JOURNAL OF VIROLOGY, Aug. 2008, p. 7897–7904 Vol. 82, No. 16
0022-538X/08/$08.00+0 doi:10.1128/JVI.00099-08

Sf29 Gene of *Spodoptera frugiperda* Multiple Nucleopolyhedrovirus Is a Viral Factor That Determines the Number of Virions in Occlusion Bodies[▽]

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Received 15 January 2008/Accepted 28 May 2008

The genome of *Spodoptera frugiperda* multiple nucleopolyhedrovirus (NPV) was inserted into a bacmid (Sfbac) and used to produce a mutant lacking open reading frame 29 (Sf29null). Sf29null bacmid DNA was able to generate an infection in *S. frugiperda*. Approximately six times less DNA was present in occlusion bodies (OBs) produced by the Sf29null bacmid in comparison to viruses containing this gene. This reduction in DNA content was consistent with fewer virus particles being packaged within Sf29null bacmid OBs, as determined by fractionation of dissolved polyhedra and comparison of occlusion-derived virus (ODV) infectivity in cell culture. DNA from Sfbac, Sf29null, or Sf29null-repair, in which the gene deletion had been repaired, were equally infectious when used to transfect *S. frugiperda*. All three viruses produced similar numbers of OBs, although those from Sf29null were 10-fold less infectious than viruses with the gene. Insects infected with Sf29null bacmid died ~24 h later than positive controls, consistent with the reduced virus particle content of Sf29null OBs. Transcripts from *Sf29* were detected in infected insects 12 h prior to those from the polyhedrin gene. Homologs to *Sf29* were present in other group II NPVs, and similar sequences were present in entomopoxviruses. Analysis of the *Sf29* predicted protein sequence revealed signal peptide and transmembrane domains, but the presence of 12 potential N-glycosylation sites suggest that it is not an ODV envelope protein. Other motifs, including zinc-binding and threonine-rich regions, suggest degradation and adhesion functions. We conclude that *Sf29* is a viral factor that determines the number of ODVs occluded in each OB.

Nucleopolyhedroviruses (NPVs) (*Baculoviridae*) cause fatal disease in arthropods. Several have been commercialized as biopesticides to control insect pests, particularly Lepidoptera (27, 40). During virus infection, NPVs produce two virion phenotypes, occluded and nonoccluded forms, which have different roles. When an insect consumes foliage contaminated with occlusion bodies (OBs), occlusion-derived virions (ODVs) are released by alkali disruption of the OB protein matrix in the insect midgut (13). The ODVs infect midgut cells by interaction with the cell surface proteins (24), virus envelope fusion with plasma membranes, and entry of nucleocapsids. Some of the nucleocapsids that have entered cells traverse the newly infected cell and are exported directly to the hemolymph via budding. Nonoccluded virus, produced subsequently, also buds out of the infected cell to allow cell-to-cell transmission of virus throughout the host (14). This virus phenotype is known as budded virus. Later, virions containing one or more nucleocapsids are occluded in protein matrices to form the OBs, which remain in the nucleus until death of the infected insect. These are then dispersed in the environment to achieve insect-to-insect transmission (14).

The isolation of individual virus genotypes by plaque purification in cell culture (31, 47) and in larvae by low-dose in vivo cloning (41, 52) has indicated that wild-type NPV populations are genetically heterogeneous (41, 47). Examination of genotypic variants has facilitated the characterization of wild-type populations, including the evaluation of their respective genetic and biological properties, contributing to our understanding of the diversity and evolution of these viruses (7, 21, 41, 47). The consequences of genetic heterogeneity in wild-type isolates for the phenotypic characteristics of the population have not been examined in great detail. Closely related genotypic variants do not usually show large differences in major phenotypic traits (54), although on occasions, small differences in the genomes of these variants may affect important biological attributes such as pathogenicity, virulence, productivity, or even host range (6, 21, 41, 47).

During plaque assay purification of the Nicaraguan isolate (SfNIC) of *Spodoptera frugiperda* multiple NPV (SfMNPV) wild-type population, nine genotypes could be differentiated by their DNA restriction patterns (47). Three genotypes were not infectious per os, which in two of them was attributed to the absence of the peroral infection factor genes, *pif* and *pif-2*. The remaining genotypic variants showed various levels of per os infectivity (47). Among the pathogenic variants, two viruses with small genome deletions were more infectious than the original SfNIC-B genotype (SfNIC-F and SfNIC-I) (48). SfNIC-F lacked open reading frame 3 (ORF3) to ORF8 of the SfNIC variable genomic region, while SfNIC-I lacked ORF2 to

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▽ Published ahead of print on 11 June 2008.

