGATAGTTT
1585	GCGCTGCAGTAAAGAGATGGAGTCTATT
1586	GCGCTAGATTAGGCGTAGTCGGGCACGTCG TAGGGGTATTTAATTTTCTGCAACATT
1587	TTATCGACAACAGCAATATGGCGA
1588	TGTGGGAATCTATCCGATG
1699	TAAGAATCTATCCGATGGCAA
1703	TTAATCGACAACAGCAATATG
1735	ATCAACTCCCTCCACGCTAACGAA
1792	AACACGCCGATTGTGTACAATTTAAAAACAAA AACTTTAACATAATACAACGAGAGAATA
1793	GTAGTCGAAATTTTCAAATCGGCTTTTGA ATTATGTTCTGAACGTGTGTGCGA
2051	GCGTCTAGATTAGGCGTAGTCGGGCACGTCG TAGGGGTATTTAATTTTCTGCAACATT

alpha- and betabaculoviruses (17). The AcMNPV *lef3* transcript is transcribed in the opposite direction but overlaps the predicted *ac68* 5' end and initiates within the *ac68* ORF (28). *lef3* was identified as one of the genes essential for baculovirus DNA replication by transient analyses and recently by construction of a KO mutant (22, 53). LEF3 has also been shown to interact with the viral proteins P143 and alkaline exonuclease, which are involved in viral DNA replication and recombination (26, 33, 34). Using plasmid transfection experiments, it has also been shown that P143, a DNA helicase with ATPase and DNA binding activities, is dependent upon LEF3 for transport to the nucleus (15, 32, 50).

In this study we have reanalyzed the function of AC68 in AcMNPV-infected cells by generating a combination of constructs with either *lef3*, *ac68*, or both genes inactivated. This approach allowed the functional characterization of AC68 by isolating any observed phenotypic changes to either AC68 or LEF3. The results showed that AC68 is a newly identified *per os* infectivity factor (PIF6) and, surprisingly, that LEF3, though very important, is not strictly essential for viral DNA replication or for nuclear transport of viral P143 to the nucleus.

MATERIALS AND METHODS

Cells and viruses. *Spodoptera frugiperda* Sf9 cells (derived from IPLB-Sf-21 cell line) were used as host cells for viral propagation and maintained at 27°C in TC100 medium supplemented with 10% fetal bovine serum and 50 µg/ml gentamicin. AcMNPV recombinant bacmids were derived from the commercially available bacmid bMON14272 (Invitrogen Life Technologies) as described previously (7, 30) and propagated in *Escherichia coli* strain DH10B.

Construction of plasmids. The AcMNPV *lef3-ac68* gene region was amplified with the primer pair 1585 and 1586 (Table 1) using bMON14272 as the template and cloned into pBS+ to generate pBS-*lef3-ac68HA*. To enable the detection of AC68, primer 1586 added the hemagglutinin (HA) epitope tag to the 3' end of the *ac68* ORF. To generate an

ac68KO virus, a series of point mutations were inserted into pBS-*lef3-ac68HA* to disrupt protein production from the four potential ATG start codons (Fig. 1). The start codon of the predicted *ac68* lies within the *lef3* ORF. Therefore, to prevent an impact on *lef3*, an A was inserted 17 bp upstream from the *lef3* start codon, generating a frameshift mutation of the predicted *ac68* ORF, using the primer pair 1587 and 1588 and pBS-*lef3-ac68HA* as the template, generating pBS-*lef3-ac68*^{M1}. The second *ac68* ATG start codon was replaced with two stop codons, TAATAA, using the primer pair 1699/1703 and pBS-*lef3-ac68HA* as the template, producing the plasmid pBS-*lef3-ac68*^{M2}. The plasmids pBS-*lef3-ac68* and pBS-*lef3-ac68*^{M2} were used as a template, respectively, to generate TAA mutations of the third and fourth *ac68* ATGs (pBS-*lef3-ac68*^{M3}) and the second, third, and fourth *ac68* ATGs (pBS-*lef3-ac68*^{M4}) using the primer pair 1792 and 1793 (Fig. 1).

The fragments containing *lef3* and *ac68* or *ac68* mutant from pBS-*lef3-ac68*, pBS-*lef3-ac68*^{M1}, pBS-*lef3-ac68*^{M2}, pBS-*lef3-ac68*^{M3}, and pBS-*lef3-ac68*^{M4} were cloned into pFAct-Tn1pA at PstI and XbaI sites, generating transfer vectors pFAct-GFP-*lef3-ac68*, pFAct-GFP-*lef3-ac68*^{M1}, pFAct-GFP-*lef3-ac68*^{M2}, pFAct-GFP-*lef3-ac68*^{M3}, and pFAct-GFP-*lef3-ac68*^{M4}. To generate an *lef3KO* virus, an *ac68* single-repair virus was constructed. *ac68* was PCR amplified using primers 2051 and 1586 (Table 1) and cloned into pFAct-Tn1pA at PstI and XbaI sites.

