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Identification of a Novel *Lymantria dispar* Nucleopolyhedrovirus Mutant That Exhibits Abnormal Polyhedron Formation and Virion Occlusion

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In previous studies on the formation of *Lymantria dispar* nuclear polyhedrosis virus (Ld*M*NPV) few polyhedra (FP) mutants, several polyhedron formation mutants (PFM) were identified that appeared to be unique. These viral mutants are being characterized to investigate the processes of polyhedron formation and virion occlusion. Ld*M*NPV isolate PFM-1 is one of these mutants, and is described in this report. Genetic techniques were used to determine if isolate PFM-1 contained a mutation in the *polyhedrin* or *25K FP* gene. Wild-type viruses were recovered after coinfection of Ld652Y cells with isolate PFM-1 and a FP mutant, and with isolates PFM-1 and PFM-C (isolate PFM-C contains a mutation in the *polyhedrin* gene). These viruses were analyzed by genomic restriction endonuclease digestion and found to be chimeras of the original PFMs used in the coinfections. Marker rescue studies mapped the mutation in isolate PFM-1 to a genomic region that does not include the *polyhedrin* or *25K FP* genes. Isolate PFM-1 produced approximately 14-fold fewer polyhedra than Ld*M*NPV isolate A21-MPV, an isolate that produces wild-type levels of polyhedra, and approximately 2-fold more polyhedra compared to the FP isolate 122-2. Polyhedra generated by isolate PFM-1 were normal in size and shape but contained very few viral nucleocapsids. The same amount of budded virus (BV) was released from cells infected with isolates PFM-1 and A21-MPV. In contrast, isolate 122-2 yielded significantly more BV than isolates PFM-1 and A21-MPV.

Key Words: **baculovirus; *Lymantria dispar M*NPV; polyhedron; polyhedron mutant; viral occlusion; *Lymantria dispar*.**

INTRODUCTION

Nucleopolyhedroviruses (NPV, family *Baculoviridae*) are infectious to insects and arthropods, and their genomes are composed of double-stranded, circular DNA ranging in size from approximately 80 to 165 kbp (Tinsley and Harrap, 1978; Harrap and Payne, 1979). NPVs produce two morphological forms, a budded virus (BV) form and an occluded form. BV, the first viral form to be generated after infection of a cell, gives rise to a systemic infection in the insect host. The occluded viral form is embedded into a paracrystalline protein matrix termed a polyhedron. Insects are infected by NPVs after ingestion of the polyhedron and release of the occluded virions through dissolution of the polyhedron in the alkaline environment of the insect midgut (reviewed by Blissard and Rohrmann, 1990).

Virion occlusion and polyhedron formation are likely to be complex processes involving a number of viral genes. Any viral gene that is necessary for basic viral processes such as viral replication and transcription is also necessary for polyhedron formation and virion occlusion. Other viral genes such as *polyhedrin* and possibly *25K FP* code for proteins that are specific for the processes of polyhedron formation and virion occlusion. In addition to these proteins other virally encoded proteins may exist that are also specific for these processes. Four *Autographa californica* NPV (Ac*M*NPV) polyhedron formation mutants (PFMs) that contain point mutations or insertions in the *polyhedrin* gene have been described. Cells infected with the Ac*M*NPV m-29 mutant generate small particles composed of the protein polyhedrin, but lacking a paracrystalline matrix (Duncan and Faulkner, 1982; Duncan *et al.*, 1983; Carstens *et al.*, 1987). The m-5 mutant produces very few polyhedra in infected cells and these are abnormally large and cuboidal in shape. In addition, the polyhedra are essentially devoid of viral nucleocapsids (Brown *et al.*, 1980; Carstens, 1982; Carstens *et al.*, 1986). Cells infected with the M276 mutant generate small amorphous condensations of polyhedrin in both the nucleus and the cytoplasm (Carstens *et al.*, 1992). M934-infected cells generate large amorphous masses of polyhedrin that lack a normal polyhedron envelope (Carstens *et al.*, 1992).

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Neither the M276 nor the M934 mutant generate polyhedra that contain viral nucleocapsids.

During serial passage of NPVs in cell culture, few polyhedra (FP) mutants arise at a high frequency. FP mutants have the characteristics of an altered plaque morphology, production of few polyhedra, occlusion of very few viral nucleocapsids, increased release of BV, and, in Ld*M*NPV, decreased percentage of cells that produce polyhedra (MacKinnon *et al.*, 1974; Hink and Strauss, 1976; Potter *et al.*, 1976; Fraser and Hink, 1982; Slavicek *et al.*, 1992, 1995). As a consequence of an increase in BV release, FP mutants become the predominant virus type after a few serial passages in cell culture. In Ac*M*NPV, DNA insertions and deletions in the *25K FP* gene are often the basis for the FP mutant phenotype (Fraser *et al.*, 1983; reviewed by Fraser, 1987). The Ld*M*NPV *25K FP* gene has been characterized and exhibits a 52% amino acid identity with the Ac*M*NPV *25K FP* gene (Bischoff and Slavicek, 1996). In contrast to Ac*M*NPV FP mutants characterized to date, most Ld*M*NPV FP mutants do not contain large DNA insertions or deletions. Of five Ld*M*NPV FP mutants analyzed, two were found to contain single base pair insertions, two had small deletions, and one had a deletion of 1.3 kbp (Bischoff and Slavicek, 1997). The *25K FP* gene is essential for correct polyhedron formation and virion occlusion, and its deletion is sufficient to generate the FP mutant phenotype (Beames and Summers, 1988, 1989).