TABLE 1. Primers used in this study

Primer	Sequence	Amplification purpose
Sf29del.1	5′-TGATTTATTTATATTTTATTTACCCGGGTG GACAGCAAGCGAACCGGAATTGC-3′	*Sf29* deletion from SfMNPV bacmid; forward primer with 25-nt homolog to Tn*5*-neo sequence (underlined) and 25-nt homolog to 3′ untranslated *Sf29* region
Sf29del.2	5′-GTATTCATAACGCACCAACCCGGGTCAGA AGAACTCGTCAAGAAGGCG-3′	*Sf29* deletion from SfMNPV bacmid; reverse primer with 25-nt homolog to Tn*5*-neo sequence (underlined) and 25-nt homolog to 5′ untranslated *Sf29* region
Sf29del.3	5′-AACTTTTAGGAAAATTATACACAATCAA TGATTTATTTATATTTTATTTACCC-3′	*Sf29* deletion from SfMNPV bacmid; forward primer with 25-nt homolog to Sf29del.1 (underlined) and 25-nt homolog to 3′ untranslated *Sf29* region
Sf29del.4	5′-ATAAAATACTGCATCGTCGATGATTTTTAT GTATTCATAACGCACCAACCC-3′	*Sf29* deletion from SfMNPV bacmid; reverse primer with 25-nt homolog to Sf29del.2 (underlined) and 25-nt homolog to 5′ untranslated *Sf29* region
Sf29reg.1	5′-ATCTCAGACCATCTTGCCAA-3′	*Sf29* insertion into Sf29null bacmid; forward primer that amplifies 250 bp upstream of the *Sf29* stop codon
Sf29reg.2	5′-GTATAGCGTCGACGACATCG-3′	*Sf29* insertion into Sf29null bacmid; reverse primer that amplifies 250 bp downstream of the *Sf29* start codon
Sf29.1	5′-CTACTCAACGCGTTCAACGA-3′	*Sf29* transcription analysis (RT-PCR); forward primer that amplifies 250 bp downstream of the *Sf29* stop codon
Sf29.2	5′-TTAGTCAAATAACGGCAATG-3′	*Sf29* transcription analysis (RT-PCR); reverse primer that amplifies in the *Sf29* stop codon
Sfpolh.1	5′-CCCGACACCATGAAGCTGGT-3′	*Sfpolh* transcription analysis (RT-PCR); forward primer that amplifies 500 bp upstream of the *Sfpolh* stop codon
Sfpolh.2	5′-TTAGTACGCGGGTCCGTTGTA-3′	*Sfpolh* transcription analysis (RT-PCR); reverse primer that amplifies in the *Sfpolh* stop codon

-9. Variants with larger deletions, such as SfNIC-A and SfNIC-H, exhibited lower pathogenicity (48). The principal difference between the SfNIC-A and -I variants was that SfNIC-I contained an ORF that was absent in SfNIC-A, although other minor mutations may have gone undetected and contributed to biological differences between the viruses. Recently the SfMNPV genome was sequenced, and this ORF was designated ORF29 (*Sf29*) (17) (accession number NC-009011). The *Sf29* gene is homologous to *Se30* from the closely related *Spodoptera exigua* MNPV. It has an early-promoter motif, suggesting that it is transcribed early in the infection. The SfMNPV genotypes lacking this gene are able to produce a systemic infection but are less pathogenic in terms of lethal concentration and speed of kill than those with *Sf29* in their genome, suggesting that this gene could play an important role in infectivity (48). Other genes have been described as viral infectivity factors, i.e., *Ac23* (35), *pe38* (38), *Ac150* and *Ac145* (29, 57), or the *enhancins* (32, 51). Interestingly, *Ac23* is homologous to the F proteins of group II NPVs but plays a role different from that of F proteins in accelerating mortality in the host insect. *pe38* and its homologs are found primarily in group I NPVs. In contrast, *Sf29* and its homologs are found in group II NPVs, suggesting that in this group, other genes might act as infectivity factors.

In this study, we examined the role of *Sf29* in SfMNPV replication by using PCR and bacmid-based recombination systems to delete the *Sf29* gene from a complete SfMNPV bacmid genome. Different aspects of OB morphology and pathogenesis were studied.

MATERIALS AND METHODS

Insect cells and larvae. *Spodoptera frugiperda* Sf21 cells were maintained in TC100 medium containing 10% fetal calf serum at 28°C (28). *Spodoptera frugiperda* larvae in a laboratory colony were maintained on a wheat germ-based semisynthetic diet (15) at 25°C.

Construction of SfMNPV bacmid. A wild-type isolate of SfMNPV (SfWT) was collected in Nicaragua and characterized by Simon et al. (47). The SfMNPV-B genotype (SfB), with a complete genome, was selected for insertion in pBACe3.6 (GenBank accession no. U80929). Empirical analysis of SfB DNA with restriction enzymes identified a single AscI site. The pBACe3.6 vector was modified by replacing the pUC19 element with pBluescriptKS I containing AscI sites. The SfWT DNA was purified by CsCl gradient (28), digested with AscI, and ligated with the modified pBACe3.6 at 16°C overnight. The ligation reaction mixture was dialyzed for 4 h against Tris-EDTA (TE) buffer and used to transform DH10B GeneHogs electrocompetent cells (Invitrogen), which were then selected on agar containing chloramphenicol. Colonies were amplified and the bacmid DNAs purified by alkaline lysis. DNA was digested with PstI for comparison with the restriction profiles of each of the Nicaraguan SfMNPV genotypes (47). The bacmid with the complete genotype B (Sfbac) was selected for recombination experiments.

***Sf29* gene disruption.** The bacteria containing Sfbac were made electrocompetent and transformed with the Red/ET plasmid pSC101-BAD-gbaA (Gene Bridges GmbH). These cells were also made electrocompetent and induced with arabinose (0.1 to 0.2%, wt/vol) to express the recombination proteins (gbaA). The Tn*5*-neo template was amplified sequentially using two primer sets (Sf29del.1/Sf29del.2 and Sf29del.1/Sf29del.2 [Table 1) to add 50-nucleotide terminal sequences corresponding to either the 3′ or 5′ untranslated region of *Sf29*. The DNA product was used to transform the electrocompetent cells containing Sfbac and pSC101-BAD-gbaA and producing gbaA. Recombinants were selected as resistant colonies on medium containing chloramphenicol and kanamycin. To confirm the deletion of the *Sf29* gene, restriction endonuclease analysis of the bacmid DNA and PCR amplifications were performed.

