Characterization of the Role of Very Late Expression Factor 1 in Baculovirus Capsid Structure and DNA Processing

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Very late expression factor 1 (VLF-1) of *Autographa californica* multiple nucleopolyhedrovirus is a putative tyrosine recombinase and is required for both very late gene expression and budded virus production. In this report, we show that a *vlf-1* knockout bacmid was able to synthesize viral DNA at levels similar to that detected for a *gp64* knockout bacmid that served as a noninfectious control virus. Additionally, analysis of replicated bacmid DNA by field-inversion gel electrophoresis indicated that VLF-1 is not required for synthesizing high-molecular-weight intermediates that could be resolved into unit-length genomes when cut at a unique restriction site. However, immunoelectron microscopic analysis revealed that in cells transfected with a *vlf-1* knockout bacmid, aberrant tubular structures containing the capsid protein vp39 were observed, suggesting that this virus construct was defective in producing mature capsids. In contrast, rescuing the *vlf-1* knockout bacmid construct with a copy of VLF-1 that carries a mutation of a highly conserved tyrosine (Y355F) was sufficient to restore the production of nucleocapsids with a normal appearance, but not infectious virus production. Furthermore, the results of a DNase I protection assay indicated that the DNA packaging efficiency of the VLF-1(Y355F) virus construct was similar to that of the *gp64* knockout control. Finally, a recombinant virus containing a functional hemagglutinin epitope-tagged version of VLF-1 was constructed to investigate the association of VLF-1 with the nucleocapsid. Analysis by immunoelectron microscopy of Sf-9 cells infected with this virus showed that VLF-1 localized to an end region of the nucleocapsid. Collectively, these results indicate that VLF-1 is required for normal capsid assembly and serves an essential function during the final stages of the DNA packaging process.

The family *Baculoviridae* consists of a diverse group of rod-shaped viruses that contain circular covalently closed (ccc) double-stranded DNA genomes that range in size from 80 to 180 kbp. Baculovirus infections are restricted to invertebrates, with the most well-studied example, *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV), being pathogenic for insects of the order Lepidoptera. Upon entry of AcMNPV into a susceptible host, replication occurs in cell nuclei to generate two virus phenotypes. Budded virions (BV) are produced from nucleocapsids that become enveloped during egress through the plasma membrane, which is modified by the viral fusion protein GP64. In contrast, occlusion-derived virions are produced from nucleocapsids that remain in the nucleus, where they are enveloped prior to becoming occluded within a crystalline matrix comprised of polyhedrin. BV are associated with systemic infection, whereas occlusion-derived virions mediate lateral transmission between insects when released into the environment upon the death of the host.

It has been determined through the use of a transient replication assay that six baculovirus gene products are required for viral DNA replication (12, 28). These include an activator of transcription (*ie-1*), a helicase (*p143*), a DNA polymerase (*dnapol*), and three late expression factors (*lef* genes), i.e., a primase (*lef-1*), a primase accessory factor (*lef-2*), and a single-stranded DNA binding protein (*lef-3*). More recently, the *lef-11* gene product was shown to be required for the replication of

bacmid DNA in tissue culture (18). Although these data provide insight into the putative baculovirus replisome required to synthesize viral DNA, very little is known about the mode by which baculoviruses replicate their DNA or about the gene products involved in processing DNA intermediates to generate infectious genomes. Previous reports have suggested that replication may proceed via a rolling-circle mechanism that could generate large head-to-tail concatemers (14, 24). Additionally, the fact that many baculovirus genomes contain homologous regions and encode enzymes related in both sequence and function to the λ Red homologous recombination system entices one to envisage that baculoviruses employ a recombination-based replication strategy like that described for other large double-stranded DNA viruses that generate highly branched intermediate structures (6, 13, 16, 19, 20). Shared between both models is the requirement of processing DNA intermediates in order to generate monomeric genomes that can be packaged. A viral protein that might be involved in this process is very late expression factor 1 (VLF-1). Found in all baculovirus genomes sequenced to date, VLF-1 was originally identified from a temperature-sensitive mutant defective in occlusion body formation and subsequently shown to serve as a transcriptional activator by stimulating the expression of two very late genes, *p10* and *polh* (21, 31). Sequence analysis indicated that VLF-1 is a member of the tyrosine recombinase family of proteins, represented by the integrase (*int*) of λ phage, that function to catalyze DNA rearrangements through recombination (1, 21). A common feature among members of this family is an absolutely conserved nucleophilic tyrosine responsible for forming a covalent phosphodiester bond with substrate DNA and initiating strand exchange between two

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homologous target sites. Attempts to rescue a virus containing a mutation of this conserved tyrosine in VLF-1 were unsuccessful, suggesting that VLF-1 likely retains the catalytic activity that defines this family of proteins (32). Investigations into this putative activity through *in vitro* studies revealed that VLF-1 could bind to DNA substrates that mimicked recombination junctions in a non-sequence-specific manner, but they failed to exhibit enzymatic activity (22).