Construction of an *lef3-ac68* knockout, mutant, and repair viruses. A *lef3-ac68* double-KO mutant (*lef3-ac68* 2×KO) was made by replacing the *lef3-ac68* coding sequences with a zeocin resistance gene cassette (Fig. 1). Primer pair 1271 and 1564 (Table 1) was used to amplify the zeocin resistance gene expression cassette using p2ZeoKS (Invitrogen) as the template. Primers 1271 and 1564 each harbor 50 and 45 bp of flanking sequences homologous to the 5' of *lef3* and 3' of *ac68*, respectively. The fragment was transformed into competent *E. coli* BW25113 cells (7) containing bMON14272 and the pKD46 helper plasmid. *lef3-ac68* 2×KO mutants were identified by selection on zeocin- and kanamycin-containing medium (7). A positive colony (*lef3-ac68*null) was selected and verified by PCR for the correct insertion of zeocin cassette. The deletion of *lef3-ac68* was confirmed by PCR using the primer pairs 1273/1014 and 1239/1265. To enable the phenotype analysis of the *lef3-ac68* 2×KO virus, bacmid *lef3-ac68*null was transformed into *E. coli* DH10B competent cells containing the transposase helper plasmid (30). Repair constructs were made by transforming the DH10B competent cells containing *lef3-ac68*null bacmid and helper with the transfer vectors described above to generate the bacmid viruses *lef3-ac68* 2×KO, *lef3KO*, the double-repair virus *lef3-ac68*Rep, *ac68KO1*, *ac68KO2*, *ac68KO3*, and *ac68KO4*. The bacmid *lef3KO* was generated by repair with just *ac68* (Fig. 1). In *ac68KO1*, a frameshift mutation was inserted downstream from the putative first methionine codon (M1) to stop translation of *ac68* but without altering the *lef3* ORF. The second methionine codon (M2) of *ac68* was mutated to TAA in *ac68KO2*. The second and third methionine codons (M2 and M3) were mutated to TAA in *ac68KO3*, and the second, third, and fourth methionine codons (M2, M3, and M4) were mutated to TAA in *ac68KO4*.

Western blotting. Total protein from infected Sf9 samples or purified virions were resuspended in phosphate-buffered saline ([PBS] 137 mM NaCl, 10 mM phosphate, 2.7 mM KCl, pH 7.4) containing 1% protease inhibitor cocktail (Sigma), mixed with 2× SDS protein sample buffer ([2×PSB], 0.25 M Tris-Cl, pH 6.8, 4% SDS, 20% glycerol [vol/vol], 10% 2-mercaptoethanol [2-ME], 0.05% bromophenol blue), boiled for 10 min, and loaded onto SDS-PAGE gels using a Bio-Rad mini-protein apparatus for separation (23). Separated proteins were transferred to Millipore polyvinylidene difluoride (PVDF) membrane using a Bio-Rad wet transfer unit. Western blotting was conducted following standard protocols (16). Primary antibodies used include monoclonal antibodies anti-HA (dilution, 1:1,000; Covance), *Orgyia pseudotsugata* M nucleopolyhedrovirus (OpMNPV) anti-VP39 (1:3,000) (41), anti-IE1 (1:10,000) (47), and anti-GP64 (1:500) (18) and polyclonal antibodies anti-P143 (1:2,000) (19) and anti-LEF3 (1:20,000) (4). Secondary antibodies were goat anti-mouse IgG (1:10,000) and goat anti-rabbit IgG (1:10,000). A

FIG 1 Schematic diagram of the bacmids *lef3-ac68* 2×KO, *lef3*KO, *ac68*KO1, *ac68*KO2, *ac68*KO3, *ac68*KO4, and *lef3-ac68*Rep. The majority of *lef3* and *ac68* genes were deleted by replacement with a zeocin gene resistance cassette via homologous recombination in *E. coli*, which generated bMON14272^{*lef3-ac68*null}, leaving 357 bp and 222 bp of the C termini of the *lef3* and *ac68* ORFs, respectively. The lower part of the figure shows the genes inserted in the *polyhedrin* (*polh*) locus of bMON14272^{*lef3-ac68*null} by Tn7-mediated transposition for each of the repair viruses generated. The virus *lef3-ac68* 2×KO was repaired with just *polyhedrin* and *gfp*. The remaining viruses were repaired with transfer vectors containing *polyhedrin*, *gfp*, and various *lef3-ac68* ORFs to generate *lef3*KO, *ac68*KO1, *ac68*KO2, *ac68*KO3, *ac68*KO4, and *lef3-ac68*Rep. Both *lef3* and *ac68* were driven by their own promoters, and *ac68* was tagged with sequences encoding the HA epitope tag at the 3' end of the ORF. *gent*, gentamicin cassette; MCS, multiple cloning site.

Perkin Elmer enhanced chemiluminescence (ECL) kit was used to visualize the results of Western blotting.

5' RACE. The detection of AC68 using an anti-HA antibody as a predominant 16-kDa-band product prompted the analysis of the *ac68* 5' transcription start site. Total RNA was isolated from mock-infected Sf9 cells or Sf9 cells infected by AcMNPV E2 at 4 and 24 h postinfection (hpi) using a Qiagen RNeasy kit. 5' RACE was performed as previously described using an Invitrogen GeneRacer Kit (11). Gene-specific primer 1735 combined with GeneRacer 5' primer was used to amplify the 5' end of *ac68*-specific transcript.