The polyhedral envelope protein (PEP) and p10 are other proteins that either are involved in polyhedron formation or are part of the polyhedron. PEP is a major component of the polyhedron envelope, and the gene encoding this protein is present in Ld*M*NPV, Ac*M*NPV, and *Orgyia pseudotsugata* NPV (Op*M*NPV) (Whitt and Manning, 1988; Gombart *et al.*, 1989; Russell and Rohrmann, 1990; Bjornson and Rohrmann, 1992). Deletion of the *PEP* gene from the Ac*M*NPV and Op*M*NPV genomes produced viruses that were capable of generating polyhedra and of occluding viral nucleocapsids (Zuidema *et al.*, 1989; Gross *et al.*, 1994). p10 is also found in the polyhedron, and is necessary for normal polyhedron envelope formation (Vlak *et al.*, 1988; Williams *et al.*, 1989). However, deletion of the p10 gene from the Ac*M*NPV and Op*M*NPV genomes did not prevent wild-type levels of polyhedron formation or virion occlusion (reviewed by Rohrmann, 1992; van Oers *et al.*, 1993).

In this report we describe the isolation and phenotypic characterization of a Ld*M*NPV PFM, isolate PFM-1, which exhibits a novel phenotype. Genetic analysis and marker rescue of isolate PFM-1 suggest that the mutation in this isolate is not in the *polyhedrin* or *25K FP* genes.

MATERIALS AND METHODS

Cell culture and viral isolates. The *L. dispar* 652Y (Ld652Y) cell line (Goodwin *et al.*, 1978) was used for *in vitro* studies, and was propagated as previously described (Slavicek *et al.*, 1992). Ld*M*NPV isolates PFM-1, PFM-C, A21-2, B21-1, 122-2, 163-2, 5-6, A21-MPV, PFM-1 × 122-2-G5, and PFM-1 × PFM-C-C4 were used in this study. Isolates A21-2, B21-1, 122-2, 163-2, and 5-6 are plaque-purified FP mutants (Slavicek *et al.*, 1992, 1995; Bischoff and Slavicek, 1997). The mutations in the *25K FP* gene in these FP isolates have been characterized (Bischoff and Slavicek, 1997). Ld*M*NPV isolate 122-2 produces a 25K FP protein with an 8-amino-acid in-frame deletion (amino acids 106 to 113), which is in the center of the 217-amino-acid protein (Bischoff and Slavicek, 1997). Isolate PFM-C is a PFM that produces large polyhedra that are cuboidal in shape. This isolate has a mutation in the *polyhedrin* gene region (data not shown). Isolate A21-MPV exhibits wild-type polyhedron production characteristics and enhanced polyhedron production stability during serial passage in Ld652Y cells (Slavicek *et al.*, 1996a). Isolate PFM-1 was identified during a serial passage study of isolate 163 (Slavicek *et al.*, 1995). Isolates PFM-1 × 122-2-G5 and PFM-1 × PFM-C-C4 were generated as described below.

Viral DNA isolation, restriction site mapping, coinfections, and marker rescue. BV from plaque-purified Ld*M*NPV lines was isolated from infected Ld652Y cells, and viral genomic DNA was isolated as previously described (Bischoff and Slavicek, 1994). Viral genomic DNA was used for restriction site mapping and marker rescue experiments. *Bgl*II restriction maps of isolates PFM-1 and 122-2 were generated by comparison of viral genomic digests with a *Bgl*II digestion profile of isolate 5-6 (Riegel *et al.*, 1994). The locations of heterologous fragments were mapped by Southern analysis using DNA probes generated from selected genomic regions of isolate 5-6. DNA probes were labeled using the Bionick labeling system (Gibco BRL), and DNA fragments were visualized with the PhotoGene nucleic acid detection system (Gibco BRL).

For coinfection studies, Ld652Y cells were coinfected with all combinations of Ld*M*NPV isolates PFM-1, PFM-C, and the FP mutants as previously described (Bischoff and Slavicek, 1997). Cosmid clones from Ld*M*NPV isolate A21-MPV were cotransfected with PFM-1 genomic DNA into Ld652Y cells for marker rescue studies as previously described (Bischoff and Slavicek, 1996). Transfections were performed with 2.5 μg of isolate PFM-1 DNA and 2.5 μg of each cosmid using the Lipofectin reagent (Gibco BRL). Isolates PFM-1 × 122-2-G5 and PFM-1 × PFM-C-C4 were isolated from Ld652Y cells coinfected with isolates PFM-1 and 122-2, and PFM-1 and PFM-C, respectively.

Characterization of polyhedra, polyhedron synthesis, and budded virus release. Polyhedron production characteristics were analyzed through *in vitro* infections. Viral isolates A21-MPV, 122-2, and PFM-1 were used to infect Ld652Y cells, in triplicate, at 10 50% tissue culture infectious dose ($TCID_{50}$) units per cell in T25 flasks seeded with 1×10^6 cells. The cells from each flask were harvested at 7 days postinfection (p.i.) and pelleted, the supernatant containing the BV was removed, and the polyhedra pellet was resuspended in 5 ml of distilled water and sonicated for 30 s. A hemacytometer was used to count the number of polyhedra present. Polyhedra were then examined by transmission electron microscopy (TEM) as described below. Polyhedron diameter was determined through measurement of photographed polyhedra and application of a mm-to-μm conversion factor derived from the measurement of photographed microspheres of known size (2.02 ± 0.05 μm microspheres, Coulter Electronics, Inc., Hialeah, FL). The supernatant medium containing BV was removed and the budded virus $TCID_{50}$ determined by the end-point dilution assay as previously described (Slavicek *et al.,* 1992). Statistical analysis of data was performed using the StatView program from Abacus Concepts (Berkeley, CA).