***Sf29* repair in an Sf29null bacmid.** The *Sf29* coding region was amplified using PCR with primers outside the coding region, Sf29reg.1 and Sf29reg.2 (Table 1), and the SfWT DNA as template. Fourth-instar *S. frugiperda* larvae were injected with 5 μl from a mixture containing 50 μl of Sf29null bacmid DNA (100 ng/μl), 50 μl of the PCR product that covered the *Sf29* region (500 ng/μl), and 50 μl of Lipofectin reagent (Invitrogen). Inoculated larvae were transferred to a 25-compartment petri dish with diet and reared at 25°C. Virus-induced mortality was recorded daily. The OBs were purified from cadavers and virus DNA extracted as described below. A PCR was performed using viral DNA from OBs and primers outside the coding region (Sf29reg.1 and Sf29reg.2) to determine whether or not recombination had replaced *Sf29*. Viral DNAs from the complementation assay were transfected into DH5α electrocompetent cells. Colonies were grown in the selected medium, and bacmid DNAs were purified by alkaline lysis. Restriction endonuclease analysis and a PCR were performed to confirm

the transposition of the *Sf29* gene into the Sf29null bacmid to derive the Sf29null-repair bacmid.

OB purification and DNA extraction. The SfWT OBs were produced by per os infection of *S. frugiperda* fourth-instar larvae with 10^8 OBs/ml. To produce OBs from the Sfbac, Sf29null, and Sf29null-repair bacmids, fourth-instar larvae were injected with a DNA suspension including bacmid DNAs and Lipofectin reagent in a 2:1 proportion. A 200-μl volume of each bacmid DNA containing 100 ng/μl was mixed with 100 μl of Lipofectin. After 10 min, 5 μl of this suspension was injected into each larva (333 ng/larva). Per os and intrahemocelically inoculated larvae were transferred to diet. Larvae were reared at 25°C, and virus-induced mortality was recorded every day. The OBs obtained from inoculated larvae were extracted and filtered through cheesecloth. These were washed twice with 0.1% sodium dodecyl sulfate and once with 0.1 M NaCl and finally resuspended in double-distilled water. The virus suspensions were quantified using a bacterial counting chamber and stored at 4°C. Virions were released and lysed from 5×10^8 OBs of SfWT, Sfbac, Sf29null, and Sf29null-repair bacmids by mixing with 100 μl of 0.5 M Na_2CO_3 50 μl of 10% sodium dodecyl sulfate in a final volume of 500 μl and incubating for 10 min at 60°C. Undissolved OBs and other debris were removed by low-speed centrifugation (3,800 × *g*, 5 min). The supernatant fraction containing the virions was treated with 25 μl of proteinase K (20 mg/ml) for 30 min at 50°C. Viral DNA was extracted twice with TE buffer (pH 8.0)-saturated phenol and once with chloroform. Viral DNA was isolated by alcohol precipitation. The pellet was resuspended in 100 μl of TE buffer for 10 min at 60°C. The DNA concentration was estimated at 260 nm, and 10 μl was used for restriction endonuclease analysis.

Nucleocapsid packaging characteristics. To compare the nucleocapsid packaging characteristics of the SfWT, Sfbac, Sf29null, and Sf29null-repair viruses, ODVs were harvested by adding 5×10^8 OBs of each virus to an equal volume of 0.1 M Na_2CO_3. These suspensions were layered onto continuous 30 to 60% sucrose gradients and centrifuged at 76,800 × *g* for 1 h at 4°C in a Beckman SW28 rotor. The banding patterns of each virus were visually inspected and photographed.

ODV content. The mean virion titer per OB was determined by end point dilution as described previously (36, 37). First, purified OB suspensions were quantified by counting in triplicate using an improved Neubauer hemocytometer, and then 500 μl of a suspension of 10^9 OBs/ml was mixed with an equal volume of 0.1 M Na_2CO_3. A 30-μl volume of 5.4 M HCl was added to decrease the pH. This suspension was filtered through a 0.45-μm filter and diluted in medium (1:5, 1:25, 1:125, and 1:625). Each dilution (10 μl) was used to infect 10^4 Sf21 cells in 96-well plates. Twenty-four independent infections were performed for each dilution in triplicate. The dishes were sealed with masking tape, incubated at 28°C, and examined for virus infection for 1 week. The resulting data were analyzed by the Spearman-Kärber method (37) to provide tissue culture infectious dose estimates for 50% of the wells. These values were subsequently converted to infectious units per 5×10^8 OBs for presentation in the figures.

Infectivity of OBs. The 50% lethal concentration (LC_{50}) and mean time to death (MTD) for the SfWT, Sfbac, Sf29null, and Sf29null-repair viruses were determined by per os bioassay in second-instar *S. frugiperda* following the droplet feeding technique (26). For the SfWT, Sfbac, and Sf29null-repair viruses, fivefold dilutions of between 1.2×10^6 and 1.9×10^3 OBs/ml were used, whereas for the Sf29null virus, similar dilutions of between 6.0×10^6 and 9.6×10^3 OBs/ml were employed. Each dose range was previously determined to kill between 95 and 5% of the insects. Bioassays with 25 larvae per virus concentration and 25 larvae as controls were performed four times. Larvae were reared at 25°C, and virus mortality was recorded every 12 h until the insects had either died or pupated. Virus-induced mortality was subjected to logit analysis using the generalized linear interactive modeling (GLIM) program (8). Time-mortality data were subjected to Weibull analysis in GLIM. The OB concentrations used for the time-mortality analysis were those that resulted in ~65 to 80% larval mortality, namely, 2.4×10^5 OBs/ml for SfWT, 1.2×10^6 OBs/ml for Sfbac, 6.0×10^6 OBs/ml for Sf29null, and 1.2×10^6 OBs/ml for Sf29null-repair. These concentrations resulted in 80%, 74%, 65%, and 68% mortality, respectively.

