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Genetic Structure of a *Spodoptera frugiperda* Nucleopolyhedrovirus Population: High Prevalence of Deletion Genotypes

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A Nicaraguan field isolate (SfNIC) of *Spodoptera frugiperda* nucleopolyhedrovirus was purified by plaque assay on Sf9 cells. Nine distinct genotypes, A to I, were identified by their restriction endonuclease profiles. Variant SfNIC-B was selected as the standard because its restriction profile corresponded to that of the wild-type isolate. Physical maps were generated for each of the variants. The differences between variants and the SfNIC-B standard were confined to the region between map units 9 and 32.5. This region included PstI-G, PstI-F, PstI-L, PstI-K and EcoRI-L fragments. Eight genotypes presented a deletion in their genome compared with SfNIC-B. Occlusion body-derived virions of SfNIC-C, -D and -G accounted for 41% of plaque-purified clones. These variants were not infectious per os but retained infectivity by injection into *S. frugiperda* larvae. Median 50% lethal concentration values for the other cloned genotypes were significantly higher than that of the wild type. The variants also differed in their speed of kill. Noninfectious variants SfNIC-C and -D lacked the *pif* and *pif-2* genes. Infectivity was restored to these variants by plasmid rescue with a plasmid comprising both *pif* and *pif-2*. Transcription of an SfNIC-G gene was detected by reverse transcription-PCR in insects, but no fatal disease developed. Transcription was not detected in SfNIC-C or -D-inoculated larvae. We conclude that the SfNIC population presents high levels of genetic diversity, localized to a 17-kb region containing *pif* and *pif-2*, and that interactions among complete and deleted genotypic variants will likely influence the capacity of this virus to control insect pests.

The family *Baculoviridae* comprises a large group of viruses that are pathogenic to invertebrates and are characterized by enveloped rod-shaped virions with large (85 to 166 kb) circular double-stranded DNA genomes (2). These viruses have been reported from numerous insect species, particularly Lepidoptera (28). Due to their restricted host range (16) and high pathogenicity to a number of insect pest species, they have received considerable attention as microbial insecticides (31).

Baculoviruses produce two kinds of particles, one occluded and the other nonoccluded. Usually, both forms of virus are produced in a sequential manner in the same cell. When a susceptible insect consumes foliage contaminated with occlusion bodies (OBs), occlusion derived virions are released from the OB by alkali disruption of the OB protein. The occlusion-derived virions infect midgut cells, resulting in a primary infection. Subsequently, nonoccluded budded virus is produced, which buds out of the infected cell to allow cell-to-cell spread of the infection throughout the host. Budded virus particles consist of a nucleocapsid containing a single genome, enveloped by a membrane in which some virus-encoded proteins are anchored. Recent work indicates that each cell within the insect is infected by an average of four to five budded viruses (3). Later, the OB is produced, which stays inside the cell until the death of the infected insect and is then dispersed in the environment to achieve insect-to-insect transmission (13).

For members of the genus *Nucleopolyhedrovirus*, numerous virions are occluded in each OB. Importantly, in some nucleopolyhedroviruses (NPVs), each occlusion-derived virion can contain several nucleocapsids; thus, a single virion contains multiple genomes. These genomes may be identical or not but must be present in the same cell, as the OBs are formed by occlusion of virions within the cell nucleus. The occlusion-derived virions comprise nucleocapsids enveloped by a membrane assembled in the nucleus. This membrane contains various occlusion-derived virion-specific proteins that are crucial during the process of infection of the insect midgut cells.

Studies on genotypic heterogeneity within NPV populations have been performed by cloning individual genotypes in cell culture (24, 27, 29) or, less frequently, in insects (32, 44). Such studies have revealed that NPV populations often comprise a number of different genotypes that may differ in the presence of certain genes (8, 22). Certain genotypic variants may also be more prevalent in the population than the associated variants (29, 33). The fact that minority genotypes are not eliminated suggests that this heterogeneity is important for virus survival. However, most of our understanding of NPV biology is derived from the study of the behavior of clonal isolates (38). Isolation of individual genotypes has also allowed the evaluation of the relative pathogenicity and virulence of the different variants present in NPV populations (32) and facilitated the characterization of NPV genes (22), contributing to our understanding of their diversity and evolution. Moreover, the presence of certain genotypes has been shown to affect the pathogenicity of the virus population (32).

A multinucleocapsid NPV of the fall armyworm *Spodoptera frugiperda* (SfMNPV) has been isolated from this pest in different regions throughout the Americas (43). A Nicaraguan isolate (SfNIC) of this virus has been compared with other isolates (9) and tested as a potential bioinsecticide (46). Re-

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striction enzyme analysis of the genome of SfNIC shows characteristic submolar bands, indicating the presence of genotypic heterogeneity in this isolate. The aim of this study was to evaluate the degree of heterogeneity present in the wild-type virus population and to establish the possible genetic and phenotypic relationships among the variants. In total, nine different genotypic variants were isolated. Three of these were not infectious per os. The wild-type isolate presented greater insecticidal activity than any of the individually isolated variants, including the most prevalent variant in the population.

MATERIALS AND METHODS

Insects, cells, and viruses. *S. frugiperda* larvae were obtained from a laboratory culture that was started with pupae received from El Zamorano, Honduras, in 1997 and refreshed periodically with pupae from Tapachula, Mexico. The colony was continuously maintained in a growth chamber at 26 ± 1°C, 85% relative humidity, and a photoperiod of 16:8 (light-dark) on a semisynthetic diet (15). *S. frugiperda* Sf-9 ATTC cells were maintained in TC-100 medium supplemented with 10% fetal bovine serum (Gibco). The *S. frugiperda* nucleopolyhedrovirus (SfMNPV) was collected from diseased *S. frugiperda* larvae infesting maize plants in Nicaragua (9). This virus isolate, named SfNIC, was amplified by feeding OBs to fourth-instar *S. frugiperda* from the laboratory colony (18).