Real-time qPCR analysis of BV production and viral DNA replication. To assess the impact of deleting *lef3-ac68*, BV production and viral DNA replication were determined. Briefly, Sf9 cells were transfected with various bacmid constructs, and BV supernatants were collected at different times posttransfection; cell debris was removed by centrifugation at $8,000 \times g$ for 5 min, and samples were stored at 4°C until analysis. BV titers were determined by quantitative PCR (qPCR) as previously described (37). For DNA replication analysis, transfected cells were washed once with PBS and scraped from the plate surfaces. Cells were pelleted by centrifugation at $500 \times g$ for 5 min and stored at -80°C until analysis. DNA was extracted and analyzed by qPCR as previously described (36, 49).

Plaque assays. Sf9 cells (1.5×10^6 per well of a six-well plate) were transfected with 15 ng of bacmid DNA of *lef3-ac68* 2×KO, *lef3*KO, *lef3-ac68*Rep, and bMON14272 repaired with pFAct-GFP (designated the WT AcBac; GFP is green fluorescent protein). Cells were incubated for 4 h with the liposome-DNA mixture, followed by two washes with Grace's medium. SeaPlaque (1.5 ml of 1.5%) low-melting-point agarose medium

preequilibrated to 37°C was layered onto transfected cells. TC100 medium (0.3 ml) was added on top of the agarose medium at 24 h posttransfection (hpt). The flasks were kept in a sealed moisturized container, and plaque formation as indicated by GFP expression was observed at 72 hpt and 120 hpt using a fluorescence inverted microscope.

Cytoplasmic and nuclear fractionation of Sf9 cells. Infected or transfected Sf9 cells were resuspended in 1 ml of NP-40 lysis buffer (10 mM Tris, pH 7.9, 10 mM NaCl, 5 mM MgCl₂, 1 mM dithiothreitol, 0.5% NP-40 [vol/vol]) at a density of 1×10^7 cells per ml and incubated on ice for 5 min. Separation of cytoplasmic and nuclear fractions was confirmed by microscopy. Cell lysates were spun at $1,000 \times g$ for 3 min. The cytoplasm fraction, the supernatant after centrifugation, was transferred to new micro-test tubes, and the nuclear fraction (pellet) was resuspended in an equal volume of NP-40 lysis buffer.

Immunofluorescence confocal microscopy. Cellular localization of AC68 was determined by confocal microscopy of cells infected at a multiplicity of infection (MOI) of 5 by *lef3-ac68*Rep, fixed at 24, 48, and 72 hpi using 4% paraformaldehyde. For the analysis of P143 cellular distribution, Sf9 cells at a density of 1×10^6 cells per well in a six-well plate were transfected with 1 μ g of bacmid DNA of *lef3-ac68* 2×KO and *lef3*KO. Cells were fixed at 24 and 48 hpt for immunofluorescence. Fixed cells were washed three times with PBS for 10 min after paraformaldehyde was removed, followed by permeabilization with 0.15% Triton X-100 in PBS for 20 min. Nonspecific binding was blocked by incubation with 2% bovine serum albumin (BSA) in PBS for 1 h. The cells were then subjected to 1 h of incubation with anti-HA (1:100 for AC68 analysis) or anti-P143 (1:200 for P143 distribution). Alexa Fluor 635-conjugated goat anti-mouse IgG

(1:500) and Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:500) were used.

Purification of BV and ODV. Sf9 cells were infected with *lef3-ac68Rep* at an MOI of 0.1 in suspension culture using spinner flasks. At 6 days pi, 100 ml of BV supernatants and infected cells was harvested. The purification of BV and ODV and the fractionation of envelope and nucleocapsids of BV and ODV were performed as previously described (9).

In vivo infectivity assays. The *in vivo* infectivity of BV was examined by injecting 10, 25, or 38.5 50% tissue culture infective doses (TCID₅₀) of BV of *ac68KO*, *lef3-ac68Rep*, or the WT AcBac into the hemocoel of fourth instar *Trichoplusia ni* larvae. Grace's medium was used as a negative control. For the oral infectivity assay, OBs of *ac68KO*, *lef3-ac68Rep*, or WT AcBac were purified from Sf9 cells and diseased larvae as described previously (8, 27). Oral infection assays were conducted with fourth-instar *T. ni* larvae as previously described (10).

TEM. Sf9 cells (1.5×10^6 cells/60-mm-diameter plate) were transfected with 1.0 μ g of *lef3-ac68 2 $\times$ KO*, *lef3KO*, *ac68KO*, *lef3-ac68Rep*, or WT AcBac. At 48 and 96 hpt, the supernatant was removed, and cells were washed once with PBS and then fixed in 2.5% glutaraldehyde in PBS for 2 h. Samples were processed for transmission electron microscopy (TEM) analysis as previously described (9). Images were obtained using a Hitachi transmission electron microscope.

RESULTS

Construction of *lef3-ac68 2 $\times$ KO* and repair viruses. Due to the intimate association of the *lef3* ORF and promoter with *ac68*, it is not possible to generate a KO of the *ac68* gene without potentially impacting *lef3* expression. Therefore, in this study we generated a double KO of both *ac68* and *lef3* which permitted the repair of the KO virus with either just *ac68* (a *lef3KO* virus) or both genes but with point mutations in the *lef3* promoter region which would be predicted to have minimal or no impact on *lef3* expression but eliminate *ac68* expression. The majority of the sequence of both *ac68* and *lef3* was replaced by a zeocin cassette, generating the bacmid bMON14272^{*lef3-ac68null*} (Fig. 1). The deletion region was designed to conserve the putative late promoter ATAAG of *ac69* and the polyadenylation signal of *ac66* so as not to impact their expression. Two pairs of primers, 1273/1014 and 1239/1565 (Table 1), were used to confirm the insertion of zeocin cassette into the *lef3-ac68* locus as expected by amplifying the recombination junctions of upstream and downstream flanking regions generating 436-bp and 376-bp fragments.