OB yields. Larvae were injected in the hemocoel with 5 μl SfWT, Sfbac, Sf29null, and Sf29null-repair DNAs (100 ng/μl) and Lipofectin reagent in a 2:1 proportion. Larvae were then incubated at 25°C on a semisynthetic diet until death. Each corpse was homogenized in 100 μl distilled water and OB production per larva estimated by counting in a Neubauer hemocytometer. For each larva the OB count was performed in triplicate. The results were subjected to Kruskal-Wallis and Mann-Whitney nonparametric analyses using the SPSS program (SPSS version 10.0).

Protein analysis. A variety of tools were used to determine the nature of the SF29 protein. Amino acid sequence comparisons were performed using BLAST services from the Expasy (http://www.expasy.org/tools/BLAST) and Sanger protease database MEROPS (http://merops.sanger.ac.uk/) web pages. Protein domain analysis was performed using Pfam (http://www.sanger.ac.uk/software/pfam/), signalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/), and TMHMM server v 2.0 software (http://www.cbs.dtu.dk/services/TMHMM-2.0/) (11, 12). Multiple-sequence alignment of the catalytic conserved regions (Zn-binding motif) of the SfMNPV *Sf29*, *Vibrio cholerae* metalloprotease gene, and *Amsacta moorei* entomopoxvirus (AmEPV) ORF3 was performed using the MultAlin service (http://bioinfo.genopole-toulouse.prd.fr/multalin/multalin.html) (5).

Temporal expression of *Sf29* gene. Total RNA was extracted from SfWT-infected larvae at 0, 2, 4, 6, 8, 10, 12, 24, 48, 72, and 96 h postinfection (hpi) with Trizol reagent (Gibco). The RNA samples were treated with DNase prior to reverse transcription-PCR (RT-PCR) using the Access RT-PCR system kit (Promega). First-strand cDNA synthesis was performed using avian myeloblastosis virus reverse transcriptase and the internal oligonucleotide Sf29.2 (Table 1). The cDNA mixtures were then amplified by PCR using the Sf29.1 and Sf29.2 primers (Table 1), and the products were analyzed in 1% agarose gels. To verify the absence of contaminant DNA in the RNA samples, a PCR was performed after treatment with DNase. For comparison, RT-PCR was also done with two primers specific for the very late *polyhedrin* gene (*Sfpolh*), Sfpolh.1 and Sfpolh.2 (Table 1).

RESULTS

Generation of Sfbac, Sf29null, and Sf29null-repair viruses. To determine the effects of disrupting *Sf29* on virus structure and replication, we deleted it from the SfMNPV genome inserted into a bacmid (Sfbac) to produce Sf29null. A Sf29null-repair bacmid was constructed by coinfection of *S. frugiperda* larvae with Sf29null bacmid DNA and a PCR product that spanned the *Sf29* region. Recombinant virus DNA from insects was then used to transform bacteria to recover genetically homogeneous genomes. Replacement of the *Sf29* gene with the kanamycin cassette in Sf29null and reinsertion of *Sf29* in Sf29null-repair was confirmed by restriction endonuclease analysis and PCR with specific primers for deletion of predicted recombinant junction regions (data not shown).

Sf29null bacmid OBs have a reduced DNA content. An SfWT OB stock was produced by per os infection with OBs, whereas Sfbac, Sf29null, and Sf29null-repair bacmid OBs were produced by intrahemocelic infection with the respective DNAs. The OBs were purified from cadavers. DNA was extracted from 5×10^8 OBs from SfWT, Sfbac, Sf29null, and the Sf29null-repair bacmid. Restriction endonuclease analysis of similar volumes of DNA rather than absolute amounts using PstI confirmed the expected SfWT, Sfbac, Sf29null, and Sf29null-repair bacmid profiles. The restriction profile of the Sf29null bacmid was clearly less intense than the SfWT, Sfbac, and Sf29null-repair profiles (data not shown), suggesting the presence of a reduced quantity of DNA in the OBs. Further DNA extractions were performed from 5×10^8 OBs and the yields subjected to Kruskal-Wallis and Mann-Whitney nonparametric analyses. No significant differences in the mean amounts (± standard deviations) of DNA in OB samples were detected between the SfWT (165 ± 32 ng/μl), Sfbac (160 ± 28 ng/μl), and Sf29null-repair (156 ± 42 ng/μl) bacmids. However, Sf29null OBs yielded significantly less DNA; 27 ± 13 ng/μl was obtained from 5×10^8 OBs (Fig. 1), representing an approximately sixfold reduction in the DNA content of Sf29null bacmid OBs compared with the other viruses studied ($P < 0.05$).

Packaging of nucleocapsids in Sf29null ODVs. To determine whether differences in nucleocapsid packaging among ODV populations could account for the reduced DNA content of Sf29null OBs, virus particles isolated from equal numbers of

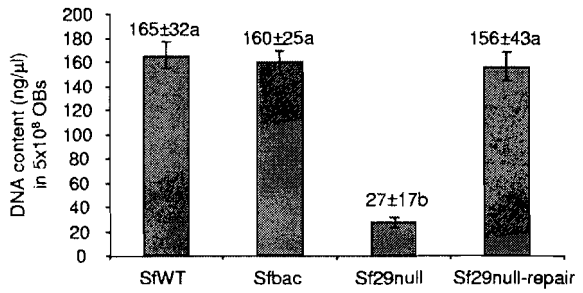

FIG. 1. Mean amounts of DNA extracted from samples of 5×10^8 OBs of SfWT, Sfbac, Sf29null, and Sf29null-repair bacmids. Values above the bars indicate means, and those labeled with different letters are significantly different (Mann-Whitney test, $P \leq 0.05$). Error bars indicate standard deviations.