Analysis of virion occlusion and intranuclear nucleocapsid envelopment. Polyhedra produced by Ld*M*NPV isolates A21-MPV, 122-2, PFM-1, PFM-1 × 122-2-G5, and PFM-1 × PFM-C-C4 were examined by TEM for the presence of viral nucleocapsids. Polyhedra were prepared and sectioned for electron microscopic analysis as previously described (Slavicek *et al.,* 1992). Polyhedra cross sections were photographed, and the number of virions present was quantified by counting and expressed as the number of viral nucleocapsids present per square micrometer of cross-section area. Intranuclear nucleocapsid envelopment was examined by electron microscopic analysis of infected cells. Ld652Y cells were infected with 10 $TCID_{50}$ units per cell of viral isolates A21-MPV or PFM-1. Five days p.i. the cells were fixed *in situ* by replacing the medium with sodium cacodylate buffer (SCB, 0.05 M sodium cacodylate, 0.5 mM HCl, pH 7.0) containing 3% glutaraldehyde and incubating the cells at 4°C for 1 h. The cells were then washed 3 times in SCB, and incubated in SCB containing 2% osmium tetroxide for 2 h at 4°C. The cells were washed 3 times with SCB, scraped off the flasks, and then processed as previously described (Slavicek *et al.,* 1992).

RESULTS

Isolation of LdMNPV isolate PFM-1. In the course of previous studies on FP mutant formation (Slavicek *et al.,* 1995, 1996a) several PFMs were identified which appeared to be different from FP mutants. One of the PFMs was identified during a serial passage study of Ld*M*NPV isolate 163. This PFM appeared to produce far fewer polyhedra compared to wild-type Ld*M*NPV and yet more than a FP mutant. This isolate was plaque-purified and designated as PFM-1. The designation of PFM-1 was chosen since it is the first PFM other than mutants with an altered *polyhedrin* or *25K FP* gene that has been described to date.

Restriction endonuclease digestion patterns of isolates 163 and PFM-1 were compared in order to confirm that isolate PFM-1 was derived from isolate 163, and to identify genotypic differences between the isolates. The genomic DNA restriction profiles of isolates 163 and PFM-1 were identical after digestion with *Bam*HI, *Bgl*II, *Cla*I, *Eco*RI, *Eco*RV, *Hin*dIII, *Nde*I, or *Pst*I (data not shown). This result indicates that isolate PFM-1 is derived from isolate 163 and the mutation responsible for the altered phenotype of isolate PFM-1 is not a large DNA insertion or deletion.

Genetic analysis of polyhedron formation mutants. A genetic approach was used initially to place Ld*M*NPV PFMs into complementation groups. Most of the PFMs were placed into a single group, and these were found to contain mutations in the *25K FP* gene (Bischoff and Slavicek, 1997). Isolate PFM-C was included in the genetic analysis of isolate PFM-1. Ld*M*NPV isolate PFM-C produces large cuboidal polyhedra (hence the designation PFM-C, where C stands for cuboidal) lacking viral nucleocapsids. This isolate was found to contain a mutation in the *polyhedrin* gene region (4.0 to 6.5 map units) through marker rescue studies (unpublished data). Isolate m-5, an Ac*M*NPV mutant with a similar phenotype, has been previously described (Brown *et al.,* 1980). The mutation in the m-5 isolate was localized to the *polyhedrin* gene (Carstens, 1982; Carstens *et al.,* 1986).

Since the genetic analysis included viruses with mutations in the *polyhedrin* or the *25K FP* gene, it allows the identification of PFMs containing mutations in genes other than *polyhedrin* or *25K FP* that affect polyhedron formation. This approach allows the rapid screening of PFMs for mutations in previously unidentified genes. A wild-type polyhedron formation phenotype would be present in cells coinfected with PFMs that contain mutations in different genes, if the functional gene products complement *in trans.* In addition, if the mutation in isolate PFM-1 was not in the *polyhedrin* or *25K FP genes,* wild-type virus could be generated by recombination between the mutant viral strains used in the coinfections.

Ld652Y cells were coinfected with all combinations of isolates PFM-1, PFM-C, and the FP mutants. In all the coinfections, the cells were found to contain few polyhedra at 7 days p.i. (data not shown). BV from the coinfections was plaque purified and the plaques used to infect Ld652Y cells to determine if wild-type virus

was generated through recombination between the mutant viruses. Virus exhibiting a wild-type polyhedron synthesis phenotype was found after coinfection of isolate PFM-1 with the FP isolates and PFM-C (data not shown). Approximately 14 and 3% of the virus plaques analyzed from the PFM-1 and PFM-C, and PFM-1 and FP isolate 122-2 coinfections, respectively, exhibited a many polyhedra (MP) phenotype. Wild-type virus was not detected after coinfection of cells with combinations of the FP mutants (Bischoff and Slavicek, 1997) or in control infections with the Ld*M*NPV PFMs used alone.

Analysis of wild-type virus derived from LdMNPV PFM coinfections. The viral coinfections were performed with Ld*M*NPV genotypic variants, thus allowing for the identification of chimeric viruses generated through genomic recombination. Viruses exhibiting a MP phenotype are expected to be genomic chimeras consisting of genomic segments from the viruses used in the coinfections. The genomic DNA restriction profiles of two viruses exhibiting a MP phenotype isolated after coinfection of isolate PFM-1 with isolate PFM-C or FP isolate 122-2 were analyzed to determine if these viruses were chimeras. Genomic *Bgl*II restriction profiles of isolates PFM-1 × 122-2-G5 and PFM-1 × PFM-C-C4 contained DNA fragments that originated from each of the two viruses used in the coinfection (data not shown).