The bacmid bMON14272^{*lef3-ac68null*} was repaired with a series of constructs using the transposition transfer vector pFAct-GFP (6) which inserts *polyhedrin*, the marker gene *gfp*, and any repair genes (Fig. 1). Repair of bMON14272^{*lef3-ac68null*} with pFAct-GFP generated the double-KO virus *lef3-ac68 2 $\times$ KO*. The double-repair virus *lef3-ac68Rep* was generated by repairing bMON14272^{*lef3-ac68null*} with *lef3* and *ac68HA* driven by their native promoters. The *lef3KO* virus was generated by repairing bMON14272^{*lef3-ac68null*} with only *ac68HA*. To inactivate *ac68*, point mutations were introduced to generate *ac68* mutants to minimize any impact on *lef3* expression. From the published AC-MNPV genomic sequence, there are four ATG methionine codons (Fig. 1, M1 to M4) in the predicted *ac68* ORF (1). A series of four mutants was generated to disrupt translation of *ac68* initiating from each of the methionines. The virus *ac68KO1* inserts a frameshift mutation between M1 and M2, which are upstream of *lef3* and therefore do not disrupt the *lef3* ORF. The virus *ac68KO2* mutates M2 to two stop codons. *ac68KO3* mutates M3 and M4 to stop codons, and *ac68KO4* mutates M2, M3, and M4 to stop codons (Fig. 1). WT AcBac is the bacmid bMON14272 repaired

FIG 2 Analysis of AC68 and LEF3 expression and BV titers in *lef3* and *ac68* knockout and repair viruses. (A) Western blot analysis to detect AC68 (top panel) or LEF3 (bottom panel) from each of the *lef3-ac68* constructs. AC68 and LEF3 were detected with anti-HA mouse monoclonal antibody and anti-LEF3 rabbit polyclonal antibody, respectively. Sizes of the various forms of AC68 detected are shown on the right of the top panel. (B) Titration of BV production at 72 hpt from Sf9 cells transfected with the indicated bacmids. Titers were determined by endpoint dilution assays. Error bars indicate standard error.

with pFAct-GFP and serves as the WT control as previously described (6).

To compare the AC68 translation products from different *lef3-ac68* constructs, each virus was transfected into Sf9 cells, and the expressed gene products and BV titers were examined at 72 hpi. The predicted size of AC68 would be 23.4 kDa if translation initiates at M1, but Western blot analysis showed that for the double-repair virus *ac68* is translated as a 16.8-kDa protein (Fig. 2A), which is the predicted size if translation initiates from M2. Disrupting translation from M1 (*ac68KO1*) by insertion of a frameshift mutation results in no change in the expression of the 16.8-kDa AC68. This shows that M1 is not the translation start site of *ac68*. Mutation of M2 results in two smaller proteins of 11.4 and 8.3 kDa, which would agree with predicted proteins with translation initiating from M3 and M4, respectively (Fig. 2A, *ac68KO2*). Mutation of M3 and M4 and of M2, M3, and M4 (*ac68KO3* and *ac68KO4*, respectively) results in no detectable AC68 translation. These results therefore indicate that the initiation codon of *ac68* is M2, and to ensure that no translation of AC68 occurs, M2, M3, and M4 must be mutated. Therefore, for further investigation of the impacts of *ac68* disruption on the virus life cycle, *ac68KO4* was used in all additional analyses. Western blot analysis with LEF3 antibody confirmed the expression of LEF3 from *lef3-ac68Rep* and *ac68KO1*, -2, -3, and -4 viruses, but not from *lef3-ac68 2 $\times$ KO* or *lef3KO*, as expected (Fig. 2A). The BV production at 72 hpt from different *lef3-ac68* constructs was analyzed by TCID₅₀s (Fig. 2B). All *ac68* mutant viruses, *ac68KO1*, -2, -3, and -4, produced similar levels of BV as *lef3-ac68Rep* and the WT AcBac. This suggests that *ac68* is not required for the normal production of BV which has been previously reported (25, 52). BV titers were, however, significantly reduced, as expected for both *lef3-ac68 2 $\times$ KO*