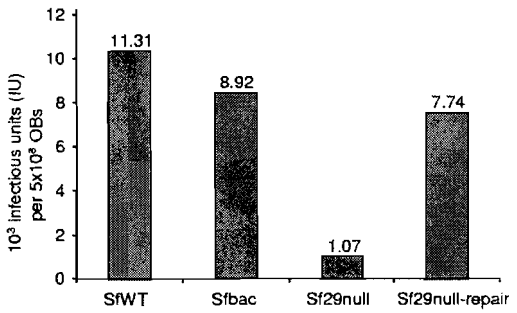

FIG. 3. ODV contents in 5×10^8 OBs of SfWT, Sfbac, Sf29null, and Sf29null-repair bacmid viruses. Sf21 cells were serially infected (1:5, 1:25, 1:125, and 1:625) with ODVs released from OBs. ODV titers (ODV/ml) were calculated by end point dilution.

OBs of SfWT, Sfbac, Sf29null, and Sf29null-repair bacmids were fractionated using continuous sucrose density gradients. The ODV banding pattern of the Sf29null bacmid virus could not be seen with 5×10^8 OBs. A second sucrose gradient was performed with five times more Sf29null bacmid OBs (2.5×10^9 OBs). No apparent differences in banding patterns of virus particles containing different numbers of nucleocapsids were observed between the four viruses (Fig. 2).

In vitro infectivity of ODVs. The reduced intensity of the ODV banding pattern in the Sf29null bacmid-produced OBs suggested differences in ODV content per OB. Therefore, the infectivity of ODVs derived from equal numbers of OBs of SfWT, Sfbac, Sf29null, and Sf29null-repair bacmids was compared by end point dilution (Fig. 3). Samples of 5×10^8 OBs of SfWT, Sfbac, and Sf29null-repair bacmid viruses produced titers of 1.13×10^4, 9.92×10^3, and 7.74×10^3 infectious units/ml, respectively. In contrast, the same number of OBs of the *Sf29* deletion mutant virus had a titer of 1.07×10^3 infectious units/ml, around 8 times fewer ODVs than the SfWT, Sfbac, and Sf29null-repair OBs.

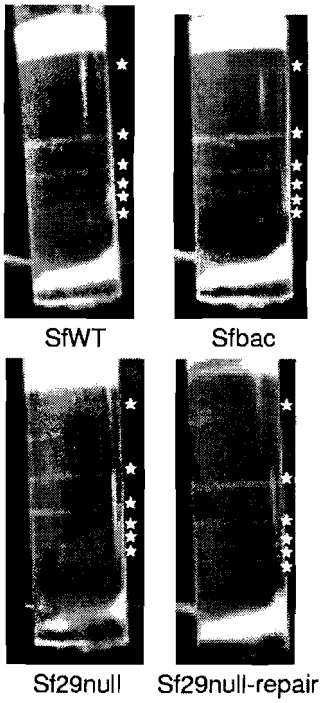

FIG. 2. ODV banding patterns of SfWT, Sfbac, Sf29null, and Sf29null-repair bacmid viruses after sucrose gradient separation of ODVs from similar quantities of OBs (5×10^8 OBs). For visualization, Sf29null ODVs were harvested from a fivefold-greater quantity of OBs (2.5×10^9). Stars indicate the positions of the observed ODV bands.

Infectivity of viruses in vivo. Transfection of insects with Sfbac, Sf29null, and Sf29null-repair DNAs resulted in similar percentages of mortality and MTD for the three viruses. Mortalities of 70%, 75%, and 73% were observed when larvae were injected with 333 ng of Sfbac, Sf29null, and Sf29null repair bacmid DNAs, respectively. All larvae died between days 10 and 13 postinoculation; the MTDs for the viruses were 9, 10, and 9 days, respectively. The LC_{50}s and MTDs for SfWT, Sfbac, Sf29null, and Sf29null-repair bacmid OBs were determined in *S. frugiperda* second-instar larvae. The value for the SfB genotype from a previous study (47) was included for comparison. SfWT was more infectious than all of the other viruses. The 95% confidence limits of the relative potencies, representing the ratio of effective concentrations (44), overlapped broadly in the SfB, Sfbac, and Sf29null repair viruses, suggesting no significant differences in OB infectivity in *S. frugiperda* larvae (Table 2). The LC_{50}s of SfB, Sfbac, and Sf29null-repair OBs in *S. frugiperda* larvae were 9.3×10^4, 1.0×10^5, and 2.1×10^5 OBs/ml, respectively. In contrast, the LC_{50} estimated for Sf29null bacmid OBs was 1.5×10^6 OBs/ml, approximately 10-fold greater than the LC_{50}s of the other OBs (Table 2).

The MTDs for SfWT, SfB, Sfbac, and Sf29null-repair ranged from 110 to 132 h, and overlapping 95% confidence limits indicated no significant differences in the speed of kill among these viruses (Table 2). The MTD for the Sf29null bacmid OBs (157 h) was statistically higher than those for the SfWT, SfB, Sfbac, and Sf29null-repair viruses.

Yields of OBs in virus-infected insects. No significant differences were found in OB production when larvae were infected with the same quantity of DNA from viruses either lacking or containing native *Sf29* ($\chi^2 = 2.45$, df = 3, $P = 0.485$) (Fig. 4). The SfWT, Sfbac, Sf29null, and Sf29null-repair viruses produced 5.61×10^8, 5.60×10^8, 5.10×10^8, and 5.50×10^8 OBs/larva, respectively.