Polyhedra generated by Ld*M*NPV isolates PFM-1 × 122-2-G5 and PFM-1 × PFM-C-C4 were analyzed by electron microscopy to determine if the polyhedra contained viral nucleocapsids. The confirmation of a wild-type polyhedron phenotype is necessary since we have isolated a PFM that produces normal numbers of polyhedra but in which the polyhedra lack viral nucleocapsids (Slavicek *et al.*, 1996b). Polyhedra generated by

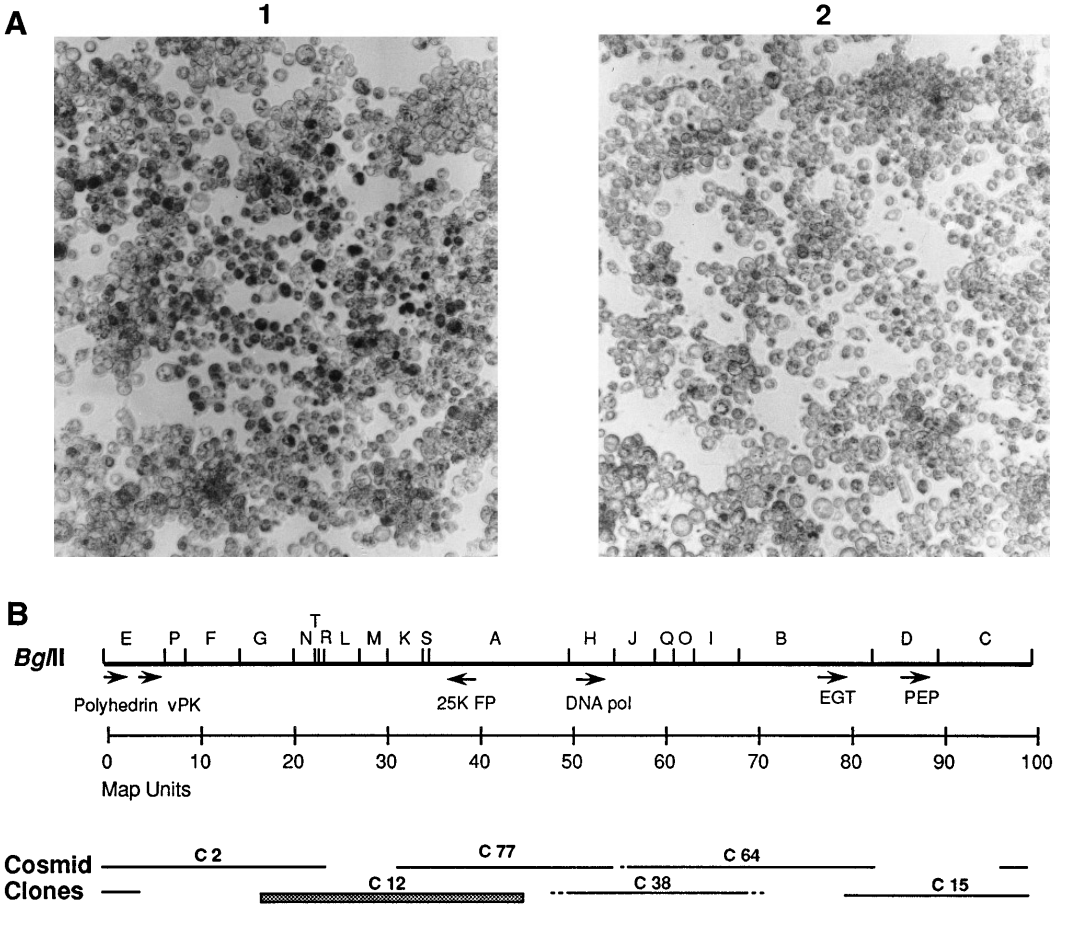

FIG. 1. Marker rescue of Ld*M*NPV isolate PFM-1. (A) Ld652Y cells cotransfected with PFM-1 viral DNA and cosmid C12 (1) or cosmid C15 (2). The cells were visualized with a Nikon Diaphot-TMD inverted microscope at 100× magnification. (B) The location of clones used to map the mutation in isolate PFM-1 are shown below the *Bgl*II restriction map of this isolate. Cosmid clones are depicted as lines, and the clone that rescued the mutant phenotype of PFM-1 is shown as a hatched bar. The locations of the Ld*M*NPV *polyhedrin* (Smith *et al.*, 1988), *vPK* (*viral protein kinase;* Bischoff and Slavicek, 1994), *25K FP* (Bischoff and Slavicek, 1996), *DNA pol* (*DNA polymerase;* Bjornson *et al.*, 1992), *PEP* (*polyhedral envelope protein;* Bjornson and Rohrmann, 1992), and *EGT* (*ecdysteriod UDP-glucosyltransferase;* Riegel and Slavicek, 1994) genes are shown.

isolates PFM-1 × 122-2-G5 and PFM-1 × PFM-C-C4 exhibited a normal morphology and contained viral nucleocapsids (data not shown). The amount of polyhedra produced by the MP recombinant isolates PFM-1 × 122-2-G5 and PFM-1 × PFM-C-C4 was determined and compared to polyhedra production by isolates A21-MPV, PFM-1, 122-2, and PFM-C. The recombinant MP viral isolates PFM-1 × 122-2-G5 and PFM-1 × PFM-C-C4 produced significantly more (ANOVA Fisher's PLSD, $P < 0.05$) polyhedra (2.3×10^8 and 1.6×10^8 polyhedra/flask, respectively) than isolates PFM-1, 122-2, and PFM-C (1.6×10^7, 7.9×10^6, and 3.5×10^6 polyhedra/flask, respectively). There was no significant difference in the amount of polyhedra produced by the recombinant MP viral lines and isolate A21-MPV (2.0×10^8 polyhedra/flask).

Marker rescue of isolate PFM-1. Marker rescue studies were performed to identify the genomic region containing the mutation(s) responsible for the abnormal polyhedron formation phenotype of isolate PFM-1. Transfection of Ld652Y cells with isolate PFM-1 and cosmid C12 resulted in the formation of focal areas of cells containing a wild-type number of polyhedra (Fig. 1A). Cells transfected with isolate PFM-1 and the other cosmids exhibited a typical PFM-1 infection (Fig. 1A). These results indicate that the mutated gene(s) responsible for the abnormal polyhedron formation phenotype of isolate PFM-1 is located within the region unique to cosmid C12. This region is from approximately 23.5 to 32.7 map units on the viral genome (Fig. 1B). By themselves, the marker rescue results do not eliminate the 25K FP gene since cosmid C12 contains this gene. Cosmid C77 also contains the 25K FP gene and failed to rescue the mutant phenotype of isolate PFM-1. However, this was a negative result. The genetic analysis described in the previous section corroborates the marker rescue results indicating that the mutation in isolate PFM-1 is not in the 25K FP gene. In addition, the marker rescue results indicate that the mutation in PFM-1 is not in either the *polyhedrin* or *PEP* genes (Fig. 1B).