FIG 3 Temporal expression and subcellular localization of *ac68*. (A) The transcriptional start site of *ac68* was determined by 5' RACE analysis of RNA isolated from WT-infected Sf9 cells at 4 and 24 hpi. The late promoter (ATAAG) and the translation start codon (ATG) are shown in bold. The arrowhead shows the location of the transcription initiation site of *ac68*. Also shown in bold are the locations of the predicted start codons M1 and M2 (Fig. 1) and the location of the theoretical early site identified by Li et al. (24). (B) Time course analysis of AC68. Sf9 cells were infected with *lef3-ac68Rep* at an MOI of 5 and harvested at the various times p.i., and total protein was analyzed by SDS-PAGE and Western blotting with anti-HA antibody. (C) Cellular localization of AC68. Sf9 cells were infected with *lef3-ac68Rep* at an MOI of 5. Cells were harvested and separated into the nuclear (N) and cytoplasmic (C) fractions. Fractions were analyzed by SDS-PAGE and Western blotting with anti-HA, anti-IE1, and anti-GP64 monoclonal antibodies to detect HA-tagged AC68, IE0 and IE1, and GP64, respectively. GP64 and IE0/1 are shown as fractionation controls. GP64 is found only in the cytoplasm up to 24 hpi, and IE0/1 is solely found in the nucleus. (D) Confocal analysis of AC68 in infected Sf9 cells at 72 hpi. The left panel shows a light micrograph of infected cells, and the right panel shows a merged confocal fluorescence image of the same cells. Location of AC68 is shown in green, and nuclei were identified by staining with 4',6'-diamidino-2-phenylindole. (E) Purified BV and ODV were analyzed by SDS-PAGE and Western blotting, and AC68 was detected both in BV and ODV (arrow). AC68 was detected with anti-HA antibody.

and *lef3KO*, showing that LEF3 is critical for virus replication. Unexpectedly, however, extremely low levels of BV, approximately 1×10^3 TCID₅₀s, were produced by the *lef3 KO* viruses, *lef3-ac68 2×KO* and *lef3KO*. This result suggested that *lef3*, though critical, may not be absolutely essential for virus replication. The production of BV by the *lef3KO* and *lef3-ac68 2×KO* viruses is a different result than previously reported for *lef3 KO* viruses (53). We therefore further analyzed the *lef3 KO* viruses as described below along with the *ac68 KO* viruses.

Expression and subcellular localization of AC68 during infection. Previously *ac68*, using cDNA sequence data, was reported to be an early gene transcribed from a CATT motif upstream of the M1 start codon of *ac68*. However, in contrast, Western blotting did not detect AC68 until late times, i.e., 36 hpi (25). The mutational analysis above indicated that M2 and not M1 was the *ac68* start codon. Therefore, to further characterize *ac68*, the transcription start site was mapped using 5' RACE. No RACE product was amplified at early times postinfection, but a transcriptional start site was mapped at 24 hpi (Fig. 3A). The result showed that in AcMNPV-infected cells, *ac68* transcription initiates from the canonical baculovirus late motif ATAAG, 65 nucleotides upstream from the M2 start codon of *ac68* and downstream from the ATG codon M1 of *ac68*. No early or late transcription was observed to initiate from the previously mapped CATT site.

To establish the temporal expression and cellular localization of AC68, Sf9 cells were infected with *lef3-ac68Rep* at an MOI of 10. Infected cells were collected at various times postinfection and analyzed by Western blotting. AC68 was detected from 24 to 96

hpi and peaked at 72 hpi (Fig. 3B). Cellular localization was analyzed by biochemical cytoplasmic and nuclear fractionation of infected cells (Fig. 3C). From 24 to 72 hpi, AC68 localized to a greater extent in the cytoplasm but was also detected in the nuclear fraction at 48 and 72 hpi. The subcellular localization of AC68 was also analyzed by immunofluorescence confocal microscopy at 48 and 72 hpi; AC68 fluorescence signal was detected at the periphery of the virogenic stroma in the nucleus and also in cytoplasm (Fig. 3D).

Previous proteomic studies of ODV did not identify AC68, but the *Culex nigripalpus* nucleopolyhedrovirus (CuniNPV) homolog, orf58, was reported to be present in ODV (2, 43). To investigate whether AC68 is also a virion structural protein, BV and ODV were purified and analyzed by Western blotting for the presence of AC68. The results showed that AC68 could be detected easily in BV and weakly in ODV (Fig. 3E, lanes BV and ODV).

Analysis of *lef3KO* and *ac68KO* viruses. To quantitatively analyze the impact of deleting *lef3* or *ac68* on viral growth kinetics, a temporal analysis of viral DNA replication and BV production was performed using qPCR (Fig. 4A). Sf9 cells were transfected with *lef3-ac68 2×KO*, *lef3KO*, *ac68KO4*, *lef3-ac68Rep*, and WT AcBac, and samples were analyzed at various times posttransfection. The temporal profiles of DNA replication were similar for *ac68KO*, *lef3-ac68Rep*, and WT AcBac. This indicates that loss of AC68 does not have any impact on viral DNA replication. For *lef3KO* and *lef3-ac68 2×KO* viruses, the viral DNA levels were substantially reduced compared to those of WT AcBac, *ac68KO*, and *lef3-ac68Rep* from 48 to 96 hpi. However, a small but consis-

FIG 4 Replication kinetics of *lef3-ac68 2xKO*, *lef3KO*, *ac68KO*, *lef3-ac68Rep*, and WT AcBac in transfected Sf9 cells. (A) Quantitative real-time PCR analysis of viral DNA replication. Sf9 cells were transfected with bacmid DNA of *lef3-ac68 2xKO*, *lef3KO*, *ac68KO*, *lef3-ac68Rep*, and WT AcBac. At various hours posttransfection, total DNA was extracted, and viral DNA levels were analyzed by qPCR. Each time point represents the average of two independent replication assays. (B) BV growth curves determined by quantifying the number of viral genomes by qPCR in the extracellular medium from Sf9 cells transfected with *lef3-ac68 2xKO*, *lef3KO*, *ac68KO*, *lef3-ac68Rep*, and WT AcBac at the designated time points. Error bars indicate standard error. (C) Time course analysis of IE0/1, VP39, and LEF3 expression profiles in Sf9 cells transfected with *lef3-ac68 2xKO*, *lef3KO*, *ac68KO*, *lef3-ac68Rep*, and WT AcBac. Cells were harvested at various times p.i., and total protein was analyzed by SDS-PAGE and Western blotting with anti-IE1 and anti-VP39 monoclonal antibodies and LEF3 rabbit polyclonal antibody.