TABLE 2. Logit regression and time-to-death analysis for the SfB, Sfbac, S29null, and Sf29null-repair bacmids compared with the SfWT isolate in second-instar *Spodoptera frugiperda* larvae

Virus	Logit regression[a]					Time to death[b]		
	LC_{50} (OBs/ml)			Relative potency	*P*	Mean (h)	95% Confidence limit	
	Mean	95% Confidence limit						
		Low	High				Low	High
SfWT	2.9×10^4	1.7×10^4	4.8×10^4	1.0		110 a	105	116
SfB[c]	9.3×10^4	5.5×10^4	1.1×10^5	0.31	<0.001	120 a	114	125
Sfbac	1.0×10^5	6.1×10^4	1.7×10^5	0.28	<0.001	123 a	116	130
Sf29null	1.5×10^6	8.2×10^5	2.9×10^6	0.02	<0.001	157 b	147	167
Sf29null-repair	2.1×10^5	1.2×10^5	3.5×10^5	0.13	<0.001	132 a	125	140

[a] Logit regressions were fitted using the GLIM program with a common slope of 0.7847 ± 0.0695 (mean ± standard error). A test for nonparallelism was not significant ($\chi^2 = 6.374$, df = 4, $P = 0.173$). Relative potencies were calculated as the ratio of effective concentrations relative to the SfWT isolate. *P* values were calculated by a *t* test of the differences between regression intereepts compared to that of the SfWT isolate.
[b] MTDs were estimated by Weibull analysis (8). MTDs labeled with different letters were significantly different.
[c] SfB data are from reference 48.

Predicted protein sequence analysis. Amino acid sequence comparison of SF29 revealed that this protein is present in group II NPVs and an EPV. The SF29 showed the highest degree of similarity with the ORF30 of *S. exigua* MNPV (66%). Surprisingly, the SF29 protein shares a high degree of similarity in the catalytic conserved region with two copies of this gene in AmEPV (genus *Betaentomopoxvirus*): ORF3 (45%) and ORF258 (45%) (1). Both ORFs are predicted to encode proteins (16). Phylogenetic analysis revealed that similar SF29 proteins in group II NPVs and EPVs originated from a common ancestor and clearly separated a long time ago (data not shown).

Analysis of SF29 revealed at least 12 probable N-glycosylation sites, as well as domains characteristic of extracellular proteins such as a signal peptide, a transmembrane domain, and an intracellular domain (Fig. 5A). The putative amino acid sequence of SF29 showed high similarity with a region of a metalloprotease from *Vibrio* spp. that included a HEXXH zinc-binding motif characteristic of metalloproteases (18, 55), and this region is also conserved in AmEPV ORF3 (Fig. 5B). A threonine-rich region was also identified near the C terminus (Fig. 5A) that is present in different bacterial pathogens, including *Vibrio* spp., *Anthrax* spp., *Pseudomonas* spp., *Legionella* spp., and *Serratia* spp. (18), and which plays an important role in adhesion to cell membrane glycoproteins, as described for the herpesviruses (25).

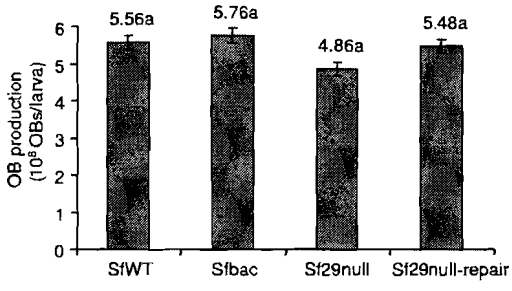

FIG. 4. OB production values in larvae infected with SfWT, Sfbac, Sf29null, and Sf29null-repair bacmid DNAs (100 ng/μl). Values above the bars indicate means. Mean values followed by the same letter do not differ significantly (Mann-Whitney test, $P > 0.05$). Error bars indicate standard deviations.

Temporal expression of *Sf29*. Temporal regulation of the *Sf29* transcript was examined by RT-PCR using total RNA isolated from *S. frugiperda* larvae that had been orally infected with SfWT. The very late-transcribed gene Sfpolh was included for reference. Total RNA extracted from *S. frugiperda* larvae at different intervals postinfection was treated with DNase prior to RT-PCR assay. Control amplifications performed to ensure the absence of contaminant DNA proved negative (data not shown). Single RT-PCR products of the expected size were obtained following amplification of Sf29 and Sfpolh. An *Sf29* amplification product was detected at a very low level at 12 hpi, increased at 48 hpi, and remained at a steady state up to 96 hpi. Similarly, an Sfpolh amplification product was detected at a very low intensity at 24 hpi, increased at 48 hpi, and remained at a steady-state level up to 96 hpi (Fig. 6). Transcription of *Sf29* commenced 12 h before that of Sfpolh.

DISCUSSION

The role of *Sf29* in the structure and replication of SfMNPV was studied by producing virus mutants lacking the gene using bacmid technology and the λ Red homologous recombination system. The Sf29null bacmid virus had eightfold-fewer ODVs per OB, which resulted in a lower virus DNA content. However, no difference in infectivity between viruses lacking or retaining *Sf29* was observed when genomic DNA was used to infect insect larvae by injection. This suggested that once inside the virus-infected insect, the *Sf29* deletion mutant was fully replication competent. These results are consistent with earlier studies in which a number of SfMNPV mutants lacked various amounts of DNA in the region of *Sf29* and displayed reduced infectivity as a consequence (34, 47, 48).