Phenotypic characterization of LdMNPV isolate PFM-1. The attributes of polyhedron morphology, size, production, and virion occlusion exhibited by isolate

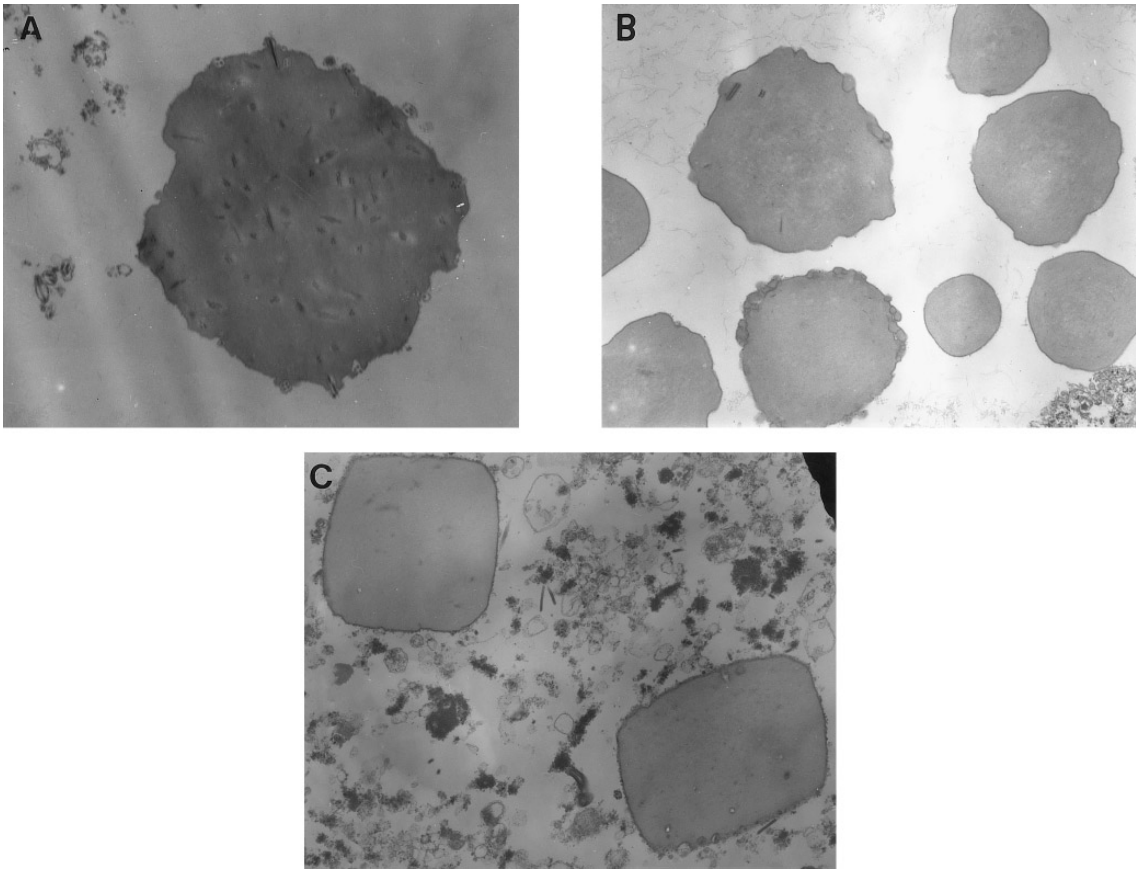

FIG. 2. Electron micrographs of polyhedra cross-sections of (A) A21-MPV, which produces wild-type polyhedra, (B) PFM-1, and (C) 122-2, a FP mutant. The bar represents 1 μm.

PFM-1 were characterized and compared to those traits exhibited by a Ld*M*NPV isolate that produces wild-type polyhedra (isolate A21-MPV) and a FP mutant (isolate 122-2). The morphology of isolate PFM-1 polyhedra was similar to that of A21-MPV and 122-2 polyhedra (Fig. 2). Similar to 122-2 and in contrast to A21-MPV, polyhedra produced by PFM-1 contained very few viral nucleocapsids. The diameter of PFM-1 polyhedra was significantly smaller (ANOVA, Fisher's PLSD, $P < 0.05$) than the diameters of A21-MPV and 122-2 polyhedra (Fig. 3A). Isolate PFM-1 produced approximately 14-fold fewer polyhedra than isolate A21-MPV (Fig. 3B). The difference in polyhedra production exhibited by these isolates was significant (ANOVA, Fisher's PLSD, $P < 0.05$). Isolate PFM-1 produced approximately 2-fold more polyhedra compared to isolate 122-2. This difference was not significant when analyzed by Fisher's PLSD test; however, it was significant when analyzed by the two-tail unpaired t test ($P < 0.0014$). The relative number of virions present within polyhedra generated by isolates PFM-1, A21-MPV, and 122-2 was determined through electron microscopic examination of sectioned polyhedra. The polyhedra were sectioned randomly with respect to the cutting plane, thereby generating representative cross sections from all areas of the polyhedra. Most cross sections of polyhedra generated by isolates PFM-1 and 122-2 were devoid of viral nucleocapsids. Polyhedra generated by isolates PFM-1 and 122-2 contained significantly fewer (ANOVA, Fisher's PLSD, $P < 0.05$) viral nucleocapsids than poly-

