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Isolation of a Baculovirus Variant That Exhibits Enhanced Polyhedra Production Stability during Serial Passage in Cell Culture

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The formation of few polyhedra mutants during serial propagation of baculoviruses in cell culture encumbers commercial scale production in this system. A *Lymantria dispar* nuclear polyhedrosis virus (Ld*M*-NPV) variant (isolate A21-MPV) has been isolated and the traits of budded virus (BV) production, synthesis of polyhedra, the percentage of cells that produce polyhedra, and the formation of few polyhedra (FP) mutants were investigated during serial passage of the variant in *L. dispar* 652Y cells. Isolate A21-MPV exhibited stable levels of BV production and polyhedra production and a small increase in the percentage of cells that produced polyhedra during five serial passages in cell culture. In contrast, isolate A21, a wild-type isolate, exhibited a significant increase in BV production and a decrease in polyhedra production and in the number of cells that produced polyhedra during five serial passages. The traits exhibited by isolate A21 are typically observed when FP mutants are formed and become predominate in a virus population. After five serial passages, greater than 95% of isolate A21 virus exhibited a FP phenotype. In contrast, less than 8% of isolate A21-MPV exhibited a FP phenotype after five serial passages. These results indicate that isolate A21-MPV exhibits enhanced polyhedra production stability during serial passage in Ld652Y cells. Consequently, this isolate could be useful for large-scale production in cell culture systems.

KEY WORDS: ***Lymantria dispar; L. dispar* 652Y cell line; *L. dispar M* nuclear polyhedrosis virus; few polyhedra mutant; serial passage; biological control.**

INTRODUCTION

Insect baculoviruses are one group of insect biological control agents that have received considerable attention. These viruses have been isolated from hundreds of insect species, exhibit relatively narrow host ranges, can be aerially applied to agricultural crops and forests, and can be manipulated through genetic engineering to enhance viral efficacy (Tinsley and Harrap, 1978; Maramorosch and Sherman, 1985; Granados and Federici, 1986; Bishop *et al.,* 1992; Bonning and Hammock, 1992; Winstanley and Rovesti, 1993; and Miller, 1995 for reviews). The *Lymantria dispar* nuclear polyhedrosis virus (Ld*M*NPV) product Gypchek is used as a biocontrol agent for the gypsy moth, *L. dispar* (Reardon and Podgwaite, 1994). The gypsy moth is a serious tree defoliating insect pest in the Northeastern United States and is spreading into the South and Midwest (Blackburn *et al.,* 1994; Twardus, 1994).

The Ld*M*NPV has the significant advantage of specificity for the gypsy moth in contrast to other control agents (Lewis and Podgwaite, 1981). Consequently, Ld*M*NPV is the agent of choice for use in environmentally sensitive areas. However, Ld*M*NPV is not used extensively for gypsy moth control because of high production costs and low potency (Podgwaite, 1981). Production of virus in cell culture systems is an alternative to *in vivo* production. *In vitro* production has several advantages including a controllable system, product purity, and lower costs. However, one particular problem with baculovirus production in cell culture is the propensity of the virus to mutate into a form that produces few polyhedra (MacKinnon *et al.,* 1974; Potter *et al.,* 1976; Tramper and Vlak, 1986).

Nuclear polyhedrosis viruses have two distinct morphological forms (Blissard and Rohrmann, 1990). The infection cycle begins with ingestion of a polyhedron, a polyhedral-shaped crystalline protein matrix that contains virus particles. The polyhedron is dissolved within the insect midgut, thereby releasing the occluded virus particles that then infect the insect. Early after infection a budded form of virus is produced from an infected cell and gives rise to a systemic infection within the insect larval host. Late in infection virions are produced that become occluded into polyhedra, which is the form of the virus used for insect control.

Baculovirus few polyhedra (FP) mutants arise at a high frequency during serial passage in cell culture. In

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contrast to wild type or many polyhedra (MP) virus, FP mutants produce few polyhedra and these are essentially noninfectious. The lack of polyhedra potency is probably due to the occlusion of very few virus particles into polyhedra (Hink and Strauss, 1976; Potter *et al.,* 1976; Fraser and Hink, 1982; Slavicek *et al.,* 1995). FP mutants also synthesize greater amounts of budded virus (BV) than wild-type virus. Increased production of BV results in the conversion of the virus population from one exhibiting a MP phenotype to one with FP phenotype during serial passage in cell culture. FP mutants have been identified in the *Autographa californica M*NPV (Ac*M*NPV; Hink and Strauss, 1976), *Trichoplusia ni M*NPV (Tn*M*NPV; Mackinnon *et al.,* 1974; Potter *et al.,* 1976), *Galleria mellonella M*NPV (Gm*M*NPV; Fraser and Hink, 1982), and the Ld*M*NPV (Slavicek *et al.,* 1992, 1995).