In vitro virus cloning. For the isolation of individual genotypes, SfNIC-infected larvae were surface decontaminated with 70% ethanol, and hemolymph was taken by bleeding at 48 h postinfection. The hemolymph was diluted in sterile phosphate-buffered saline and used to infect *S. frugiperda* cells. Sf-9 cells were plated at 2×10^6 cells/well in six-well tissue culture plates and incubated at 27°C for 3 h with the appropriate TC100 medium supplemented with 1% penicillin-streptomycin (Biowhittaker) and 1% amphotericin B (Gibco). The medium was removed, and 0.1 ml of serial 10-fold dilutions (from 10^{-2} to 10^{-6}) of the hemolymph were inoculated onto cells. After 1 h, the inoculum was removed, and 3 ml of 50% TC100 medium, 5% fetal calf serum (Gibco) supplemented with 3% (wt/vol) SeaPlaque agarose, and the antibiotics described above were added to each well. Once the agarose had solidified, it was overlaid with 3 ml of TC100 medium supplemented with the antibiotics. The liquid overlayer was replaced with fresh medium every day. After 10 days, cells were stained with 0.001% neutral red overnight (34). One hundred sixty-four well-isolated plaques were picked individually with a sterile Pasteur pipette and transferred to a vial containing 0.1 ml of phosphate-buffered saline. Volumes of 5 μl of this suspension were used for the amplification of the clones in 1.8×10^4 Sf-9 cells in 24-well tissue culture plates. Ten days after infection, the medium was collected and centrifuged to separate the cells, and the supernatants containing the budded virus were stored at 4°C.

In vivo virus production. Healthy fourth-instar *S. frugiperda* were individually injected through a proleg with 8 μl of budded virus suspension. Five larvae were injected per clone. After injection, larvae were reared individually on a semisynthetic diet under controlled conditions. Mortality was recorded daily. Dead infected larvae were individually transferred to a microcentrifuge tube and stored at −20°C until required.

Purification of OBs, DNA extraction, and restriction endonuclease analysis. Viral OBs were purified from dead diseased larvae by titration and centrifugation (8). Purified OBs were stored at 4°C in distilled water. OB concentration was calculated with an improved Neubauer hemacytometer (20). For viral DNA extraction, virions were released from OBs by mixing 100 μl of OB suspension 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 containing the virions was treated with 25 μl of proteinase K (20 mg/ml) for 15 min at 50°C. Viral DNA was extracted twice with Tris-EDTA (TE) buffer (pH 8.0)-saturated phenol and once with chloroform. Viral DNA was isolated from the aqueous phase by alcohol precipitation. The pellet was resuspended in 50 to 100 μl of 0.1× TE for 10 min at 60°C. The DNA concentration was estimated by reading the optical density at 260 nm and by agarose gel electrophoresis after restriction endonuclease digestion of an aliquot.

For restriction endonuclease analysis, 2 μg of viral DNA was mixed with 10 U of one of the restriction enzymes EcoRI, HindIII, and PstI (Amersham) and incubated for 4 to 12 h at 37°C. These enzymes were selected because they had proved to be informative in a previous study (8). The reaction was stopped by a 15-min treatment at 65°C, and 4 μl of loading buffer (0.25% bromophenol blue, 40% sucrose in water) was added. Electrophoresis was performed with horizontal 1% agarose gels in TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA, pH 8.0) at 20 V for 10 to 24 h. DNA fragments were stained with ethidium bromide and visualized on a UV transilluminator.

Construction of physical maps. Nine different genotypes were characterized by restriction endonuclease analysis and named alphabetically (SfNIC-A to SfNIC-I). Once the nine different genotypes were identified, a physical map was constructed for each one. The construction of the physical map was achieved by ordering the restriction fragments on the viral variant's genome according to the Southern blot hybridization data and multiple digestion of cloned viral restriction endonuclease fragments. Restriction fragments obtained from each genotypic variant were compared with the physical map previously determined for the SfNIC isolate (Simon et al., unpublished data). Genotype-specific fragments were isolated from agarose gels with the gel band purification kit (Amersham) and cloned into the pUC19 vector. The cloned fragments were labeled with digoxigenin by random priming (42).

The wild-type isolate and pure genotype DNAs were digested with restriction endonucleases. The fragments were separated in agarose gels and transferred to Nylon+ membranes (Roche) or Hybond-N+ (Amersham) by bidirectional (sandwich) transfer.

Hybridization of digoxigenin-labeled viral DNA probes to separated SfNIC and genotypic variant DNA fragments was performed as described previously (7). Prehybridization, hybridization, and probe detection were performed following the manufacturer's instructions in the DIG-DNA labeling kit (Roche). Finally, the nylon sheet was washed, dried, sealed in a plastic bag, and exposed to Hyperfilm-ECL (Amersham) for 2 to 24 h with a Lightning-Plus intensifier screen (DuPont). The cloned fragments were double digested to determine the restriction sites present within each fragment.

Insect bioassays. The 50% lethal concentration (LC_{50}) and mean time to death of the wild-type isolate and each cloned genotype were determined by the insect per os bioassay following the droplet feeding technique (18). Second-instar *S. frugiperda* were starved for 8 to 12 h at 23°C and then allowed to drink from an aqueous suspension containing 10% (wt/vol) sucrose, 0.001% (wt/vol) Fluorella blue, and either 1.2×10^6, 2.4×10^5, 4.8×10^4, 9.6×10^3 or 1.9×10^3 OBs/ml. This concentration range was previously determined to kill between 5 and 95% of the experimental insects. Larvae that ingested the suspension within 10 min were transferred to individual wells of a 25-well tissue culture plate with a semisynthetic formaldehyde-free diet plug.

Bioassays with 25 larvae per virus concentration and 25 larvae as controls were performed three times. Larvae were reared at 26°C, and larval mortality was recorded every 12 h until the insects had either died or pupated. All the statistical analyses were performed with the generalized linear interactive modeling program (5). Virus-induced *S. frugiperda* mortality was subjected to logit analysis. Time mortality data of the per os infectious genotypes were subjected to Weibull analysis in the generalized linear interactive modeling program. The time mortality characteristics of SfNIC-C, -D, and -G could not be determined because these variants were not infective per os. The OB concentration used for the time mortality analysis was that which produced ≈70% larval mortality: for SfNIC, 2.4×10^5 OBs/ml (68%); for SfNIC-A, 1.2×10^6 OBs/ml (73%); for SfNIC-B, 1.2×10^6 OBs/ml (73%); for SfNIC-E, 1.2×10^6 OBs/ml (67%); for SfNIC-F, 1.2×10^6 OBs/ml (73%); for SfNIC-H, 1.2×10^6 OBs/ml (73%); and for SfNIC-I, 1.2×10^6 OBs/ml (75%).

Intrahemocelic injections were performed to determine the infectivity of nonoccluded virions. Occlusion-derived virions suspensions from all genotypic variants were obtained by alkali disruption of OBs with a dissociation buffer (1 volume of OBs:1 volume of 0.5 M Na_2CO_3:5 volumes of water). Fifty larvae were individually injected with a volume of 8 μl of occlusion-derived virion suspension. After injection, larvae were reared individually under the conditions indicated above.

Dead larvae were examined microscopically to determine the presence of OBs. When present, OBs were purified from each group of dead larvae. Viral DNA was extracted from these larvae and subjected to restriction endonuclease analysis to determine the identity of the genotypic variant.