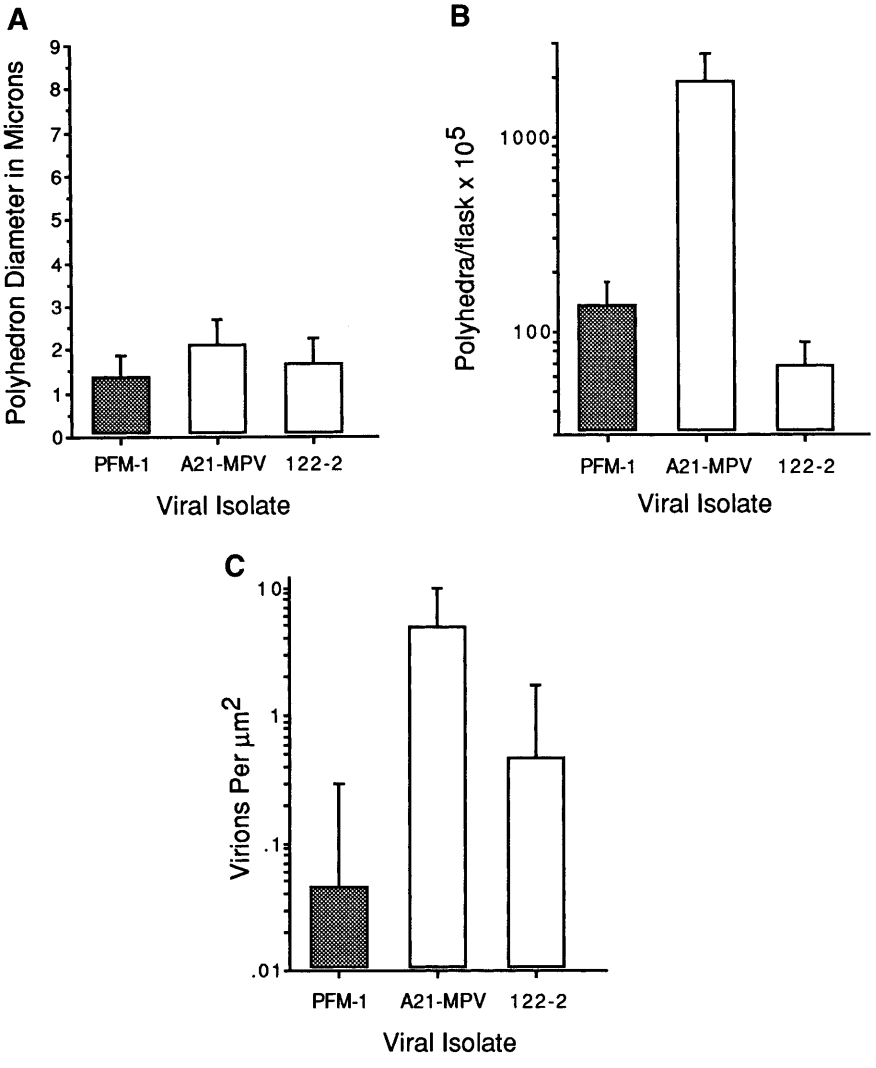

FIG. 3. Characterization of polyhedra and polyhedron generation by Ld*M*NPV isolates PFM-1, A21-MPV, and 122-2. (A) Diameter of polyhedra generated by the indicated Ld*M*NPV isolates. The values are the averages of 101 to 157 determinations. One standard deviation is shown. (B) Number of polyhedra produced per flask at 7 days p.i. The values are averages (±SD) of three determinations. (C) Virion occlusion within polyhedra generated by the indicated Ld*M*NPV isolates. The number of viral nucleocapsids present within cross-sections was quantitated for each isolate and expressed as the number of viral nucleocapsids per square micrometer of polyhedra cross-section surface area. The values are the averages (±SD) of 25 to 50 cross-sections.

hedra produced by isolate A21-MPV (Fig. 3C). Isolate PFM-1 polyhedra contained fewer viral nucleocapsids compared to 122-2 polyhedra; however, the difference was not significant (ANOVA, Fisher's PLSD and the two-tailed unpaired *t* test).

Analysis of BV formation. The amount of BV released by cells infected with Ld*M*NPV isolates PFM-1, A21-MPV, and 122-2 was determined by $TCID_{50}$ analysis. Cells infected with isolate PFM-1 released essentially the same amount of BV as isolate A21-MPV, and approximately fourfold less than cells infected with isolate 122-2 (Fig. 4). The difference in the amount of BV released by cells infected with isolates PFM-1 and A21-MPV compared to isolate 122-2 was significant (ANOVA, Fisher's PLSD, $P < 0.05$). There was no significant difference in the amount of BV released by cells infected with isolates PFM-1 and A21-MPV.

Analysis of virion encapsulation. Ld652Y cells infected with isolate PFM-1 were analyzed to determine if intranuclear nucleocapsid envelopment occurred. The majority of intranuclear viral nucleocapsids present in cells infected with isolate PFM-1 were not enveloped (Fig. 5A). In contrast, the majority of intranuclear nucleocapsids present in cells infected with isolate A21-MPV were enveloped (Fig. 5B). In addition, there were many more unoccluded viral nucleocapsids present in cells infected with isolate PFM-1 compared to cells infected with A21-MPV (Figs. 5A and 5B).

DISCUSSION

In previous studies on the formation of Ld*M*NPV FP mutants during serial passage (Slavicek *et al.*, 1995), we identified several PFMs that appeared to be different from FP mutants. We have chosen to study these putative unique PFMs in order to identify viral genes affecting polyhedron formation. This approach targets viral genes known to impact the process of polyhedron formation. Alternatively, viral genes could be systematically mutated to investigate gene function; however, this approach is more costly and time consuming compared to using viral mutants exhibiting alterations in the phenotype of interest. A genetic approach was used to place the PFMs into complementation groups and to identify PFMs containing mutations in genes other than *polyhedrin* and *25K FP*. This approach allows for the rapid and inexpensive screening of PFMs to identify those with mutations in novel genes that impact polyhedron formation.