Previous characterization of a bacmid lacking VLF-1 indicated that it was not infectious when transfected into Sf-9 cells due to an apparent defect in budded virus production, although DNA synthesis and late gene expression were observed (17, 29). This phenotype suggests that VLF-1 may be involved in DNA transactions during the late stages of the replication cycle, namely, processing of DNA intermediates, or may facilitate packaging into viral capsids. Therefore, to assist in elucidating the essential function of VLF-1 and to advance our understanding of the mechanisms involved in baculovirus replication, we continued our investigation of VLF-1 using the AcMNPV bacmid system. The results described herein demonstrate that VLF-1 is not required for normal levels of DNA synthesis or for the production of replication intermediates that display a similar pattern to that of a wild-type (wt) control virus when analyzed by field-inversion gel electrophoresis (FIGE). However, VLF-1 is required for the production of normal capsid particles that contain nuclease-resistant viral DNA, and this requirement appears to be exclusive of its activity as a putative recombinase. In addition, our results indicate that the association of VLF-1 with the nucleocapsid is localized to an end region.

MATERIALS AND METHODS

Cells and antibiotics. *Spodoptera frugiperda* (Sf-9) cells were cultured in Sf-900 II serum-free medium (Invitrogen) with added penicillin G (50 units/ml), streptomycin (50 units/ml; Whittaker Bioproducts), and amphotericin B (Fungizone [375 ng/ml]; Invitrogen) as previously described (10).

Bacmid construction, purification, and transfection. A *vlf-1* knockout bacmid was generated using the λ Red homologous recombination system in *Escherichia coli* and has been described previously (29). For this study, the same methods were used to construct a *gp64* knockout bacmid. Briefly, primers *gp64catF* (5'-TACAATTTTATTATTACATTAATAATGATACAACATATGAATATCCTCCTAG-3') and *gp64catR* (5'-TACTAGTAAATCAGTCACACCAAGGCTTCAATAAGGTGTAGGCTGGAGCTGCTTC-3') were used to generate a linear chloramphenicol acetyltransferase (CAT) marker cassette from plasmid pKD3 (7) and to include 50 nucleotides of sequence homology to the 5' and 3' regions of the *gp64* open reading frame (ORF). This fragment was subsequently electroporated into *E. coli* DH10B cells containing bacmid bMON14272 (Invitrogen) and plasmid pKD46 encoding the λ Red recombination genes *gam*, *beta*, and *exo* (7). Potential bacmid knockout clones were selected on LB plates containing chloramphenicol, and isolated colonies were screened by PCR with primers Ac107700 (5'-GCCTATTCTATGGCCATA-3') and Ac110240 (5'-TGAGCGCCATAAGCAAGTC-3').

Several transfer plasmids were constructed for transposing gene sequences into the polyhedrin (*polh*) locus of bacmid constructs following the Bac-to-Bac protocol (Invitrogen), and these are outlined in Fig. 1A. The transfer plasmid pfbie-GFP was used to transpose a green fluorescent protein (GFP) marker gene into *vlf-1* and *gp64* knockout bacmids to generate constructs *vlf-1*-KO and *gp64*-KO, respectively. This entailed generating a 1.3-kb fragment from *pegfp* (25) with primers *ieGFPF* (5'-GGTACCGATGTCTTTGTGATGCGCG-3') and *ieGFPR* (5'-GGTACCTTACTTGTACAGCTCGTCC-3'). This fragment included a GFP reporter gene derived from plasmid pEGFPN-1 (Clontech) under the control of the AcMNPV immediate early (*ie-1*) promoter (AcMNPV coordinates 126600 to 127197). The resulting fragment was cloned into pCR2.1-TOPO (Invitrogen), excised by KpnI digestion, and ligated into the KpnI site of pFastBac Dual (Invitrogen), which had previously been digested with SmaI and

BamHI to remove the *polh* and *p10* promoters. The transfer plasmid pfbie-GUS(Eco81I) was used to transpose a β -glucuronidase (GUS) reporter gene, in addition to an Eco81I restriction site, into the *vlf-1* and *gp64* knockout bacmids to generate the constructs *vlf-1*-KO(Eco81I) and *gp64*-KO(Eco81I), respectively. The transfer plasmid pfbie-GUS (29) was modified to include an Eco81I restriction site that is not otherwise found in the AcMNPV bacmid. (Previously, AvrII has been used for such analyses [22], but the bacmid construct contains two such sites.) This was accomplished by annealing the oligonucleotide 5'-TCGACCCCTAAGGG-3', followed by ligation into the XhoI restriction site of pfbie-GUS. To generate an epitope-tagged VLF-1 repair construct (VLF-1-HA), a *vlf-1* knockout bacmid was transposed with the transfer plasmid pfbvlf-1 as described previously (22). A second VLF-1 repair construct, VLF-1(Y335F), was made in which tyrosine 355 of VLF-1 was changed to phenylalanine. For this construct, a region of the *vlf-1* ORF was excised from plasmid pXA7(Y335F) (32) by digestion with HpaI and BstEII, ligated into plasmid pfbvlf-1, described above, to generate the transfer plasmid pfbvlf-1(Y335F), and transposed into a *vlf-1* knockout bacmid. To generate the *gp64* repair construct (*gp64*-repair), primers Ac108179 (5'-TTAATATTGTCTATTACGGT-3') and Ac110240 (5'-TGAGCGCCATAAGCAAGTC-3') were used to amplify the *gp64* ORF and native promoter region. This PCR fragment was cloned into plasmid pCR2.1-TOPO (Invitrogen), excised by XbaI and HindIII digestion, inserted into pfbie-GFP as described above to generate the transfer plasmid pfbgp64-repair, and subsequently transposed into a *gp64* knockout bacmid. The table in Fig. 1A summarizes the transfer plasmids used to transpose DNA fragments into parental bacmids to generate the virus constructs used for this study.