The presence of fewer ODVs in each Sf29null occlusion suggests that the SF29 protein could be involved in ODV formation or packaging. Currently, we can only speculate about SF29 function, as we have no experimental data on its location in virus particles or virus-infected cells. We do know that multiple encapsidation within virus particles, the occlusion

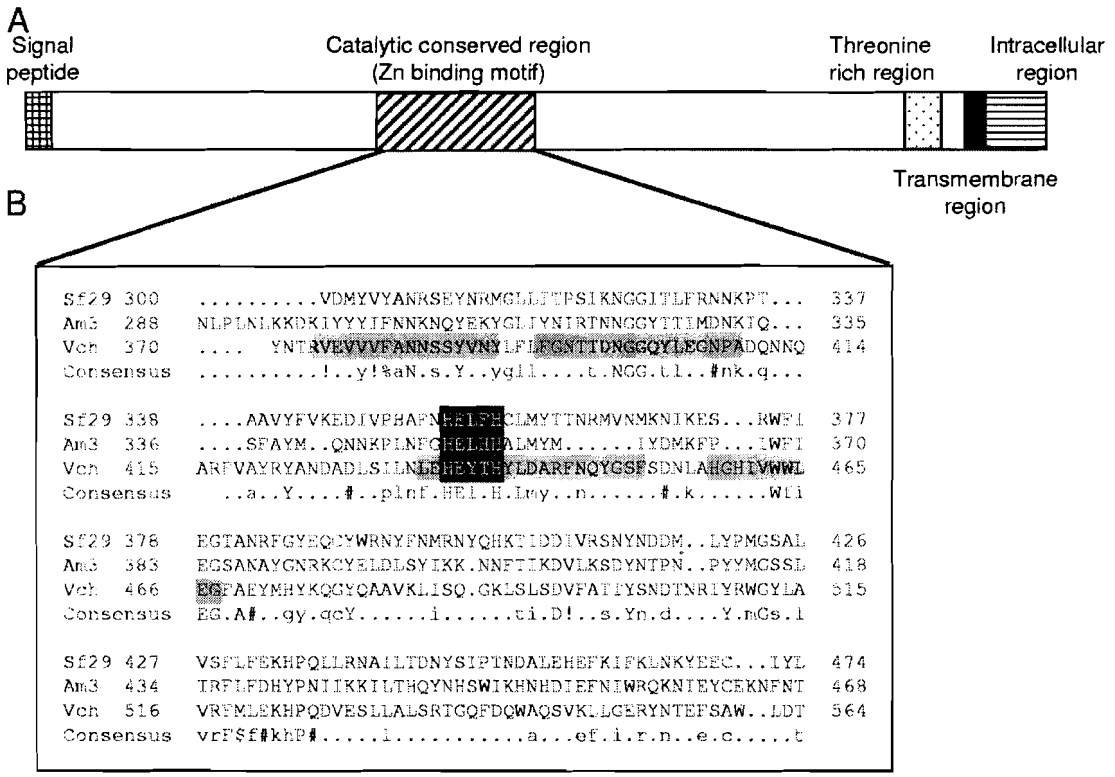

FIG. 5. (A) Schematic representation of the SF29 protein and its domains. The signal peptide, the transmembrane domain, the intracellular domain, the threonine motif, and the zinc-binding site homolog to metalloproteases are indicated. (B) Comparison between the catalytic conserved regions (Zn binding motifs) of *Vibrio cholerae* (Vch) (TrEMBL accession number A3EN11), AmEPV ORF3 (Am3) (PubMed accession no. NC_002520), and SfMNPV SF29 (Sf29) (27% and 25% identity and 43% and 45% similarity in putative amino acid sequence with *Vibrio cholerae* metalloprotease and AmEPV ORF3). The catalytic conserved region of the M9A metalloproteases family (IPR002169 MEROPS) is shaded in gray. The zinc-binding site (HEXXH motif) is shaded in gray with white letters. Consensus symbols: !, I or V; $, L or M; %, F or Y; #, N, Q, E, B, or Z.

process, a diverse viral protein composition, and the structure of the tegument all contribute to the complexity of ODVs (50). Braunagel et al. (3) identified 44 ODV-associated proteins in AcMNPV ODVs. When sequences and annotated genomes of NPVs are compared, it becomes apparent that a large number of the genes encoding ODV-associated proteins are conserved (20, 49). However, an increasing number of differences between group I and II NPVs in the conservation of ODV and

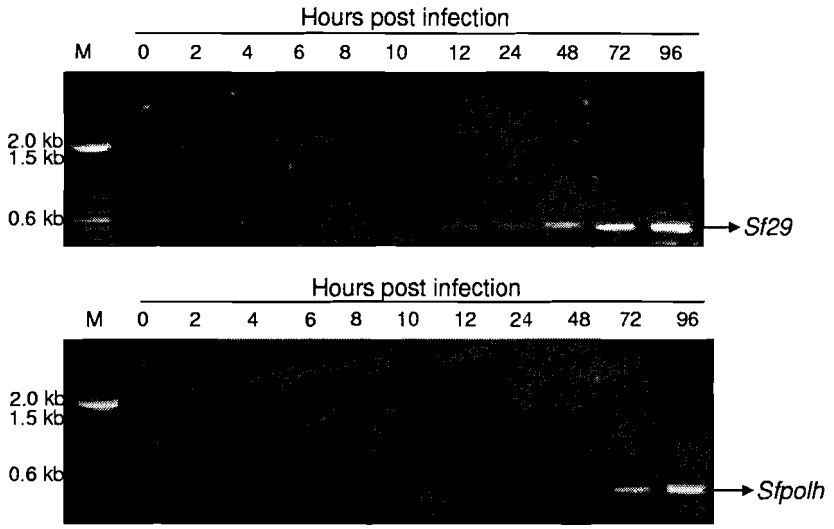

FIG. 6. Temporal expression of SfMNPV *Sf29* and *polyhedrin* (*Sfpolh*) genes. RT-PCR analysis of *Sf29* and *Sfpolh* was performed on total RNA extracted from infected larvae at indicated times postinfection. Transcript amplifications were performed using primers Sf29.1 and Sf29.2 for *Sf29* and primers Sfpolh.1 and Sfpolh.2 for *Sfpolh*. The same amount of RNA was used for both *Sf29* and *Sfpolh* amplifications. M, molecular DNA marker of 100 bp Stratagene.

envelope proteins is becoming apparent (10, 49), and it is possible that SF29 is an additional example of an ODV-associated structural protein that is restricted to group II NPVs.