The formation of FP baculovirus mutants during propagation in cell culture is a significant impediment to development of cell culture virus production systems that would facilitate availability of these viruses for biocontrol purposes. During previous studies on Ld*M*-NPV we noted a putative FP mutant revertant (isolate A21-MPV) that exhibited a MP phenotype. Isolate A21-MPV was purified and the stability of its MP phenotype was investigated through serial passage in Ld652Y cells. Our findings suggest that isolate A21-MPV exhibits enhanced stability of the MP phenotype during serial passage.

MATERIAL AND METHODS

Insects, cell lines, and virus isolates. *L. dispar* neonate larvae were obtained from the U.S. Department of Agriculture, Animal and Plant Health Inspection Service rearing facility at Otis Air Force base (MA) and were reared to the fourth instar on gypsy moth diet (Bell *et al.,* 1981). The *L. dispar* 652Y (Ld652Y) ovarian cell line was used for serial passage studies (Goodwin *et al.,* 1978). The cells were propagated in complete medium (Goodwin's IPL-52B medium, JRH Biosciences, Lexena, KS; with 10% heat-inactivated fetal bovine serum, Hyclone, Logan, UT; and 6.25 mM glutamine, Gibco, BRL). Ld*M*NPV isolate A21 was used in this study as a wild-type control (Slavicek *et al.,* 1992). Isolate A21-MPV was plaque purified from isolate A21, and viral isolates B21, 122, and 163 used in this study were generated as previously described (Slavicek *et al.,* 1995).

Serial passage of isolates A21 and A21-MPV and determination of the percentage of cells containing polyhedra and the number of polyhedra produced per cell. Fourth instar gypsy moth larvae were infected *per os* with isolates A21 and A21-MPV by placing them on a diet containing surface applied polyhedra (2.5×10^5 per square millimeter of diet surface). Larvae were bled on Day 6 postinfection (pi), and the hemolymph was spun in an Eppendorf table top centrifuge at 14,000 rpm for 10 min to remove large debris. Hemolymph was collected from virus-infected larvae, diluted with complete cell culture medium, and passed through a series of syringe filters (5, 1.2, and 0.45 μm) for further purification. The medium and hemolymph mixtures were used as inoculum at 0.2 50% tissue culture infectious dose ($TCID_{50}$) units per cell for three separate infections of Ld652Y cells plated at a density of 2×10^5 cells/ml. The cells were allowed to adsorb virus for 1 hr at 27°C. At the end of the incubation period, the inoculum was removed, the cells were rinsed four times with complete medium, and 5 ml of the complete medium containing 50 μg/ml gentamycin was applied after the final rinse. This infection constituted the first passage of virus in cell culture.

Passage 1 material was harvested on Day 7 pi. The cells from each flask were evenly dispersed and at least three aliquots were counted using a hemacytometer to determine the number of cells per milliliter and the number of cells with polyhedra per milliliter. These numbers were then used in calculating the percentage of cells with polyhedra. Polyhedra were pelleted by centrifugation at 550*g* for 5 min. The supernatant containing the BV was removed and used to determine BV $TCID_{50}$, for plaque assays to determine the percentage of virus that exhibited a MP or FP phenotype and for inoculum for passage 2. The polyhedra pellet was resuspended in complete medium and sonicated for 30 s and the number of polyhedra present was determined using a hemacytometer. This number and the number of cells that contained polyhedra were used to determine the average number of polyhedra per cell. Polyhedra were purified by centrifugation at 113,000*g* for 2 h at 15°C on sucrose step gradients (58 and 52% wt/wt). Serial passages 2 through 5 were performed as described above using BV from the previous passage as inoculum. Statistical analysis of data was performed using the StatView program from Abacus Concepts (Berkeley, CA).

$TCID_{50}$ determination, plaque assay, and DNA analysis. Viral titers from cell culture medium were determined by the end-point dilution assay first described by Reed and Muench (1938) and adapted by Summers and Smith (1987). Ld652Y cells (1×10^4 per well) were seeded in P96 plates and allowed to attach for 1 h. The cells were infected with virus at 10^{-1} to 10^{-11} dilutions and the plates were incubated at 27°C. The plates were scored 2 weeks after infection, and the viral titer was expressed as the $TCID_{50}$ per milliliter of cell culture medium.