Detection of *pif*, *pif-2*, and *p74* genes in the SfMNPV genotypic variants. Three genes have been described as being responsible for oral infectivity: *p74* (23), *pif* (22), and *pif-2* (36). All these genes are present in the SfNIC genome. The *pif* and *p74* genes sequences from *Spodoptera exigua* nucleopolyhedrovirus (SeMNPV) (21) were used as probes for the homologous genes in SfMNPV. Sequences internal to both genes were obtained by PCR amplification from SeMNPV DNA with specific primers. Purified PCR products were labeled with digoxigenin by random priming with the Klenow enzyme (Amersham) (42). Southern blots were processed as previously described (8). The fragment that contained the SfMNPV *pif* gene was cloned into the pSP70 vector (Promega) to produce p264.306. This fragment was sequenced, and *pif-2* was located to the left of the *pif* gene.

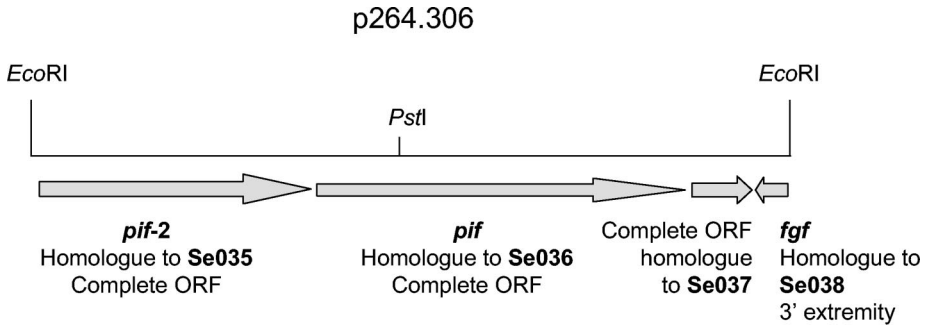

FIG. 1. Arrangement of ORFs within restriction fragment EcoRI-N of the SfNIC isolate. This fragment is included in the p264.306 plasmid. Shown is a schematic representation of the organization of the SfNIC EcoRI-N restriction fragment.

RNA purification. Total RNA was isolated at 48 h postinfection from fourth-instar *S. frugiperda* following mock inoculation or inoculation with SfNIC, SfNIC-C, -D, or -G. Larvae were inoculated per os with approximately 10^9 OB/ml, estimated to kill ≈90% of the larvae (9). Larval tissues were triturated, suspended in 500 to 1,000 μl of Trizol (Gibco) and 200 μl of chloroform, incubated for 10 min, and centrifuged at 12,000 × *g* for 15 min at 4°C. The water fraction was precipitated with isopropanol, centrifuged at 12,000 × *g* for 10 min at 4°C, washed with 70% ethanol, and resuspended in approximately 50 μl of water, depending on the size of the final pellet observed. RNA solutions were incubated at 60°C for 10 min to assist resuspension of the RNA; in some cases it was necessary to freeze and thaw the samples various times. RNA was quantified by absorbance at 260 nm and stored at −80°C until used.

RT-PCR assay of orally noninfectious genotypic variants. Reverse transcription (RT)-PCR was performed to determine the capacity of the virus to enter midgut epithelial cells and initiate expression of viral genes. Specific primers for the *ie-0* gene of SfNIC were designed to detect the presence or absence of an *ie-0* transcript for the SfNIC isolate and the SfNIC-C, -D, and -G variants. The *ie-0* gene is a very early transcribed gene and is the first gene expressed in the viral infection (11, 39).

RT-PCR was performed with the Access RT-PCR system kit (Promega). First-strand cDNA synthesis was performed with avian myeloblastosis virus reverse transcriptase and the internal oligonucleotide Sfie0.2. As oligo(dT) was not used for the amplification, the samples were treated with DNase (Promega) prior to reverse transcription. cDNA mixtures were amplified by PCR with Sfie0.1 and Sfie0.2. PCR products were analyzed in 1% agarose gels. A normal PCR was carried out with the samples previously treated with DNase to confirm the absence of DNA. SfNIC DNA was used as the template for the positive PCR control. The fragments obtained by RT-PCR were blotted onto Nylon+ charged membranes (Roche). The nylon membrane was air dried and exposed to a UV transilluminator. Hybridization was carried out overnight at 60°C with a probe obtained by PCR amplification of SfNIC DNA with the Sfie0.1 and Sfie0.2 primers labeled with digoxigenin (DIG DNA labeling kit, Roche) by random priming.

Complementation analysis and rescue. The following procedure was used to determine the ability of the p264.306 vector that includes both *pif* and *pif-2* to rescue infectivity of noninfectious genotypic variants and to determine whether the absence of *pif* and *pif-2* was responsible for the lack of per os infectivity. For this, 5×10^5 cells were transfected with 800 ng of DNA of the perorally noninfectious variants SfNIC-C, -D, or -G, and the perorally infectious clone SfNIC-B alone or mixed with p264.306 (2 μg) that covered the *pif* and *pif-2* genes region (Fig. 1) with DOTAP (*N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulfate) (Roche). SfNIC-B DNA was used as a positive control in the transfection and cotransfection. Cotransfected cells were harvested once the OBs were produced (9 days after infection). Cells were then rinsed with phosphate-buffered saline and suspended in 20 μl of phosphate-buffered saline. These samples were frozen to inactivate budded virus that could have been present and to disrupt the cells and release OBs. This OB suspension was used to inoculate neonatal *S. frugiperda* with the droplet feeding method. Larvae that had fed on the suspension during a 10-min period were individually placed in rearing boxes with the usual diet. Mortality was recorded daily. Dead larvae were checked for OBs, and the viral DNA was extracted, digested with restriction endonuclease, analyzed by electrophoresis, and subjected to PCR amplification with primers designed for the SeMNPV *pif* gene. OBs were also used in an additional passage in neonatal larvae to test for per os infectivity.

RESULTS

Identification of genotypic variants. The genotypic variants present in the wild-type population of SfNIC were isolated in cell culture. Of 164 plaque picks, 104 were successfully amplified and caused fatal infection upon injection in insect larvae. The plaques produced in Sf9 cells were small. It is therefore possible that a certain proportion of the plaques were not actually the result of virus infection but just holes in the cell monolayer. Another possibility is that the small agarose cylinder taken did not contain enough virus to kill the insect larvae. As the objective was to obtain a representative sample of the virus genotypes capable of autonomous replication, the nonproductive plaques were not analyzed further. Nine different genotypes were identified after restriction analysis of the individual plaque picks amplified in insects. They were named SfNIC-A, SfNIC-B, SfNIC-C, SfNIC-D, SfNIC-E, SfNIC-F, SfNIC-G, SfNIC-H, and SfNIC-I. All of these variants could be differentiated with the PstI enzyme (Fig. 2) except variants SfNIC-C and SfNIC-D, which were distinguished by EcoRI (data not shown).