Bacmid DNAs were purified from 0.5-liter cultures by using a Large-Construct purification kit (QIAGEN) according to the manufacturer's instructions, and 2 μ g of purified DNA was used for transfecting Sf-9 cells (0.9×10^6 cells/well) seeded in a six-well plate via a cationic liposome method (4). Briefly, bacmid DNA was mixed with 200 μ l of Sf-900 II medium containing 10 μ l of liposomes and incubated at 27°C for 30 to 45 min. After incubation, the DNA solution was increased to 1 ml with Sf-900 II medium, overlaid onto freshly plated Sf-9 cells, transferred to 27°C, and allowed to incubate for 4 h. After 4 h of incubation, the transfection medium was removed, and the cells were replenished with 2 ml of fresh Sf-900 II medium and returned to 27°C.

Q-PCR DNA replication assay. To assess viral DNA replication, a quantitative real-time PCR (Q-PCR) assay was performed as previously described (30). To prepare total DNA for analysis, transfected Sf-9 cells were harvested in 1 ml phosphate-buffered saline (PBS), lysed in 500 μ l cell lysis buffer (10 mM Tris, pH 8.0, 100 mM EDTA, 20 μ g/ml RNase A, 0.5% sodium dodecyl sulfate), and incubated for 30 min at 37°C before the addition of 80 μ g/ml of proteinase K and continued incubation overnight at 65°C. Total DNA was phenol extracted, ethanol precipitated, and suspended in 300 μ l of water. Prior to PCR, 10 μ l of total DNA from each time point was digested with 10 units of DpnI restriction enzyme (Fermentas) for 24 h in a 50- μ l total reaction volume. Quantitative PCR was performed with 1 μ l of digested DNA added to Platinum SYBR green qPCR SuperMix UDg (Invitrogen) according to the manufacturer's instructions and was analyzed on an ABI Prism 7000 sequence detection system under the following conditions: 50°C for 2 min, 95°C for 2 min, and 45 cycles of 95°C for 30 s and 60°C for 30 s, with a 500 nM concentration of each primer.

Field inversion gel electrophoresis. Sf-9 cells were transfected as described above, and at the indicated time points, cells were harvested, washed once with PBS, and mixed with 1% low-melting-point agarose to a final cell density of 3×10^6 cells/200 μ l. The mixture was poured into a plug mold, resulting in a 20- by 9- by 1.5-mm agarose plug that was cut into 8 to 10 pieces. The agarose plugs were treated with 10 mM Tris-HCl (pH 8.0), 100 mM EDTA, 1% N-lauroyl sarcosine, and 200 μ g/ml proteinase K at 50°C overnight. After being washed several times with 10 mM Tris-HCl (pH 8.0), samples were stored at 4°C until further use. Digestion of DNA was performed with 20 U of DpnI and 30 U of Eco81I per sample in a 100- μ l reaction volume and incubated at 37°C overnight. Finally, the plugs were inserted into the loading wells of a 1% pulsed-field-certified agarose gel (Bio-Rad) in 0.5 $\times$ TBE buffer (45 mM Tris-borate, pH 8.0, 1 mM EDTA). DNAs were separated by FIGE using an MJ Research PPI-200 programmable pulse inverter with program 4 (initial reverse time, 0.05 min; reverse increment, 0.01 min; initial forward time, 0.15 min; forward increment, 0.03 min; number of steps, 81; reverse increment, 0.001 min; forward increment, 0.003) run at 8 V/cm for 17 h at 4°C. MidRange PFG marker I (New England Biolabs) was used as a DNA size marker. The DNAs were transferred to a nylon membrane and hybridized with viral genomic DNA as described previously (29).

Immunoelectron microscopy. Sf-9 cells were either transfected as stated above or infected at a multiplicity of infection of ~ 5 , harvested at 72 h posttransfection (hpt) or postinfection (hpi), and prepared for immunoelectron microscopy as described previously (26). A mouse monoclonal antibody to the hemagglutinin

FIG. 1. Organization of transfer plasmids used to generate virus constructs and confirmation of a *gp64* knockout bacmid. (A) Organization of transfer plasmids used for transposing gene sequences into the *polh* locus of bacmid constructs. The diagrams outline the transfer plasmids containing the selected marker genes, the *vlf-1* and *gp64* repair genes, and the Eco81I restriction site; *pie-1* refers to the AcMNPV immediate-early promoter, *ppolh* refers to the AcMNPV polyhedrin promoter, and *pgp64* refers to the native promoter located upstream of the *gp64* ORF. The table summarizes the bacmid constructs used for this report that were derived by transposing parental bacmids with the indicated transfer plasmids. The virus construct wt-

(HA) epitope tag (Babco) was used at a dilution of 1:50, and the monoclonal antibody to AcMNPV, vp39 (a generous gift from Loy Volkman), was used as an undiluted tissue culture supernatant. The goat anti-mouse immunoglobulin G 10-nm-gold-conjugated secondary antibody was used at a dilution of 1:50. Images were obtained with a Phillips EM 300 electron microscope.