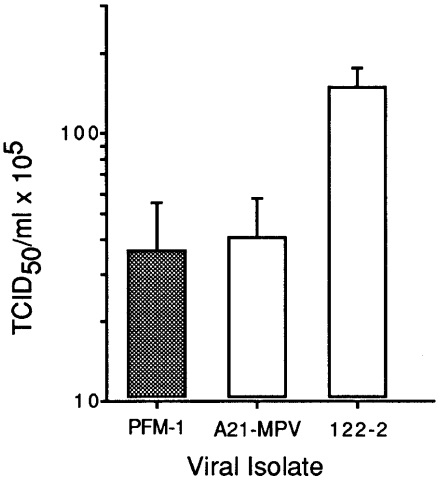

FIG. 4. BV release by cells infected with Ld*M*NPV isolates PFM-1, A21-MPV, and 122-2. The amount of BV present 7 days p.i. is expressed as the $TCID_{50}$/ml of cell culture medium. The values are the averages (±SD) of three determinations.

The genetic analysis included PFM-1, PFM-C, five FP mutants, and several other PFMs currently under investigation. Through this analysis the FP mutants were placed into a single complementation group (Bischoff and Slavicek, 1997). DNA sequence analysis of the FP mutants revealed mutations in the *25K FP* genes, and all of the mutants were rescued with a wild-type *25K FP* gene (Bischoff and Slavicek, 1997). The results of the genetic analysis of PFM-1 are described in this report.

A wild-type polyhedron formation phenotype would be present in cells coinfected with isolates PFM-1 and PFM-C, and with PFM-1 and the FP mutants, if the mutation in PFM-1 was not in the *polyhedrin* or *25K FP* genes, and if the functional gene products complement *in trans*. In addition, wild-type virus could be generated through recombination between the mutant viral strains if the mutation in isolate PFM-1 was not in the *polyhedrin* or *25K FP* genes. Wild-type virus was recovered after coinfection of Ld*M*NPV isolate PFM-1 with FP mutants (isolate PFM-1 × 122-2-G5) and isolate PFM-C (isolate PFM-1 × PFM-C-C4). These results indicate that the mutation in isolate PFM-1 was not located in the *polyhedrin* gene region or the *25K FP* gene. Genotypic analysis of the wild-type virus isolates PFM-1 × 122-2-G5 and PFM-1 × PFM-C-C4 revealed that they were chimeras of the two viral strains used in the coinfection.

Of interest was the observation that cells coinfected with isolate PFM-1 and PFM-C, and with PFM-1 and the FP mutants, did not produce wild-type amounts of polyhedra. This result could suggest that the abnormal proteins produced by the mutants interfered with the function of the wild-type proteins, and consequently prevented wild-type levels of polyhedron formation. Alternatively, the lack of wild-type polyhedron formation could be an experimental artifact (e.g., the majority of cells were not coinfected with both viral mutants, or one viral mutant was preferentially replicated within the cell).