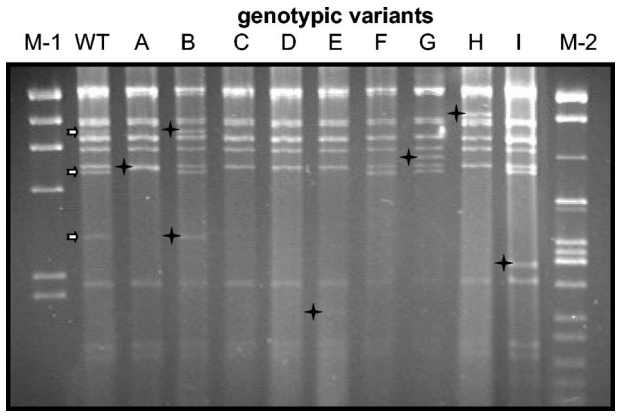

FIG. 2. PstI restriction endonuclease profiles of DNA of the SfNIC isolate and nine genotypic variants isolated by plaque assay from the wild type. Arrowed fragments are submolar bands present in the wild type, and asterisks indicate restriction fragment length polymorphism (RFLP) bands unique to one particular genotype. The first and last lanes represent lambda DNA digested with HindIII (M-1) and with EcoRI, HindIII, and PstI (M-2) as molecular size markers. (From López-Ferber et al. [25], with permission.)

TABLE 1. Restriction fragments generated by PstI in the genomic DNA of the SfNIC isolate and its genotypic variants

Fragment	Size (kb) in SfNIC and genotypic variant:									
	SfNIC	A	B	C	D	E	F	G	H	I
A	27	27	27	27	26.2	27	27	27	27	27
B	24	24	24	24	24	24	24	24	24	24
C	16.7	16.7	16.7	16.7	16.7	16.7	16.7	16.7	16.7	16.7
Additional									9.6	
D	9	9	9	9	9	9	9	9	9	9
E	8.2	8.2	8.2	8.2	8.2	8.2	8.2	8.2	8.2	8.2
F	7.8	0	7.8	0	0	0	0	0	0	0
G	6.9	6.9	6.9	6.9	6.9	6.9	6.9	6.9	0	6.9
H	6.7	6.7	6.7	6.7	6.7	6.7	6.7	6.7	6.7	6.7
I	5.9	5.9	5.9	5.9	5.9	5.9	5.9	5.9	5.9	5.9
Additional								5.4		
J	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
K	4.7	0	4.7	0	0	0	4.7	4.7	4.7	4.7
Additional		5.0					4.7			
L	2.9	0	2.9	0	0	0	0	0	0	0
Additional										2.3
M	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Additional						1.7				
N	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.3
O	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2
Total	129.3	118.9	129.3	113.9	113.1	115.6	123.3	124.0	116.6	120.9

Genotype SfNIC-B was selected as the standard because its restriction profile corresponded to the predominant profile in the SfNIC population. All the other genotypes displayed one or more deletions compared to SfNIC-B. All of the deletion variants lacked PstI-F and PstI-L, and in some variants we also identified additional deleted fragments. SfNIC-A lacked the PstI-F (7.8 kb in SfNIC-B), PstI-L (2.9 kb in SfNIC-B), and PstI-K (4.7 kb in SfNIC-B) fragments, but showed a longer fragment of 5 kb instead (Fig. 2, Table 1). SfNIC-C and -D were indistinguishable by PstI restriction. Both of these genotypes lacked PstI-F, PstI-L, and PstI-K. Analysis with EcoRI revealed that SfNIC-D presented a deletion, absent in SfNIC-C, of approximately 0.8 kb in the EcoRI-L fragment that resulted in a 5.3-kb fragment. The SfNIC-E genotype lacked PstI-F, PstI-K, and PstI-L but showed an additional 1.7-kb fragment. SfNIC-F and -G lacked PstI-F and PstI-L but displayed additional fragments of 4.7 and 5.4 kb, respectively. The PstI profile of SfNIC-H included a 9.6-kb fragment, whereas PstI-G, PstI-F, and PstI-L were absent. Finally, the SfNIC-I variant showed an additional PstI fragment of 2.3 kb and lacked PstI-F and PstI-L (Fig. 2, Table 1). The additional fragments described above were unique to each variant.

No submolar bands were observed in these genotypic variants after one passage, and the restriction profiles remained invariant for a minimum of three passages in insects, suggesting that they represented stable cloned genotypes. Most of the submolar bands that were detectable in the SfNIC DNA profiles as PstI-F or PstI-L were present only in SfNIC-B and not in the other variants. Another submolar fragment visible in the agarose gels was PstI-K, which is shared by variants SfNIC-B, -F, -G, -H, and -I. In contrast, the characteristic fragments present in variants SfNIC-A, -C, -D, -E, -F, -G, -H, and -I were not apparent in the DNA restriction profile of the wild-type SfNIC isolate by ethidium staining.

SfNIC-C was the most frequently isolated genotype, representing 33% of the cloned variants. Together with SfNIC-A (18%), -B (15%), and -H (13%), these variants accounted for the majority of the genotypes present in the population. The other genotypes appeared only in low proportions. A single clone with the SfNIC-I profile was obtained.

Mapping of a variable region in the DNA of genotypic variants. The restriction fragment length polymorphism markers of each genotype were mapped to the genome with the SfNIC physical map that represented the predominant variant SfNIC-B (Simon et al., unpublished data) and the results of Southern blot hybridization and double digestions. Genotypic variability was located in a single region of the genome, between map units 9 and 32.5 (PstI-G, PstI-F, PstI-L, PstI-K, and EcoRI-L) (Fig. 3). All genotypes presented a deletion (compared to SfNIC-B) that differed in size.

The marker fragments present in each variant hybridized to the following fragments of the SfNIC-B genome; the characteristic fragments present in SfNIC-A and -E hybridized to PstI-F and PstI-K, whereas the fragments of SfNIC-F, -G, and -I hybridized to PstI-F and PstI-L. Finally, the marker fragment of SfNIC-H following treatment with PstI hybridized to PstI-G and PstI-K. Variants could be assigned to three groups according to the size of the deletion in the genome (Table 1). The variants SfNIC-C and -D presented a large deletion of approximately 15 kb which mapped between 14.8 and 26.7 map units of the SfNIC genome (Fig. 3). A second group with a medium-sized deletion included the variants SfNIC-A, -H, and -E. The third group consisted of variants SfNIC-F, -G, and -I with the smallest deletions. Variant SfNIC-G presented a deletion of 5.3 kb localized between 14.8 and 23.1 map units with a characteristic fragment of 5.4 kb (Table 1, Fig. 3).