DNase I protection assay. Sf-9 cells were transfected in triplicate and harvested in PBS as described above, and each cell pellet was initially suspended in 50 μ l of supplemented RSB buffer (20 mM Tris-HCl [pH 7.4], 10 mM KCl, 1.5 mM MgCl, 1 mM CaCl, 0.5% NP-40, 100 μ g/ml phenylmethylsulfonyl fluoride [PMSF], 20 μ g/ml RNase A), split into two equal 25- μ l portions that were either left untreated (total DNA) or treated with DNase I (MP Biomedicals) at 100 μ g/ml (encapsidated DNA), and incubated at 37°C for 1 h. For BV controls, virions were collected in triplicate from 6 ml of budded virus stock by centrifugation at 12,000 $\times$ g at 4°C and suspended in 300 μ l RSB buffer. Each sample was subsequently split into three 100- μ l aliquots that were either untreated, treated with DNase I (100 μ g/ml), or treated with proteinase K (80 mg/ml) for 20 min followed by treatment with DNase I (100 μ g/ml). (PMSF was included in the RSB buffer, except for the final aliquot, in which PMSF was added after incubation with proteinase K.) For an intracellular capsid control, a 50-ml culture of Sf-9 cells ($\sim 1.5 \times 10^6$ cells/ml) was infected with wt-GUS virus stock at a multiplicity of infection of ~ 2 , harvested at 72 hpi, suspended in 5 ml of supplemented RSB buffer, and disrupted by sonication (three 10-s bursts). The cell lysate was then clarified by centrifugation at 8,000 $\times$ g for 20 min, and the supernatant was removed and loaded onto a 25% sucrose column. The nucleocapsids were collected by ultracentrifugation through the 25% sucrose column at 24,000 rpm in a Beckman SW28 rotor at 4°C, suspended in 350 μ l of supplemented RSB buffer, and divided into six 50- μ l aliquots. Three samples were left untreated, and three were treated with DNase I as described above for transfected cells. For all samples, after the 1-h incubation at 37°C, the suspension volume was increased twofold with RSB buffer and with EDTA to a final concentration of 40 mM. Samples were then treated with an equal volume of 2 $\times$ cell lysis buffer (20 mM Tris-HCl [pH 7.4], 80 mM EDTA, and 1% sodium dodecyl sulfate) plus 80 μ g/ml proteinase K and incubated for 4 h at 65°C. DNAs were extracted with phenol-chloroform, precipitated with an equal volume of 2-propanol, and suspended in 20 μ l water. Viral DNAs were quantified by using the conditions described for the Q-PCR DNA replication assay, with 1 μ l of the DNA sample added to the reaction mixture.

RESULTS

Construction and characterization of *gp64* knockout bacmid. To construct a *gp64* knockout bacmid, a chloramphenicol resistance marker gene was designed to recombine with the *gp64* ORF via the λ Red recombination system in *E. coli*. The target site corresponded to AcMNPV coordinates 108179 to 109729, with the intention of deleting the entire *gp64* coding sequence. Deletion of the *gp64* ORF from the bacmid was confirmed by PCR analysis with primers designed to amplify genomic DNA spanning the recombined locus outlined in Fig. 1B. The results of this analysis indicated that when PCR was performed with a *gp64* knockout bacmid as the template, a 1.9-kb PCR product was generated, whereas a 2.5-kb product

GUS has been described previously (29). (B) Confirmation of *gp64* gene deletion by recombination. The top diagram outlines the *gp64* locus in the unmodified bacmid (diagram 1), and the bottom diagram outlines the identical location in the *gp64* knockout bacmid (diagram 2) after homologous recombination with the chloramphenicol resistance marker gene (CAT). The arrows indicate the relative annealing positions for primers Ac107700 and Ac110240, used to amplify this region, and the dashed lines indicate the sizes of the expected PCR products from each template. The panel shows an ethidium bromide-stained agarose gel of the PCR products generated using the aforementioned primer set to confirm the deletion of the *gp64* ORF. Lane 1 is the PCR product generated from the unmodified bacmid, and lane 2 is the PCR product generated from the *gp64* knockout bacmid. M indicates a 1-kb DNA size marker (Invitrogen).

FIG. 2. Real-time PCR analysis of viral DNA replication in transfected Sf-9 cells. At the designated times, total DNAs were isolated from Sf-9 cells transfected with the indicated bacmid constructs, digested with the restriction enzyme DpnI to eliminate input bacmid DNA, and analyzed by real-time PCR using SYBR green I. Values are displayed as averages for transfections performed in triplicate, with error bars indicating standard deviations.

was produced when an unmodified (wt) bacmid was used as the template, confirming that the *gp64* ORF was replaced with the CAT marker gene (Fig. 1B).

To confirm that deletion of the *gp64* ORF eliminated the ability of the bacmid to propagate in cell culture, the *gp64*-KO bacmid containing the GFP marker gene was transfected into Sf-9 cells and monitored for GFP expression. The expression of GFP could only be observed in a small fraction of isolated cells within the monolayer by 96 hpt, and no GFP expression was observed in cells incubated with the transfection supernatant by 72 hpi (data not shown). To ensure that the inability of this construct to initiate cell-to-cell infection was not due to unintentional mutations acquired during the cloning procedures or to a disruption of regulatory elements, Sf-9 cells were transfected with a *gp64* repair bacmid and monitored for GFP expression as described above. Cells transfected with the *gp64* repair bacmid showed widespread fluorescence in the initial transfection at 96 hpt and in cells incubated with the transfection supernatant at 72 hpi. Additionally, a growth curve analysis showed that BV production of the *gp64* repair bacmid was similar to that of a wt control virus (data not shown). Therefore, these results confirmed that deletion of the *gp64* ORF in the context of a bacmid abolished the ability of the virus to propagate in cell culture.