tent increase was observed for both viruses from 24 to 96 hpt showing that viral replication appears to occur in the absence of *lef3* (Fig. 4A). These results suggest that *lef3* is essential for the efficient production of normal levels of viral DNA but not abso-

lutely essential for viral DNA replication, which is different from what has been previously reported (22, 53).

Virus growth curves were determined by qPCR, which detects copies of viral genomes regardless of their infectivity (Fig. 4B). The

FIG 5 Plaque assays of *lef3-ac68 2×KO*, *lef3KO*, *ac68KO*, *lef3-ac68Rep*, and WT AcBac. Plaque assays were performed on Sf9 cells transfected by *lef3-ac68 2×KO*, *lef3KO*, *ac68KO*, *lef3-ac68Rep*, and WT AcBac. Plaques were analyzed at 5 days p.i. for plaque size; *ac68KO*, *lef3-ac68Rep*, and WT AcBac formed large normal plaques and produced occlusion bodies that appeared normal. Both *lef3-ac68 2×KO* and *lef3KO* produced predominantly single-cell plaques (white arrows), but in addition many small multicell plaques were observed, which indicates limited levels of virus production and cell-to-cell transmission.

kinetics of BV production of *ac68KO4* were the same as those of *lef3-ac68Rep* and WT AcBac viruses, which again supports the conclusion that AC68 is not required for production of BV. In contrast, no exponential increase was observed at 24 hpi for *lef3KO* and *lef3-ac68 2×KO*, indicating limited, if any, production of BV. However, from 48 to 96 hpt a very small but consistent increase in BV was observed. A very low level of production of BV agrees with the results of the experiment shown in Fig. 2B, which detected production of infectious virus at 72 hpt.

To further investigate the progression of virus replication in cells infected with the *lef3* and *ac68* deletion constructs, the expression of the major early protein IE0/IE1 (IE0/1) and late protein VP39 were analyzed by Western blotting. Late gene expression in baculovirus-infected cells is dependent on viral DNA replication (45). Therefore, if LEF3 is required for DNA replication, then expression of the late gene VP39 should not be observed in cells infected with the *lef3KO* and *lef3-ac68 2×KO* viruses, whereas *ac68KO4* should not affect late gene expression. The results showed that Sf9 cells transfected with *ac68KO* and *lef3-ac68Rep* expressed levels of IE0/1 and VP39 similar to those of WT AcBac. Cells transfected with *lef3-ac68 2×KO* and *lef3KO* showed similar temporal expression levels of IE0/1 but with lower levels than WT AcBac from 24 hpt. However, expression of the late protein VP39 was observed at 72 hpt and increased in steady-state levels through to 120 hpt, but the overall levels were significantly reduced relative to those of WT AcBac-transfected cells. The expression of major late protein VP39 from the *lef3-ac68 2×KO* and *lef3KO* viruses again supports our results (Fig. 2 and 3) indicating that *lef3* is not strictly required for viral DNA replication. Western blot analysis confirmed the expression of LEF3 from cells infected with *ac68KO4*, *lef3-ac68Rep*, and WT AcBac and the absence of LEF3 in *lef3-ac68 2×KO*- and *lef3KO*-transfected cells (Fig. 4D).

To further confirm production of BV from the *lef3-ac68* constructs, plaques produced by each of the viruses were compared (Fig. 5). By 5 days posttransfection, cells transfected by *ac68KO4*, *lef3-ac68Rep*, and WT AcBac produced large plaques, as expected. In comparison, cells transfected with *lef3-ac68 2×KO* and *lef3KO* showed mostly single-cell infections, but small plaques consisting of a few adjoining cells were also observed. This result demonstrates that a proportion cells transfected with *lef3-ac68 2×KO* and *lef3KO* produced infectious virus, supporting the conclusion that *lef3* is not essential for virus replication.

Nuclear localization of P143 in the absence of LEF3. In previous studies, LEF3 was shown, using transient assays, to be required for the nuclear transport of viral P143 (5) which is required for viral DNA replication (14, 29). In this study, we deleted *lef3*, yet limited levels of viral DNA replication were observed (Fig. 2 and

4). These results raise the question of whether viral P143 is transported to the nucleus in infected cells in the absence of LEF3. To address this question, we analyzed P143 cellular localization by cell fractionation and Western blot analysis. Sf9 cells were transfected with *lef3-ac68 2×KO*, *lef3KO*, and WT AcBac. Cells were collected at various times postinfection (p.i.) and fractionated into nuclear and cytoplasmic fractions. Western blot analysis showed that P143 was detected in both nuclear and cytoplasmic fractions from 24 to 120 hpt in the WT AcBac-transfected cells (Fig. 6A). Similarly, P143 was shown to be present in both the nuclear and the cytoplasmic fractions from *lef3-ac68 2×KO*- and *lef3KO*-transfected cells. To further confirm the nuclear transport of P143 without LEF3, *lef3KO*-transfected cells were analyzed by confocal microscopy (Fig. 6B). Results showed that P143 was detected mainly in the cytoplasm of transfected Sf9 cells but was also in the nucleus of some cells, showing that LEF3 is required for the efficient nuclear transport of P143 but is not the sole mechanism by which P143 can be transported to the nucleus.