Biological activity of SfNIC genotypic variants. Three of the genotypic variants, SfNIC-C, previously reported in López-Ferber et al. (25), -D, and -G, were found to be noninfectious per os in *S. frugiperda* larvae. However, these variants retained their ability to form plaques in cell culture and to replicate and kill larvae upon injection of cell culture supernatants. The

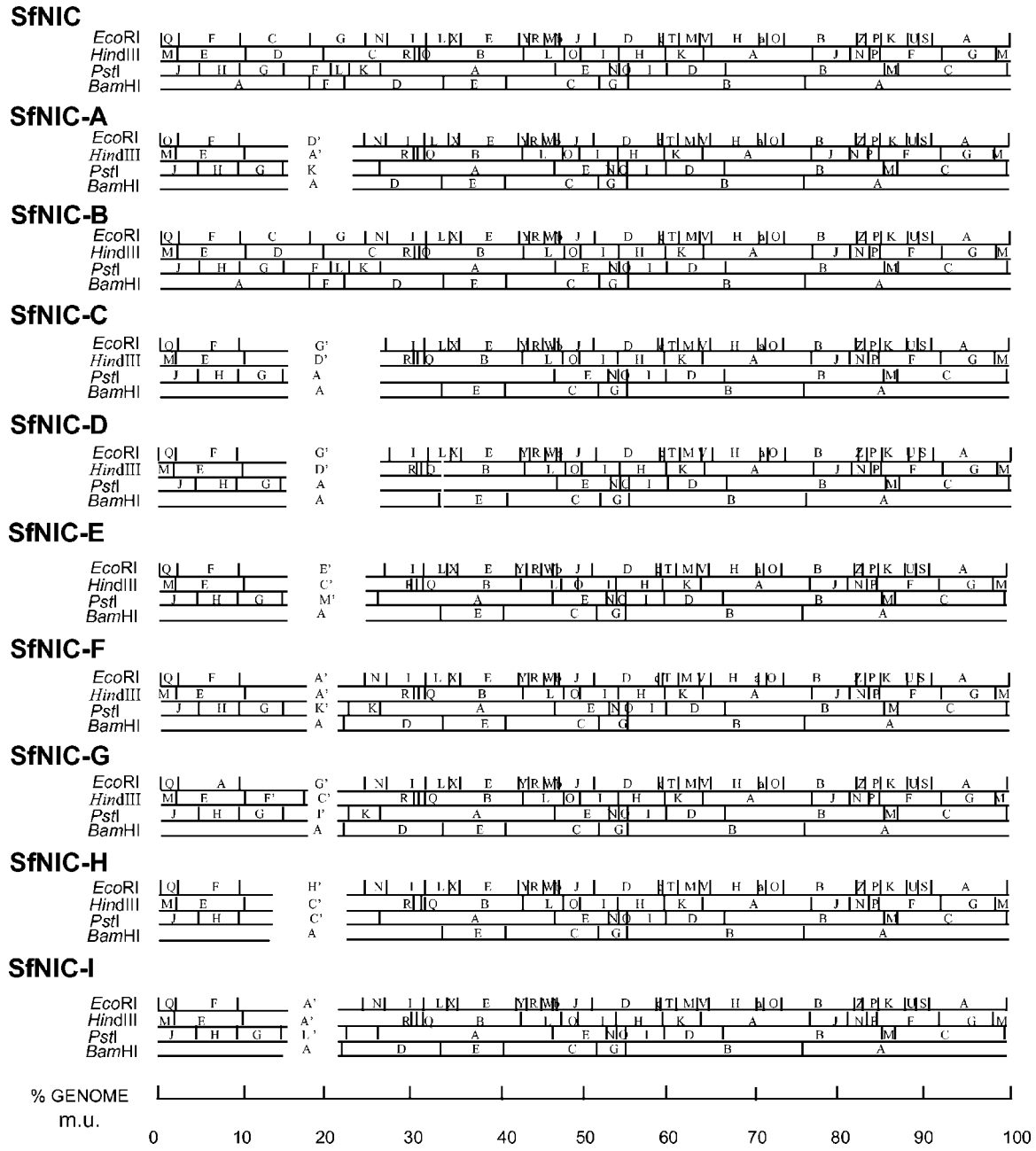

FIG. 3. Physical maps of SfNIC isolate and nine genotypic variants genomes (SfNIC-A to SfNIC-I). SfNIC-B is represented as the reference standard because of the absence of deletions in its genome. The physical map of SfNIC-B is the same as that described for the SfNIC isolate (Simón et al., unpublished). Restriction maps for EcoRI, HindIII, PstI, and BamHI are shown. The circular SfNIC DNA is represented in linear form, and the site between EcoRI-A and EcoRI-Q was designated site 1. Scales are indicated in map units (m.u.). The genome size was estimated to be 129.3 kbp.

occlusion-derived virions of SfNIC-C, -D, and -G obtained from dead insects were also able to replicate and kill *S. frugiperda* larvae when injected into the hemocoel but not when the larvae were inoculated per os. DNA extracted from OBs from these variants was infectious by transfection into Sf-9 cells.

The OBs produced from SfNIC genotypic variants that killed larvae by oral infection were purified and bioassayed for pathogenicity. The LC_{50} values of these OBs ranged from 1.56 $\times$ 10^5 to 4.04 $\times$ 10^5 OBs/ml (Table 2). These values were significantly higher than the LC_{50} observed in the SfNIC isolate, which was 5.47 $\times$ 10^4 OBs/ml, indicating that the wild-type mixture of genotypes represented the most infectious inoculum. The 95% confidence limits of the relative potencies, representing the ratio of effective concentrations (40), overlapped in SfNIC-A, -E, and -H, indicating no significant differences in pathogenicity among these variants. This group of variants was significantly less pathogenic than variants SfNIC-B, -F, and -I. None of the genotypic variants was more

TABLE 2. Relative potencies per os and time to death for cloned genotypes compared with the wild-type isolate in second-instar *S. frugiperda* larvae[a]