Analysis of viral DNA replication. To determine if VLF-1 is required for generating normal levels of nascent viral DNA within transfected cells, a DNA replication assay was performed, with the *gp64* knockout bacmid described above serving as the control virus. Because the viral fusion protein GP64 is required for nucleocapsids to egress from infected cells (23), this mutant construct is similar to a *vlf-1* knockout bacmid in that it lacks the ability to initiate cell-to-cell infection, but all other replication processes should be unaffected. DNA replication was assessed using a highly sensitive and quantitative assay involving real-time PCR and DpnI digestion to discriminate between input and replicated DNAs. The results of this analysis are shown in Fig. 2 and indicate that although some variability at early times was observed, the bacmid lacking *vlf-1* was able to synthesize similar amounts of nascent DNA as the

bacmid lacking *gp64* by 72 and 96 hpt. These data confirm that the phenotype of the *vlf-1* knockout does not result from a defect in the level of DNA synthesis.

Analysis of replicated viral DNA by FIGE. Since the data described above indicated that a *vlf-1* knockout could synthesize normal levels of viral DNA, it was then of interest to characterize the replicated DNA relative to the *gp64* knockout control. For these experiments, FIGE was performed, which is suitable for separating DNA molecules with high molecular weights. Initial experiments were intended to characterize the replicated bacmid DNA in its native form without restriction digestion. An analysis of in situ-processed Sf-9 cells transfected with the *gp64*-KO^(Eco81) control bacmid from 0 to 72 hpt under these conditions indicated that as nascent viral DNA accumulated, the majority of this DNA remained in the wells of the agarose gel (Fig. 3A). Similarly, this was the case when viral DNA was analyzed from cells transfected with the *vlf-1*-KO^(Eco81) bacmid under the same conditions (Fig. 3A). Although a band was present at 48 and 72 hpt for both the *gp64* and *vlf-1* knockout samples of the >250-kb size marker, this DNA is not predicted to represent linearized monomeric genomes due to its high molecular weight (Fig. 3A). Similarly, most of the DNA from BV was also trapped in the well (Fig. 3A). Additionally, two bands were present that migrated between 100 and 150 kb for the uncut BV control. However, it should be noted that because bacmid DNA and baculovirus genomes are circular DNA molecules, they are likely impaled by agarose fibers during electrophoresis, which prevents them from entering the gel or causes them to migrate aberrantly, as reported for other circular DNA molecules (5, 15). Therefore, it is not possible to draw conclusions regarding the sizes of patterns for uncut DNA. However, our data do demonstrate similar electrophoretic patterns of replicated DNAs from cells transfected with either the *vlf-1* knockout or the control.

The next set of FIGE experiments intended to characterize replicated bacmid DNA after treatment with the single-cutting restriction enzyme Eco81I (see Materials and Methods). An analysis of DNA from cells transfected with a *gp64* knockout bacmid after Eco81I digestion indicated that a large proportion of the viral DNA that was previously trapped in the wells was able to migrate through the gel to produce a distinct band representing unit-length genomic DNA that first appeared at 48 hpt and gradually increased in concentration by 72 hpt (Fig. 3B, *gp64*-KO^(Eco81)). A similar band was observed with DNA purified from budded virus- but not mock-transfected Sf-9 cells under the same conditions, confirming the classification of this band as unit-length genomic DNA (Fig. 3B, BV lane). Similarly, a band representing unit-length genomic DNA was observed when cells transfected with a *vlf-1* knockout bacmid were analyzed, and this band also first appeared by 48 hpt and increased by 72 hpt (Fig. 3B, *vlf-1*-KO^(Eco81)). Two additional bands were observed at 48 and 72 hpt for cells transfected with either the *vlf-1* or *gp64* knockout bacmid. These included DNA that migrated as less than unit length (Fig. 3B, *gp64*-KO^(Eco81) and *vlf-1*-KO^(Eco81)) and a relatively high-molecular-weight species that migrated >250 kb (Fig. 3B, *gp64*-KO^(Eco81) and *vlf-1*-KO^(Eco81)), the latter of which has been confirmed to consist of viral DNA that has been completely digested with Eco81I (unpublished data). Therefore, similar to the FIGE analysis of uncut DNA, these FIGE gels demonstrate that after

FIG. 3. Analysis of viral DNA by FIGE. (A) Southern blot of total DNA from in situ-processed Sf-9 cells transfected with either the *gp64*-KO^(Eco81I) or *vlf-1*-KO^(Eco81I) bacmid. The agarose-embedded plugs were processed and separated in a 1% Tris-borate-EDTA-agarose gel, using a pulsed-field gel electrophoresis apparatus as described in Materials and Methods. For all samples, the DNA was digested with the restriction enzyme DpnI to eliminate input bacmid DNA. Times posttransfection are indicated above the blot (hpt), and the Mid-Range PFG DNA size marker (New England Biolabs) is indicated in kilobases on the left. (B) An identical Southern blot to that shown in panel A, except that cell samples were treated with the restriction enzymes DpnI and Eco81I during processing. BV indicates viral DNA extracted from budded viruses, and Sf-9 indicates mock-transfected cells. The solid arrows indicate unit-length genomic DNAs, the asterisks indicate viral DNAs that migrated as larger than unit length, and the open arrowheads indicate subgenomic-sized DNA fragments generated after Eco81I digestion. The blots were probed with AcMNPV genomic DNA using the alkaline phosphatase direct labeling system (Amersham).

digestion with the single-cutting enzyme Eco81I, the *vlf-1* and *gp64* knockouts produce similar patterns of DNA.