TEM of OBs. It was previously reported that deletion of *ac68* did not impact the production of OBs, whereas deletion of the BmNPV homolog *bm56* resulted in defective OB morphogenesis (25, 52). All of our *ac68* deletion constructs produced OBs with a normal appearance, as determined by light microscopy, similar to previously reported results. In order to determine whether the disruption of *ac68* interferes with the assembly of ODV within the OBs, the OBs of *ac68KO* and *lef3-ac68Rep* were compared by transmission electron microscopy (TEM) (Fig. 7). Many ODVs were embedded in the polyhedral matrix of both *ac68KO* and *lef3-ac68Rep* viruses, and no physical differences in the OBs compared to those of the WT AcBac were observed. This result indicates that *ac68* does not appear to be required for the formation of normal OBs.

Bioassays of *ac68KO*, *lef3-ac68Rep*, and WT AcBac. Previous reports have indicated that deletion of *ac68* (C-terminal deletion) resulted only in extended LT₅₀s (25). Similar results were also reported from the study of the *ac68* homologue BmNPV *bm56* (52). To further analyze the *in vivo* effects of deleting *ac68* BVs and OBs of *ac68KO*, *lef3-ac68Rep* and WT AcBac were assayed in fourth-instar *T. ni* larvae. Intrahemocoelic injection of *ac68KO* BV supernatant into larvae resulted in mortality similar to that of injection with *lef3-ac68Rep* and WT AcBac viruses (Table 2), suggesting that AC68 does not affect BV infectivity. Bioassays of OBs, however, resulted in a significant difference in infectivities. Larvae were fed 5, 50, or 500 OBs per larva, and up to 100% mortality occurred for the WT AcBac and *lef3-ac68Rep* infections. In all assays of *ac68KO* OBs, no mortality was observed. This result in-

FIG 6 Cytoplasmic and nuclear localization of P143 in Sf9 cells transfected with WT AcBac, *lef3-ac68* 2×KO, and *lef3*KO. (A) Sf9 cells were transfected with *lef3-ac68* 2×KO, *lef3*KO, or WT AcBac and collected for nuclear (N) and cytoplasmic (C) fractionation. Fractions were analyzed by SDS-PAGE and Western blotting with anti-P143 polyclonal antibodies. Correct nuclear and cytoplasmic fractionation was confirmed by analysis of GP64 and IE0/1 antibodies (data not shown). (B) Immunofluorescence confocal analysis of P143 in *lef3*KO-transfected Sf9 cells. P143 (green) was observed in both the cytoplasm and the nucleus. Sf9 cells were transfected with *lef3*KO and fixed at 72 hpt for immunofluorescence analysis using polyclonal rabbit anti-P143 primary and anti-rabbit Alexa Fluor 635-conjugated IgG secondary antibody to label P143. Staining with 4',6'-diamidino-2-phenylindole (DAPI) was used for localization of the nucleus.

indicates that AC68 is required for the *per os* infectivity of AcMNPV and should be considered a PIF protein.

DISCUSSION

The AcMNPV gene *ac68* is a baculovirus core gene for which homologs have been identified in all baculovirus genomes completely sequenced to date (17, 46). Nearly all core genes have proved to serve critical functions in the baculovirus life cycle, but previous studies on *ac68*, however, have been contradictory. Analyses of *ac68* (or the BmNPV homolog *bm56*) have described this gene as being an early or a late gene encoding a protein of a size that differs from or is the same as predicted; and if the gene was deleted, it was shown to have variable impacts on ODV production (24, 25, 52). Possible reasons for these discrepancies are that deletions generated in the previous studies also impacted adjacent genes, potentially masking the function of AC68. To avoid these issues, in this study a *lef3-ac68* double gene knockout virus was generated that did not impact the regulatory sequences of the flanking genes *ac66* and *ac69*. This enabled the repair of the double knockout with WT *lef3* and *ac68* that contained a series of point mutations to disrupt AC68 translation without impacting *lef3* expression. In addition, it permitted the HA epitope tagging of AC68.

Transcriptional mapping and Western blot data showed that *ac68*, as reported for *bm56*, is a late gene and that AC68 migrates at the size of the predicted 134-aa, 16.8-kDa protein, not the 22.3-kDa, 192-aa protein as previously reported (24, 25). The smaller size of AC68 reported in this study agrees with all other predicted homologs. The exceptions are *Plutella xylostella* NPV, *Rachoplusia ou* NPV, and *Maruca vitrata* NPV, which are nearly identical to AcMNPV AC68 and have the same in-frame ATG sequence upstream of the conserved late promoter. This suggests that they all utilize an ATG within the ORF, thereby producing a protein smaller than what would be predicted from the ORF size. The deletion of *ac68* had no impact on viral DNA replication, BV production, or tissue culture infectivity, and, unlike deletion of *bm56*, it had no impact on OB morphogenesis. However, *ac68*KO occlusion bodies lost the ability to infect and kill *T. ni* larvae by oral ingestion, demonstrating that *ac68* encodes a previously unknown *per os* infectivity factor, and we have therefore renamed this protein PIF6 in accordance with the other previously identified PIF proteins (10, 12, 13, 21, 38, 44, 48, 51). It has been previously shown that the PIF factors form a complex, which includes at least PIF1, PIF2, and PIF3, that is distributed on the ODV surface (42). In a more recent study PIF6 (AC68) has also been shown by mass spectrometry to coimmunoprecipitate with the PIF complex (K. Peng et al., submitted for publication).