Virus	Deletion length (kb)	LC_{50} (OBs/ml)	95% confidence interval		Relative potency	*P*	Mean time to death (h)	95% confidence interval	
			Low	High				Low	High
SfNIC		5.47×10^4	3.82×10^4	7.84×10^4	1.00		131[a]	124	137
A	10.4	2.45×10^5	1.87×10^5	3.21×10^5	0.22	<0.001	130[ab]	124	136
B	0	1.99×10^5	1.52×10^5	2.58×10^5	0.27	<0.001	120[b]	114	125
C	15.4								
D	16.2								
E	13.7	4.04×10^5	3.09×10^5	5.35×10^5	0.13	<0.001	140[a]	134	147
F	6.0	1.56×10^5	1.19×10^5	2.03×10^5	0.85	<0.001	90[c]	86	94
G	5.3								
H	12.7	2.45×10^5	1.89×10^5	3.21×10^5	0.22	<0.001	136[a]	129	141
I	8.4	1.66×10^5	1.29×10^5	2.15×10^5	0.33	<0.001	90[c]	86	94

[a] Logit regressions were fitted in GLIM with a common slope of 0.5813 ± 0.034 (standard error). A test for nonparallelism was not significant ($\chi^2 = 1.37$, d.f. = 6, $P = 0.22$). Variants were subsequently assigned to four groups from higher to lower pathogenicity; the first group comprises the SfNIC isolate; the second group includes SfNIC-F, -I, and -B; the third group includes SfNIC-A, -H, and -E; and the last one includes the noninfectious variants SfNIC-C, -D, and -G. Relative potencies were calculated as the ratio of effective doses relative to the SfNIC isolate. *P* values were calculated by *t* test of the differences between regression intercepts compared to that of the SfNIC isolate. Mean time to death was estimated by Weibull analysis (5); values labeled with identical letters did not differ significantly (*t* test, GLIM, $P > 0.05$).

pathogenic than the wild-type SfNIC isolate, as demonstrated by the relative potencies of each one. Moreover, SfNIC was significantly more pathogenic than SfNIC-B, the predominant variant in the wild-type population, as demonstrated by restriction endonuclease analysis.

Analysis of the mean time to death (Table 2) revealed three distinct groups. The mean times to death for SfNIC-E, -H, and -A were the highest at 140, 135, and 130 h, respectively. The mean time to death for SfNIC (131 h) was statistically similar to those of the variants, as judged by the overlap of fiducial limits. The standard variant, SfNIC-B, presented a significantly lower mean time to death of 120 h. Finally, the lowest mean times to death were observed for SfNIC-F and -I at 90 h, which was significantly different from all the other variants.

Detection of *p74*, *pif*, and *pif-2* genes in genotypic variants. The gene *p74* was localized in the PstI-C fragment of SfNIC, and its presence was also confirmed in all SfNIC cloned genotypic variants. However the *pif* gene, located between the PstI-K and PstI-A fragments of SfNIC, was present in some variants but absent in others. In the variants SfNIC-B, -F, -G, and -I, which conserved that region of the genome intact, the *pif* gene was present and was located at the same site. In variants SfNIC-A, -E, and -H, the fragment PstI-K was absent but the *pif* gene was found to be present between fragment PstI-A and the characteristic additional fragment of each variant: SfNIC-A (5.0 kb), -E (1.7 kb), and -H (9.6 kb). The *pif* gene was not detected in variants SfNIC-C and -D, both of which were not infectious per os. The absence of the *pif* gene was confirmed by PCR analysis with oligonucleotides that spanned the complete ORF; no amplification was observed in either SfNIC-C or -D genomic DNA. By sequencing the SfNIC-B EcoRI-N fragment that included the *pif* gene, the *pif-2* gene was localized to the left of the *pif* gene (Fig. 1). EcoRI-N included the right part of PstI-K; i.e., *pif-2* was located in PstI-K. It therefore follows that *pif-2* was also absent in SfNIC-C and -D.

Detection of very early transcribed genes in SfNIC-C-, -D-, and -G-infected *S. frugiperda* larvae. RNA from larvae inoculated per os with SfNIC-C, -D, and -G was extracted and used for RT-PCR analysis. No transcription of *ie-0* was detected in larvae fed SfNIC-C or -D OBs, suggesting that these variants were not able to enter midgut epithelial cells and initiate transcription. A weak hybridization signal was observed in three of the five larvae fed SfNIC-G OBs. This observation indicated that the SfNIC-G variant was able to enter and to start replication in midgut cells. In contrast, a strong hybridization signal was observed in SfNIC-infected larvae, used as positive controls, compared with that observed in SfNIC-G-infected larvae (Fig. 4).

Complementation analysis of variants lacking per os infectivity. OBs of SfNIC-C, -D, and -G were not infectious per os, and variants SfNIC-C and -D lacked the *pif* and *pif-2* genes necessary for per os infectivity (22, 36). A complementation analysis was used to test whether the EcoRI-N fragment, that included both *pif* and *pif-2* was responsible for this phenotype. Purified genomic DNA from SfNIC-C, -D, and -G was cotransfected with p264.306. This plasmid contained the EcoRI-N fragment, which included the complete ORFs of *pif* and *pif-2* and therefore compensated for the absence of *pif* and *pif-2* in the defective variants.

Larvae inoculated with OBs obtained from transfection of viral DNA suffered the same mortality as larvae transfected with the negative control (water), indicating that SfNIC-C, -D, and -G were not infectious per os. In contrast, OBs from SfNIC-B-transfected cells were able to infect *S. frugiperda* larvae, resulting in a normal viral infection. The majority of the larvae died following ingestion of OBs produced in cells cotransfected with the SfNIC-C or -D variant plus the p264.306 plasmid. These results indicated that the EcoRI-N fragment, which included both *pif* and *pif-2*, was responsible for the observed phenotype of the SfNIC-C and -D genotypes, although we could not determine whether *pif* or *pif-2* or both genes were responsible.