Electron microscopic (EM) analysis of transfected cells. VLF-1 has previously been shown to be associated with nucleocapsids by Western blot analysis, suggesting a possible role in capsid assembly (32). To investigate this possibility, immunoelectron microscopy was performed with thin sections generated from bacmid-transfected cells. Control experiments performed with the *gp64*-KO bacmid revealed cells in which the nucleus became enlarged and reorganized into an electron-dense virogenic stroma typical of baculovirus infection. As expected, these cells also had rod-shaped nucleocapsids that reacted with a monoclonal antibody to the vp39 major capsid protein, confirming that nucleocapsid synthesis was not inhibited by their inability to egress from the cell (Fig. 4A). Nucleocapsids were also observed in bundle formations that are typically seen in baculovirus-infected cells. Observations of cells transfected with the *vlf-1*-KO bacmid also revealed cells with enlarged nuclei and virogenic stroma; however, in contrast to cells transfected with the *gp64*-KO control bacmid, these cells contained clusters of elongated tube-like structures that localized to the inner nuclear membrane and reacted specifically with the vp39 antibody, suggesting that they are related to capsids (Fig. 4B). Additionally, these structures appeared more electron translucent than capsids found in cells transfected with the *gp64* knockout bacmid, although in some cases a few electron-dense structures could be discerned within these clusters (Fig. 4C, arrows). A cross section of these structures indicated that they lack an electron-dense core that would be indicative of nucleoprotein (Fig. 4D, arrowheads).

To determine if the aberrant capsid structures observed in cells transfected with the *vlf-1* knockout bacmid were the result of a structural or enzymatic deficiency associated with VLF-1, a VLF-1(Y355F) construct containing a point mutation of the

highly conserved tyrosine 355 was analyzed by electron microscopy. Although this mutation in VLF-1 has not previously been characterized in the context of a bacmid, our analysis agreed with a previous report that suggested it could prevent infectious virus production (32; data not shown). Electron microscopic analysis of cells transfected with the VLF-1(Y355F) bacmid revealed the presence of electron-dense rod-shaped nucleocapsids that were morphologically indistinguishable from nucleocapsids observed in cells transfected with the *gp64* control bacmid (Fig. 4E). However, the nucleocapsids in these cells appeared to be more heavily concentrated within the virogenic stroma than those in control transfected cells. We also observed that, whereas in *gp64*-KO-transfected cells both single and bundled nucleocapsids could be seen dispersed between the perimeter of the virogenic stroma and the nuclear membrane, no nucleocapsids could be seen in this region in cells transfected with the VLF-1(Y355F) bacmid, suggesting that they remained tethered to the virogenic stroma (compare Fig. 4A and F). Together, these data show that VLF-1 is required for proper capsid assembly and for nucleocapsids to be released from the virogenic stroma.

DNase I protection assay. Because the results described above indicated that the VLF-1(Y355F) bacmid appeared as proficient in nucleocapsid production as a *gp64* knockout bacmid, a DNase I protection assay was performed to assess the ability of this repaired virus to encapsidate viral DNA. This involved treating transfected cells with DNase I and quantifying the encapsidated nuclease-resistant viral DNA by real-time PCR. To confirm that encapsidated baculovirus DNA is protected from nuclease digestion, control experiments were performed with budded virions isolated from infected cell supernatants as well as nucleocapsids purified from infected cell lysates. The results of this assay showed that the level of viral DNA detected from budded virions treated with DNase I was

FIG. 4. Electron microscopic analysis of transfected Sf-9 cells stained with vp39 antibody to characterize VLF-1 mutants. (A) Image showing a portion of a cell transfected with the *gp64*-KO bacmid as a control. The arrows indicate nucleocapsid bundles dispersed along the outer regions of the nucleus. The inset shows nucleocapsid bundles observed in the same cell at a higher magnification. (B) Image of the nucleus of a cell transfected with a *vlf-1*-KO bacmid showing tubular capsid-like structures staining for vp39. (C) Image of the nucleus of a cell transfected with a *vlf-1*-KO bacmid showing electron-translucent tubular structures with a few examples that are electron dense (arrows). (D) Image showing a cross section of a nucleocapsid bundle located in the nucleus of a *vlf-1*-KO-transfected cell. The arrowheads indicate tubular structures that appear to lack an electron-dense core. (E) Image of the nucleus of a cell transfected with the VLF-1(Y355F) bacmid showing abundant electron-dense nucleocapsids associated with the virogenic stroma. (F) Image showing a portion of the nucleus from a cell transfected with the VLF-1(Y355F) virus indicating that, in contrast to the case for panel A, no nucleocapsids were observed outside the virogenic stroma. For all samples, sections were generated from cells harvested at 72 hpt and stained with a primary antibody to AcMNPV p39 as an undiluted tissue culture supernatant. The secondary gold-conjugated antibody was used at a 1:50 dilution. For images B, C, and D, the bar represents 0.25 μ m; for images A and F, the bar represents 0.5 μ m; and for image E, the bar represents 1 μ m. VS, virogenic stroma; nm, nuclear membrane.