Proteins located in the ODV envelope have transmembrane motifs (49). Hydrophobic transmembrane motifs are involved in membrane insertion and anchoring and are features of certain virion membrane proteins (23, 50). SF29 possesses a prominent transmembrane domain, which is characteristic of surface proteins. However, the major ODV envelope proteins do not have N-terminal membrane insertion signal peptides, and ODV proteins are almost never N glycosylated (4). SF29 has a predicted signal peptide motif and at least 12 potential N-glycosylation sites, which might suggest that it is not located in the ODV envelope. One exception to this is the *Spodoptera litura* MNPV ORF137 ODV envelope protein, which has been shown to be N glycosylated (56).

Amino acid sequence comparisons revealed that SF29 has a high degree of identity/similarity with two AmEPV proteins encoded by ORF3 and ORF256 (1, 16). Phylogenetic analysis confirmed that the SF29 homolog in group II NPVs and in EPVs likely evolved from a common ancestor. In contrast to baculoviruses, poxviruses do not replicate in the nucleus. The EPV particles and proteins are located in the cytoplasm of infected hemocytes (30, 42). This could suggest that if protein function of SF29 is conserved across virus families, then it will not be found in baculovirus ODVs or even the nucleus. The inner nuclear membrane is presumed to be the source of ODV envelopes (22, 23). The ODV envelope proteins that originate in the endoplasmic reticulum are targeted to the inner nuclear membrane, which blebs off as ODV envelope precursor microvesicles in the nuclear ring zone (22, 23). Transcription of *Sf29* begins 12 h before that of polyhedrin, so it may be a late gene product. Without defining studies using aphidicolin to block virus DNA replication, we cannot unambiguously assign early or late status to the gene. ODV envelope proteins result from late transcripts (46), suggesting that a late SF29 could be located in the ODV envelope.

The presence of a threonine-rich region, which plays an important role in adhesion to cell membrane glycoproteins and cell matrix proteins of bacteria (25, 43), suggests an alternative role for SF29 protein. The lower ODV content of deletion mutant OBs could be a result of the adhesion capacity of this protein (18, 39), which may aggregate virions during the process of OB formation. SF29 contains a catalytic domain that includes a zinc-binding motif (HEXXH), suggesting that this protein could also have protease activity. The putative amino acid sequence of SF29 indicated similarities to metalloproteases (clan MA) of the M9A family, particularly the *Vibrio cholerae* metalloprotease (27% identity and 43% similarity). Metalloproteases are involved in matrix protein degradation and adhesion (18, 53, 55). The presence of a zinc-binding motif and catalytic domain in SF29 suggests that this protein may possess both protease and hemagglutination activities. This double characteristic may designate SF29 a putative novel NPV enhancin. The enhancins are metalloproteases (32), which degrade mucins, the major proteins of the peritrophic membrane, or increase the fusion efficiency with midgut cells through interaction between the viral envelope and the cell plasma membrane, increasing the infectivity of baculoviruses in a number of lepidopteran hosts (2, 32, 51). To date, enhancins have been described in many granuloviruses, in which they are localized in the granule matrix (19, 45). Although recent reports have identified enhancins in some group II NPVs (2, 9, 33), these proteins differ from SF29 in being late gene products and are located in ODV envelopes in association with nucleocapsids (51). However, it is difficult to see how an enhancement-like function could be reconciled with the reduced virus content in OBs observed in this study.

Finally, although SF29 deletion affects pathogenicity, it is unlikely to have a role similar that of to a number of other baculovirus gene products described as viral pathogenicity factors (35, 38, 57). *Ac23* and *pe38* are transcribed early in infection and are not essential for viral replication or pathogenesis, but they play an important role in accelerating mortality in the host. The role of *Ac23* remains unclear, but its presence on budded virions suggests that it may be involved in cell-to-cell transmission in the infected host (35). Deletion of *pe38* resulted in a reduction in oral infectivity of OBs by nearly sevenfold but had no effect on budded virus infectivity (38). However, loss of *pe38* function had no discernible effect on the numbers of ODVs within occlusions (38), in contrast to our observations on the *Sf29* deletion bacmid.

In conclusion, SF29 appears to be a protein with a role in determining the number of virus particles within OBs. Future studies need to address whether it is an early or late gene product, via transcription studies with inhibitors of virus DNA replication, and whether or not it is a structural protein, by proteomic analysis of ODVs and OBs.

ACKNOWLEDGMENTS

We thank T. Carty, C. Allen, and C. Pritchard (Centre for Ecology and Hydrology, Oxford, United Kingdom) and I. Maquirriain and N. Gorria (Universidad Pública de Navarra, Pamplona, Spain) for technical assistance and J. Slack and B. Arif (Great Lakes Forestry Centre, Canada) for valuable discussions.

This work was supported by core CEH funds. O.S. received a Spanish Ministry of Education and Culture postdoctoral fellowship.

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