No rescue was observed with OBs produced in cells cotransfected with the SfNIC-G variant plus p264.306 (Table 3). The results of the RT-PCR study agreed with the complementation assay; both indicated that the SfNIC-G variant was able to enter cells and start a primary infection. Therefore, the *pif* and

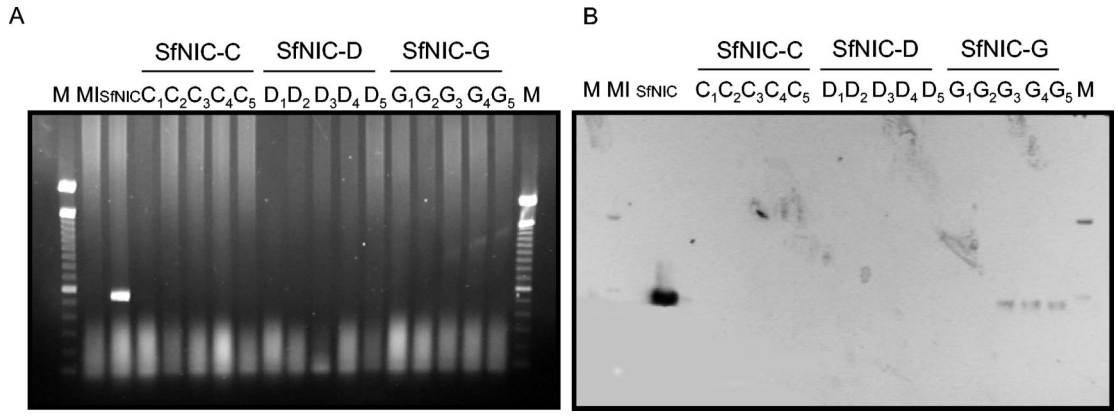

FIG. 4. (A) Agarose gel electrophoresis of RT-PCR fragments amplified with SfMNPV *ie-0* specific primers from fifth-instar *S. frugiperda* inoculated with SfNIC genotypic variants SfNIC-C, SfNIC-D, and SfNIC-G. (B) Hybridization analysis of the gel shown in A with a digoxigenin-labeled SfMNPV *ie-0* gene fragment amplified from SfNIC DNA by PCR with the same specific primers used for the *ie-0* gene. MI, mock-infected larvae (negative control); SfNIC-infected larvae were the positive control. Lane M, molecular size markers (Gibco-BRL 1-kb ladder).

pif-2 genes were functional in variant SfNIC-G and were not responsible for the nonlethal phenotype in vivo. We presumed that the absence of lethal disease observed in SfNIC-G could have been due to another gene(s) implicated in cell-to-cell transmission within the insect host.

The PstI restriction profiles of DNA obtained from OBs extracted from dead larvae were identical to that of the original variant (data not shown). Larvae fed OBs from SfNIC-C and -D cotransfection, obtained from dead larvae (second passage), were not killed, but occlusion-derived virions extracted from them were infectious by injection, confirming that the genomes amplified were similar to that of SfNIC-C or -D and that no obvious recombination had occurred during cotransfection.

DISCUSSION

Although closely related, genotypic variants can be distinguished from one another by restriction fragment length polymorphism (6) or by alterations such as additions, duplications, or deletions clustered in certain genomic regions (6, 14, 33). The high prevalence of recombination (30), together with the enveloping of multiple genomes in a single virion, may be considered evolutionary strategies to guarantee the preservation of high levels of genetic variability in NPV populations within each infected host, thereby resulting in progeny virions with a high degree of genetic diversity.

Restriction fragment length polymorphism analyses of the wild-type isolate SfNIC revealed the presence of submolar bands, indicating genetic heterogeneity. Such heterogeneity appears to be common in baculoviruses (30, 33, 43). Nine different genotypes were isolated by plaque purification, and each genotype appeared to be stable through successive infection cycles in larvae. The submolar bands observed in the SfNIC isolate corresponded to the differential restriction fragment length polymorphisms of those cloned genotypic variants. The SfNIC-B variant was the predominant variant in the wild-type SfNIC isolate, as estimated by the relative intensities of the restriction fragment length polymorphism markers (PstI-F and PstI-L) in the restriction profiles of SfNIC. The prevalence of the SfNIC-B variant was previously estimated by semiquantitative PCR at ≈78% (25). This genotype was selected as the reference standard. In contrast, the most prevalent variant isolated in cell culture was SfNIC-C (33%), whereas SfNIC-B was less abundant (15%).

In different conditions, as in cell culture, selection favors the proliferation of other variants, including certain defective genotypes (7, 18). To avoid this bias, the estimation was performed by densitometric analysis, measuring the relative proportions of a variable restriction fragment in the population as

TABLE 3. Complementation analysis of the noninfectious OB phenotype[a]

Expt	DNA used to infect cells	Plasmid	Genes complemented	Total no. of larvae	No. of dead larvae	Rescue
1	SfNIC-B			47	47	+
2	SfNIC-C or -D			37	3	−
3	SfNIC-G			35	3	−
4	SfNIC-B	p264.306	*pif*, *pif-2*, and ORF homologue to Se37	44	44	+
5	SfNIC-C or -D	p264.306	*pif*, *pif-2*, and ORF homologue to Se37	39	31	+
6	SfNIC-G	p264.306	*pif*, *pif-2*, and ORF homologue to Se37	35	1	−
7	None (mock infected)			37	2	−

[a] Sf9 cells were transfected with viral DNA alone (experiments 1, 2, and 3) or cotransfected with viral DNA and the p264.306 plasmid, which includes the *pif* and *pif-2* genes (experiments 4, 5, and 6). Neonatal larvae were fed OBs produced by the infected cells. At least two independent replicates were performed for each experiment. Dead larvae were examined for the presence of OBs.

described previously (25). With this method, the proportion of per os-noninfectious variants was estimated to comprise 19.5% of the population. This percentage was lower than that of the combined SfNIC-C and -D genotypes, which accounted for approximately 38% of the total cloned genotypes. This difference may be due to positive selection in cell culture, as suggested previously (18). However, restriction fragment length polymorphism analysis indicated that the SfNIC-B variant was predominant, since the wild-type isolate SfNIC presented the same DNA restriction profile as SfNIC-B. The causes underlying the dominance of a particular variant in natural NPV isolates (29, 32) appear to be difficult to explain.

Restriction fragment length polymorphism was associated with the fragments located between 9 and 32.5 map units (Simón et al., unpublished data). This highly variable region was a source of restriction fragment length polymorphism markers for all nine genotypic variants. This region is also variable in the genotypes isolated from the phylogenetically related SeMNPV isolate Se-US2 (31). The complete sequence of an SeMNPV clone is available (20). The genes necessary for viral replication are not located within this genomic region since all the genotypic variants could be isolated in cell culture. It has thus been possible to identify the genes most likely to be affected by the deletions that we observed. SeMNPV ORFs 15 to 35 (including genes encoding cathepsin, chitinase, and gp37 as well as *ptpt-2*, *egt*, *pkip-1*, *arif-1*, and *pif-2*) are not essential for virus replication in cell culture, and this region appears to be variable in the wild-type SfNIC isolate and also in Se-US2.

At least two genes involved in per os infectivity are located in this region, *pif* (22) and *pif-2* (36), corresponding to ORFs Se036 and Se035, respectively. Mutation or deletion of these genes apparently has no adverse effect on replication in cell culture (22, 36). The differences between the proportion of deleted genotypes obtained in vitro and their proportion deduced by densitometric analysis might indicate a selective advantage of the deleted genotypes in cell culture, such as a faster replication rate or larger plaque size. It seems likely that this advantage would also be found in natural conditions during replication in the host larva and would quickly achieve an equilibrium state, representing a balance between within-host replication and between-host transmission, where deleted genotypes alone cannot be transmitted.