Consistent with all other PIF proteins, PIF6 (AC68) is a core gene with a predicted transmembrane domain and has homologs in distantly related insect and non-insect invertebrate DNA viruses (46). This strongly suggests that PIF6 and the PIF complex exploit an ancient infection pathway that is common to insects and possibly a highly diverse group of invertebrate animals.

PIF6 (AC68) was detected in ODV as well as BV (Fig. 3E), but loss of PIF6 (AC68) did not impact ODV development and occlusion (Fig. 7). It is therefore surprising that deletion of the nearly identical *bm56* resulted in aberrant BmNPV OB production (52). In the previous study the *bm56* mutation was an insertion, and it is possible that the resultant truncated ORF could be translated as a

FIG 7 Cross-section of purified occlusion bodies from *lef3-ac68Rep*- and *ac68KO4*-infected Sf9 cells. TEM thin sections of OBs of cells infected with WT AcBac, *lef3-ac68Rep*, and *ac68KO4* show no apparent difference in the numbers of ODV or overall appearance. Scale bar, 0.5 μ M.

TABLE 2 Infectivity of WT AcBac, *ac68*KO4, and *lef3-ac68*Rep viruses in fourth-instar *T. ni* larvae

Infection type and/or dose and virus	Infection by inoculation		Infection <i>per os</i>			
			Expt 1		Expt 2	
	Total no. of larvae	No. of larvae dead by day 8 p.i.	Total no. of larvae	No. of larvae dead by day 8 p.i.	Total no. of larvae	No. of larvae dead by day 6 p.i.
25 TCID ₅₀						
No virus	24	1				
WT AcBac	24	16				
<i>ac68</i> KO4	24	18				
<i>lef3-ac68</i> Rep	24	17				
OB infection						
Control			23	1	24	0
5 OBs						
WT AcBac			22	8	24	19
<i>ac68</i> KO4			23	0	24	0
<i>lef3-ac68</i> Rep			24	5	24	24
50 OBs						
WT AcBac			22	18	24	24
<i>ac68</i> KO4			23	0	24	0
<i>lef3-ac68</i> Rep			20	18	24	24
500 OBs						
WT AcBac			21	21	24	24
<i>ac68</i> KO4			23	0	24	0
<i>lef3-ac68</i> Rep			24	24	24	24

73-aa N-terminal form of BM56. This potential protein, which would not contain the predicted transmembrane domain, may have the negative impact on the ODV and OB formation that was observed.

Due to the overlapping association with *ac68*, the impact of deleting *lef3* on virus replication was also analyzed in this study. *lef3* was originally identified as one of six genes required for viral DNA replication in transient assays (22). LEF3 has been shown to be an SSB protein and may regulate the viral protein alkaline nuclease (15, 34). Recently, a *lef3* knockout virus was constructed that deleted the *lef3* ORF and the 5' end of *ac68* (53). In bacmid-transfected cells no viral DNA replication, budded virus, or late gene expression was detected up to 96 hpi. This led to the conclusion that LEF3 was essential for viral DNA replication and virus production. In contrast, this study observed DNA replication, late gene expression, and BV and OB production, albeit at extremely low levels compared to levels in WT-infected cells. Similar to the results of Yu and Carstens (53), this study clearly shows that LEF3 is required for normal WT levels of replication but that in the absence of LEF3 the virus is able to replicate and produce infectious virus. The levels of virus produced would not be able to sustain transmission between hosts but potentially could form a persistent infection (3).

Plasmid-based transient transfection assays have also shown that P143-helicase requires LEF3 for transport to the nucleus (5). The LEF3 domain required for helicase transport was amino acids 1 to 125, which is also the domain required for DNA replication (53). However, in this study in *lef3*KO-transfected cells, P143 is readily observed in the nucleus (Fig. 6). These results indicate that alternate mechanisms involving viral factors other than LEF3 must facilitate P143 nuclear transport. Viral DNA replication in the absence of LEF3 suggests that this protein is nonessential or that there is another viral or cellular factor fulfilling the LEF3 role

in the viral replication complex. Efficiency of viral replication is obviously extremely low in *lef3*KO-infected cells compared to WT virus infection, but the ability to replicate in the absence of LEF3 might have evolutionary advantages and may indicate mechanisms for low-level replication. It is interesting that LEF3, unlike P143, is not conserved in all baculovirus genomes sequenced to date and is reported to have been a gene acquisition specific to the alpha- and betabaculoviruses, which infect primarily Lepidoptera (17, 20). The gamma- and deltabaculoviruses, which infect Hymenoptera and Diptera, appear to utilize DNA replication complexes that do not require LEF3, and the results of this study may reflect that evolutionary history.

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