FIG. 5. DNase I protection assay to detect encapsidated viral DNA. (A) Control experiments performed with budded virions or nucleocapsids isolated from infected cells. For budded virions, viral DNA was extracted from virions after they were either left untreated as a control (bar 1), treated with DNase I (bar 2), or treated with proteinase K followed by DNase I (bar 3). For purified cellular nucleocapsids, viral DNA was extracted from virions after they were either left untreated (bar 4) or treated with DNase I (bar 5). Viral DNAs were quantified by real-time PCR. The results are displayed as percentages of nuclease-resistant DNA, which were determined by comparing the amounts of DNA from treated samples with the amounts of DNA from the untreated controls, set at 100%. Values represent the averages and standard deviations from triplicate samples. (B) Percentages of nuclease-protected viral DNA from mutant bacmids at 72 hpt. Cells transfected with either the *gp64* knockout control (bar 1), the *vlf-1* knockout (bar 2), or the VLF-1(Y355F) repair bacmid (bar 3) were either left untreated (total DNA) or treated with DNase I (encapsidated DNA). The viral DNAs were subsequently isolated and quantified by real-time PCR. The values are represented as percentages of encapsidated DNA and were obtained by dividing the mean amounts of DNase I-resistant DNA by the mean of total viral DNA from triplicate samples.

essentially identical to the level of viral DNA detected from virions that were left untreated (Fig. 5A, bars 1 and 2). In contrast, when the virions were initially digested with proteinase prior to DNase I digestion, the level of nuclease-resistant viral DNA was reduced to background levels (Fig. 5A, bar 3). Similarly, nucleocapsids that were purified from cell lysates also showed nearly complete resistance to nuclease when analyzed under similar conditions (Fig. 5A, bars 4 and 5). Therefore, these results validate the experimental conditions and confirm that DNAs from both budded virions and intracellular nucleocapsids are resistant to nuclease digestion. This strategy was then extended to bacmid-transfected cells to assess the amount of encapsidated viral DNA relative to the amount of total replicated viral DNA. An analysis of cells transfected with the *gp64* knockout bacmid serving as the control showed that 28% of the total replicated viral DNA was encapsidated (Fig. 5B, bar 1). In contrast, and in agreement with the aberrant electron-translucent capsid structures observed by electron microscopy, the level of nuclease-resistant viral DNA in cells transfected with the *vlf-1* knockout was <1% (Fig. 5B, bar 2). Interestingly, when the ability of the VLF-1(Y355F) bacmid to encapsidate viral DNA was assessed under these conditions, the amount of encapsidated viral DNA was similar to that detected for the *gp64* knockout bacmid, in that ~24% of the total replicated viral DNA was resistant to nuclease digestion (Fig. 5B, bar 3). Therefore, although it is essential for budded virus production, both the EM and DNase I protection experiments clearly indicate that tyrosine 355 of VLF-1 is not required for viral DNA to be packaged into nucleocapsids.

EM analysis of VLF-1 localization on nucleocapsids. Because VLF-1 has previously been shown to be associated with nucleocapsids of both budded viruses and occlusion-derived

viruses, determining where VLF-1 localizes on the viral particle might provide insight into its essential function. Therefore, immunoelectron microscopy was performed with the virus VLF-1-HA, in which the wild-type *vlf-1* open reading frame was replaced with a version of *vlf-1* containing an HA epitope tag. Notably, this virus was able to generate titers similar to those of an unmodified control virus, indicating that the epitope tag does not interfere with the essential functions of VLF-1 (data not shown). Electron microscopic examination of thin sections generated from cells infected with VLF-1-HA at 72 hpi revealed nucleocapsids with gold labeling localized to one end (Fig. 6A), and this staining pattern could be seen among several nucleocapsids within a given field, indicating that gold labeling of the nucleocapsid was not due to random interactions (Fig. 6B). Additionally, a longitudinal section of nucleocapsids arranged in a bundled formation characteristic of baculovirus-infected cells showed gold labeling at only one end, suggesting that VLF-1 may preferentially localize to either the apical or basal end region (Fig. 6C). In contrast, although some gold-stained nucleocapsids were observed in sections from cells infected with the wt-GUS control virus that lacks the epitope-tagged version of VLF-1, this staining was less likely to be end specific and was primarily observed in regions with a high background (Fig. 6D). Therefore, these results suggest that the association of VLF-1 on the nucleocapsid is localized to an end region.

DISCUSSION

Previous data indicated that a bacmid lacking VLF-1 could only replicate DNA to 10% the level in a nonmutant control bacmid when transfected into Sf-9 cells (29). This decrease,

FIG. 6. Immunogold localization of VLF-1 in thin sections of infected Sf-9 cells at 72 hpi. (A to C) Images of cells infected with budded viruses from the recombinant bacmid (VLF-1-HA) containing a *vlf-1* ORF fused to an HA epitope tag. (D) Image of a cell infected with budded viruses from a recombinant bacmid containing the native *vlf-1* ORF without the epitope tag, serving as a control. For all samples, sections were stained with a primary monoclonal antibody to the HA epitope at a 1:50 dilution and a secondary gold-conjugated antibody at a 1:50 dilution. Bar, 0.25 μ m.