Cloned variants of *Autographa californica* NPV show no significant variation in virulence (1), whereas genotypic variants cloned from SeMNPV (33) show significant differences in virulence. In other NPV isolates, variants may show increased virulence in alternative hosts (14). The biological activity of the nine SfNIC cloned variants was determined by per os bioassay. The LC_{50} value obtained for the SfNIC isolate was the lowest and differed significantly from the rest of the orally infectious variants. Individual variants could be classified into four groups depending on their pathogenicity per os. First were SfNIC-F and -I, with relative potencies ≈3 times lower than that of the wild-type SfNIC. Second were SfNIC-A, -B, and -H, with potencies ≈4 times lower than that of the SfNIC isolate. Third was SfNIC-E, which had the lowest potency compared to that of SfNIC and also presented the largest deletion among the per os-infectious variants. The final group comprised the remaining variants, SfNIC-C, -D, and -G, which were not infectious per os.

The reduced relative potency of cloned variants compared with the wild-type population led us to hypothesize that this effect could be due to an interaction among genotypes, including defective variants, resulting in altered pathogenicity. This hypothesis was briefly examined (the interaction of SfNIC-B and -C) in a previous study (24), and deleted variants were shown to play an important positive role in the pathogenicity of the viral population. In the present study, a correlation was detected between the length of the deletion and biological activity, in terms of both lethal concentration (pathogenicity) and lethal time (virulence) (Table 2). Variants with shorter deletions, such as SfNIC-F and -I, were more pathogenic and virulent than those with longer deletions, such as SfNIC-A, -E, and -H. Two mechanisms could explain the faster killing speed of certain genotypes: quicker replication, favoring shorter genomes (11), or deletion of genes involved in host regulation (34). The replication rate hypothesis is not supported by our observations: SfNIC-A, -E, and -H, with large deletions in their genomes, had longer mean times to death than SfNIC-I and -F, with small deletions (~60-h difference in mean time to death). Variation in the LC_{50} may be related to the presence of *enh* genes. Deletion of those genes reduces the ability to establish infections per os (37). Further characterization of this genomic region, including transient replication assays, may help determine if these differences in biological activity are due to variability in certain viral genes.

Analysis of the SfNIC-C, -D, and -G deletion mutants isolated from the SfNIC isolate revealed their inability to infect *S. frugiperda* larvae per os while retaining the ability to develop a lethal infection following injection of virus particles. The deletion of SfNIC-C and -D occurred between 14.8 and 26.7 map units, whereas SfNIC-G had a deletion between 14.8 and 23.1 map units Three different genes are known to affect the per os infectivity of OBs, *p74* (10, 23), *pif* (22), and *pif-2* (36). Variants SfNIC-C and -D do not contain either *pif* or *pif-2* in their genome, but *p74* is present. Cotransfection of their DNAs with plasmid p264.306, which covered the EcoRI-N fragment, a part of the deletion that included both *pif* and *pif-2*, generated fully functional viruses. Plasmid p264.306 completely covered the SfMNPV gene homologous to *Spodoptera littoralis* NPV (SpliNPV) *pif* (22) and to SeMNPV *pif-2* (36). Hybridization of SfNIC-G DNA revealed the presence of both *pif* and *p74*, and sequencing revealed the presence of *pif-2.* Cotransfection of SfNIC-G DNA with p264.306 in conditions similar to those of SfNIC-C and -D did not allow rescue. From these results we hypothesize that the gene responsible is not fully contained in this fragment and that another gene(s) may be implicated in this phenotype. This appears to exclude *pif* and *pif-2* as causative agents.

RT-PCR analysis revealed that *pif*- and *pif-2*-deleted viruses were not able to initiate virus transcription in midgut cells, probably as a consequence of the absence of *pif* or *pif-2* or both. PIF and PIF-2 are likely to be involved in the fusion between the virus envelope and the midgut cell microvilli (22). Consequently, deletion of these genes would block occlusion-derived virion fusion to midgut cell microvilli. RT-PCR analysis of SfNIC-G resulted in the detection of transcripts from the immediate-early gene *ie-0*, suggesting that the blocking point for replication was not at the entry level. Those results indicate that one of the genes necessary for viral replication or

cell-to-cell transmission of budded virus was not functional in the SfNIC-G variant. This variant may represent a useful model for the study of the putative new gene(s). Experiments are in process to determine which gene(s) is responsible for the noninfectivity of the SfNIC-G variant.

Component genotypes of the SfNIC isolate showed a clear diversity in the *pif* gene, not all the virus genomes contained this gene. This gene is very weakly expressed in SpliNPV (17). The low degree of *pif* expression and the presence of genotypes lacking this gene indicate that *pif* may be regulated within certain limits at the transcription level and at the virus population level (17). Further studies will determine the mechanisms of this regulation.

Defective variants can replicate faster and can thereby invade a population of larger complete genotypes (11, 41). The multiple enveloping strategy has been suggested as a mechanism to increase the probability of infection by overcoming the sloughing of midgut cells, that can occur faster than the replication of the virus (45). However, multiple enveloping allows defective genomes to be cotransmitted from one host to the next. A previous study suggested that some genotypes in NPV populations can act as parasites (32), but more recently, we demonstrated that at least on some occasions, noninfectious defective variants can have a positive impact on the virus population (25).

These findings should be taken into account when developing baculovirus based insecticides. For some reason, certain genotypes are dominant in NPV populations, but minority genotypes also persist in the population. The fact that minority genotypes are not eliminated indicates that this heterogeneity is important for virus survival and suggests that field isolates containing heterogeneous populations may be more valid models for studies of baculovirus host range and pathogenicity than cloned isolates (38). Pure genotypes employed in field tests of genetically modified baculoviruses (4), or virus preparations with little genetic diversity resulting from in vitro production (26), are therefore predicted to be less effective biopesticides than genotypically heterogeneous mixtures.

In summary, the results of this study represent further evidence of the genomic diversity present in natural populations of SfMNPV. Nine different genotypes were isolated from SfNIC. Phenotypic characterization of those variants revealed a high prevalence of per os defective variants in the isolate. Why such a wide array of genotypic variants with such distinct phenotypes is present in natural NPV populations remains an open question. Our demonstration that the wild-type isolate was more infectious than any of the pure genotype components indicates that diversity and interactions among genotypes represent an important but very poorly understood aspect of baculovirus biology.

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