Additional evidence for the hypothesis that the muta-

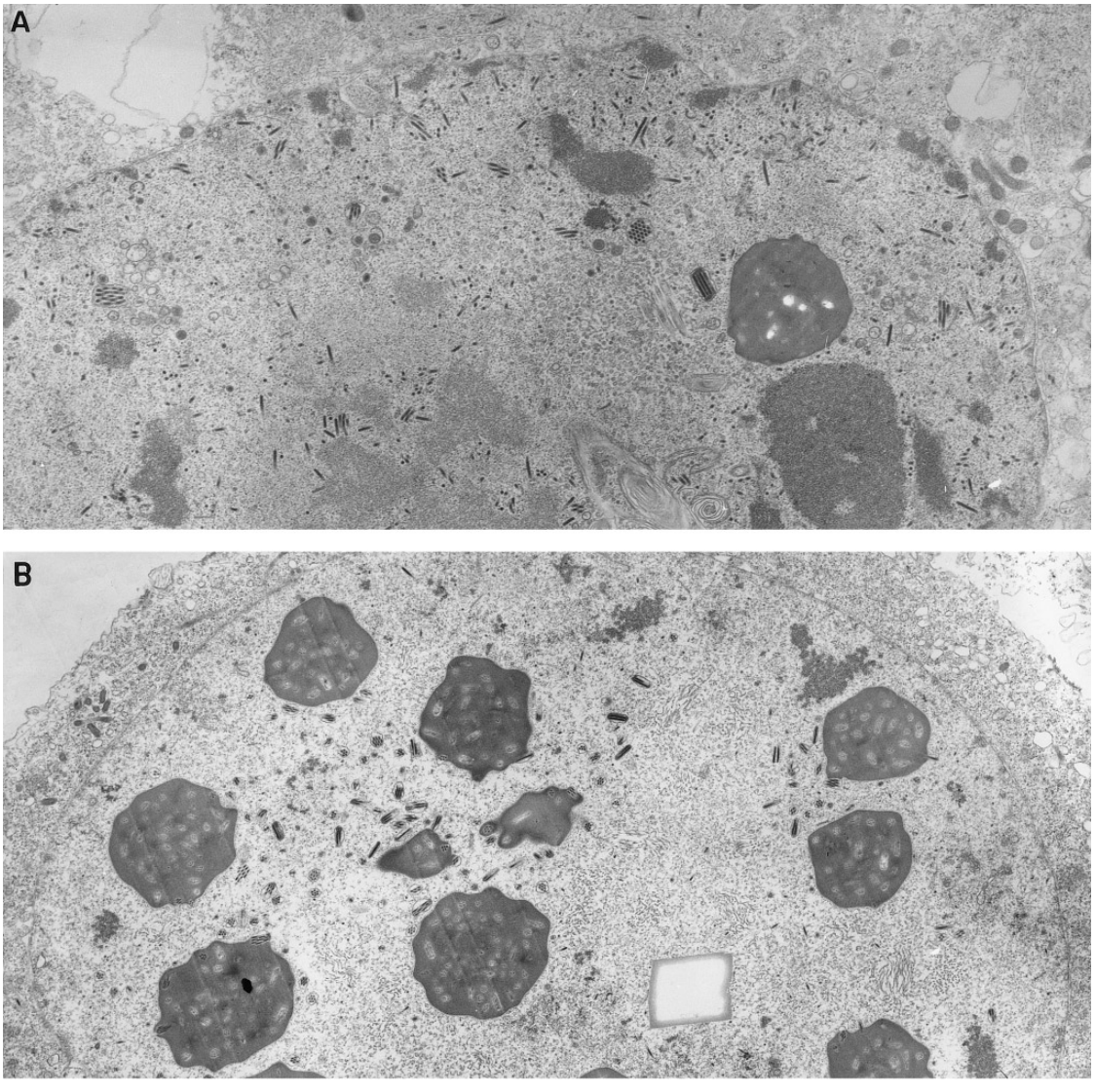

FIG. 5. Electron micrographs of cells infected with Ld*M*NPV isolates PFM-1 (A) and isolate A21-MPV (B) that were harvested 7 days p.i. The bar represents 2 μm.

tion in isolate PFM-1 was not present in the *polyhedrin* gene region or the *25K FP* gene was obtained through marker rescue experiments. The mutated gene(s) in isolate PFM-1 was localized to the genomic region from 23.5 to 32.7 map units. The *polyhedrin* and *25K FP* genes are located on the genome at approximately 4.0 and 40.0 map units, respectively. The marker rescue results would appear to rule out the involvement of these genes. However, cosmid C-12 contains a wild-type *25K FP* gene (Bischoff and Slavicek, 1996) and the failure of cosmid C77 (which contains the *25K FP* gene) to rescue PFM-1 is a negative result. Consequently, the marker rescue results in conjunction with the genetic analysis provides strong evidence that the mutation in isolate PFM-1 is not in the *25K FP* gene.

The marker rescue and genetic studies of isolate PFM-1 suggest that this isolate contains a mutation in a novel gene(s) that impacts polyhedron formation and virion occlusion. A characterization of the traits of polyhedron morphology and synthesis, and of BV release exhibited by isolate PFM-1, was performed to determine if these traits were different from those exhibited by FP mutants and PFM-C. Isolate PFM-1 produced significantly fewer polyhedra compared to a Ld*M*NPV isolate producing wild-type polyhedra, and those polyhedra that were produced were nearly devoid of viral nucleocapsids. The reduced polyhedron formation and virion occlusion traits exhibited by isolate PFM-1 are similar to those exhibited by Ld*M*NPV FP mutants (Slavicek *et al.,* 1995, 1996a; Bischoff and

Slavicek, 1997). However, isolate PFM-1 was found to produce twofold more polyhedra than a FP mutant. A significant distinction between PFM-1 and the FP mutant 122-2 was found after analysis of BV release. In contrast to FP mutants, cells infected with isolate PFM-1 did not exhibit an increase in BV release. The amount of budded virus released by cells infected with isolate PFM-1 was similar to the amount released by cells infected with wild-type virus. Taken together, these results indicate that the phenotype of isolate PFM-1 is different from that of FP mutants.

In addition to Ld*M*NPV isolate PFM-1, four Ac*M*NPV polyhedron formation mutants have been identified which are different from FP mutants. The Ac*M*NPV mutant m-29 produces large numbers of particles ranging in size from approximately 95 to 180 nm. These particles appear to be composed of polyhedrin but lack a paracrystalline matrix (Duncan *et al.*, 1983). Ac*M*NPV m-5 produces large cuboidal polyhedra, and the M276 and M934 mutants generate small and large amorphous condensations of polyhedrin, respectively (Duncan *et al.*, 1983; Carstens *et al.*, 1992). The phenotype of polyhedra generated by isolate PFM-1 is different from the size and morphology of the particles produced by the Ac*M*NPV m-5, m-29, M276, and M924 mutants. The results of genetic and marker rescue studies demonstrate that the mutation in isolate PFM-1 is not the same as the polyhedrin gene mutations in the Ac*M*NPV mutants.

Deletion of the Ac*M*NPV and Op*M*NPV *p10* and *PEP* genes results in the formation of polyhedra containing viral nucleocapsids, but lacking a complete polyhedron membrane and exhibiting reduced structural stability compared to wild-type polyhedra (Williams *et al.*, 1989; Zuidema *et al.*, 1989; van Oers *et al.*, 1993; Gross *et al.*, 1994). The mutation in Ld*M*NPV isolate PFM-1 mapped to a genomic region not containing the *PEP* gene, suggesting that a mutation in this gene is not the basis for the abnormal polyhedron formation phenotype exhibited by this isolate. The *p10* gene has yet to be identified in Ld*M*NPV. However, the differences in the morphology of polyhedra produced by isolate PFM-1 compared to Ac*M*NPV and Op*M*NPV constructs lacking the *p10* gene could suggest that the polyhedron formation phenotype of Ld*M*NPV isolate PFM-1 is not caused by a mutation in the *p10* gene.

Ld652Y cells infected with isolate PFM-1 were found to contain numerous viral nucleocapsids, and most lacked envelopes. The presence of viral nucleocapsids in the nucleus late in the infection cycle could suggest that these viral particles are deficient in a factor necessary for virion occlusion. It is tempting to speculate that the lack of viral envelopment is the basis for the lack of virion occlusion. However, some enveloped viral particles are present in cell nuclei late in infection, and yet these are not being occluded. Consequently, the presence of a viral envelope is not sufficient for virion occlusion in isolate PFM-1.

The identification of Ld*M*NPV isolate PFM-1 provides evidence for the existence of genes other than *polyhedrin* and *25K FP* that directly impact virion occlusion and polyhedron formation. Additionally, isolate PFM-1 demonstrates that an absence of virion occlusion is not concomitant with an increase in BV release. Efforts are underway to identify the mutated gene(s) that is responsible for the abnormal polyhedron formation phenotype of Ld*M*NPV isolate PFM-1, and to characterize the mutation through sequence analysis.

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