however, was at least partly due to the fact that VLF-1 is required for budded virus production and can therefore only replicate in initially transfected cells. To compensate for this limitation and to better understand the effect VLF-1 has on nascent viral DNA synthesis, comparisons in this study were made to a *gp64* knockout bacmid generated to serve as a “wild-type” control virus that should replicate its DNA normally but, similar to a bacmid lacking *vlf-1*, be limited to initially transfected cells. An analysis of DNA replication by means of a quantitative real-time PCR assay determined that by the end of a 96-h time course, a bacmid lacking *vlf-1* could replicate its DNA to similar levels to those detected for a *gp64* knockout control, indicating that VLF-1 is dispensable for normal levels of viral DNA synthesis. However, the fact that VLF-1 was not required to synthesize normal levels of viral DNA did not exclude the possibility that VLF-1 is required to generate higher-order intermediates that can then be processed into unit-length genomes. To investigate this possibility, FIGE was performed to characterize the DNA replicated by a *vlf-1* knockout bacmid. Our initial analysis of intact genomes indicated that DNA isolated from cells transfected with either

the *gp64* control or *vlf-1* knockout bacmid failed to migrate into the gel but instead accumulated in the agarose wells as the time course progressed. A similar observation has been reported for herpes simplex virus type 1 replication intermediates, and it is thought that this results from viral DNA being nonlinear or highly branched (20, 27). Additionally, when FIGE was used to analyze viral DNA from transfected cells after treatment with the single-cutting restriction enzyme Eco81I, similar DNA patterns were observed, further confirming that VLF-1 is not required to produce replication intermediates that resemble those produced by the *gp64* control virus.

To investigate whether the failure of a *vlf-1* knockout bacmid to produce budded viruses was due to a defect in nucleocapsid assembly, electron microscopy was performed with bacmid-transfected cells. The results of these experiments demonstrated that in cells transfected with the *vlf-1* knockout bacmid, elongated rod-like structures could be found grouped along the inner nuclear membrane. Since these structures were shown to react specifically with a monoclonal antibody to the major capsid protein vp39, our assessment is that these structures represent incomplete capsid particles. It was initially

thought that these aberrant capsids represent precursor structures awaiting nucleic acids and that the lack of DNA processing by VLF-1 inhibited their ability to acquire DNA. This scenario would fit well with a previously proposed model (8) which suggests that viral DNA is packaged into a preassembled capsid sheath. However, these aberrant capsid structures appeared to be substantially longer than the average length of individual capsid particles, indicating that further processing may be required to generate legitimate precursors. Additionally, it is thought that DNA replication and packaging occur in the nucleus within the centrally located virogenic stroma, yet these aberrant capsid structures remained apart from the virogenic stroma, localizing instead to the inner nuclear membrane. Therefore, a likely scenario is that these aberrant capsid structures remain incomplete due to the lack of VLF-1, suggesting that VLF-1 is an essential capsid component.

Electron microscopy was then performed with cells transfected with the recombinant virus VLF-1(Y355F). This mutant, which would be predicted to be inactive in its putative recombinase function, showed abundant nucleocapsid particles within the virogenic stroma, with no discernible difference in phenotype from nucleocapsids observed in cells transfected with the *gp64* knockout control. The VLF-1(Y355F) virus also possessed the ability to package viral DNA with an efficiency similar to that of the *gp64* knockout control, as indicated by a DNase I protection assay. These results are interesting, not only for the fact that they confirm that VLF-1 is required for capsid production, but also because they indicate that the putative enzymatic activity of VLF-1 is not required to process DNA intermediates as a prerequisite for encapsidation. However, the fact that the DNA-containing capsids observed in cells transfected with the VLF-1(Y355F) repair bacmid failed to initiate subsequent infection does confirm that the active-site tyrosine is essential for generating infectious budded virus. Possible explanations for why the repair virus was unable to generate infectious particles may be that the capsids are filled with defective genomes incapable of replicating in new cells or that the capsid-associated DNA requires additional processing by VLF-1 to complete the replication cycle. Because we were unable to detect budded virus particles in the supernatant from cells transfected with the VLF-1(Y355F) virus, the data suggest that VLF-1 is involved in a very-late-stage processing mechanism required for nucleocapsid egress. Although the type of processing mechanism this may entail remains unclear, the association of VLF-1 with the end region of the nucleocapsid suggests that VLF-1 may act in conjunction with the packaging process. It has been proposed that VLF-1 may serve as a site-specific endonuclease that cleaves concatameric DNA to generate unit-length DNA molecules for packaging (22). This would be analogous to the terminase enzyme of herpesviruses and bacteriophages (2, 3, 11). However, the observation that viral DNA could be encapsidated by the VLF-1(Y355F) bacmid indicates that genomic DNA can be packaged without such activity being performed by VLF-1. Therefore, it is likely that VLF-1 is involved in a very-late-stage processing mechanism such as terminating the packaging process. A failure to terminate this process might block nucleocapsid egress and may explain why the nucleocapsids synthesized by the VLF-1(Y355F) bacmid appeared to be heavily concentrated or trapped within the virogenic stroma, as opposed to the more

dispersed pattern observed with the control virus. Similarly, VLF-1 could function as a DNA resolvase on branched DNA structures generated during the replication process that would require processing during packaging in order to incorporate complete genomes, and the failure to resolve such structures would also likely lead to an arrest in packaging. Interestingly, a similar mechanism has been described for endonuclease VII of T4 bacteriophage and has also been shown to interact with a component of the packaging machinery (9).

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