Viral isolates were plaque purified after each serial passage as previously described (Slavicek *et al.,* 1995). After a 2-week incubation period at 27°C, the plaques that were well separated were picked and transferred to P96-well plates seeded with Ld652Y cells. After 1

week at 27°C the infections were scored as exhibiting either a FP phenotype if the cells contained from approximately 1 to 10 polyhedra or a MP phenotype if the majority of cells appeared opaque due to the presence of many polyhedra per cell.

In order to determine if FP mutant virus was present in the viral stocks used to initiate the passage studies, virus from passage 0 was plaque purified using hemolymph containing BV from infected larvae. At 7 days pi, 100 μl of hemolymph was added to 9.90 ml of complete medium and passed through the filter series as previously described. Additional dilutions were prepared and the virus was plaque purified as described above. After a 2-week incubation period, well-separated plaques were isolated and treated as described above.

Viral isolate A21-2 (a plaque-purified A21 isolate) and A21-MPV genomic DNAs were analyzed for restriction fragment length polymorphisms. BV was pelleted from medium harvested from infected cells by centrifugation at 112,700*g* for 45 min at 4°C, and the pellet was resuspended in 0.1× TE (0.01 *M* Tris, pH 8.0, 1 m*M* EDTA, pH 8.0) overnight at 4°C. Viral DNA was isolated as previously described (Slavicek *et al.,* 1995). Viral DNA was digested with the restriction enzymes *Bgl*II, *Eco*RI, *Eco*RV, *Hin*dIII, *Bam*HI, and *Pst*I and the fragments were separated by agarose gel electrophoresis and visualized after staining with ethidium bromide.

Transmission electron microscopy. Polyhedra produced by the isolates at Passages 1 through 5 were examined for the presence of viral nucleocapsids by transmission electron microscopy. The polyhedra were prepared and sectioned for electron microscopic analysis as previously described (Slavicek *et al.,* 1992). The number of virions present in polyhedra cross sections was quantified by counting and expressed as the number of viral nucleocapsids present per square micrometer of cross-section area per polyhedra cross section.

Passage of plaque-purified isolates. Plaque-purified viral lines of Ld*M*NPV genotypic variant isolates A21, B21, 122, and 163 (Slavicek *et al.,* 1995) were generated by standard plaque assay procedures. The passage study was initiated by infecting Ld652Y cells, plated at a density of 2×10^5/ml, with 0.1 $TCID_{50}$ unit per cell. The $TCID_{50}$ and the number of polyhedra produced per flask were determined at 7 days pi as described above. BV was plaque purified as described above to determine the percentage of virus exhibiting a MP or FP phenotype. BV from the first passage was used as inoculum to generate passage 2 as described above. Passages 3 through 14 were performed as described for passage 2. Once the isolates had become predominately FP, as indicated by a reduction in the number of polyhedra produced per flask, serial passage was ended.

RESULTS

Identification and isolation of LdMNPV isolate A21-MPV. During an infection with BV obtained from a transfection experiment using Ld652Y cells with a FP mutant line of Ld*M*NPV isolate A21, a few cells were noted that contained a large number of polyhedra. BV from this infection was passaged a few times in Ld652Y cells and the resulting BV was plaque purified. Viral plaques exhibiting a MP phenotype were isolated, and one of these isolates was re-plaque purified to generate isolate A21-MPV (many polyhedra variant). Isolate A21-MPV was serially passaged approximately 12 times in the Ld652Y cell line, during which the MP phenotype was maintained (data not shown). In contrast, we have previously shown that Ld*M*NPV FP mutants readily arise and become predominate after only three to four serial passages in the Ld652Y cell line (Slavicek *et al.,* 1995). In this study the stability of the MP phenotype of isolate A21-MPV during serial passage was investigated and compared to that of isolate A21 and several plaque-purified Ld*M*NPV lines.

Serial passage of isolates A21-MPV and A21. The stability of the MP phenotype of isolate A21-MPV during serial passage was investigated through analysis of BV production, the percentage of cells containing polyhedra, the number of polyhedra per cell, and the proportion of virus exhibiting the FP phenotype. Typically, viral stocks generated from plaque-purified material would be used to initiate studies. However, after generation of Ld*M*NPV viral stocks from plaques, FP mutants were evident. Since a homogeneous MP viral stock was needed to initiate the passage study, BV preparations from infected larvae were used as inoculum. Polyhedra produced in Ld652Y cells from a plaque-purified line of A21-MPV were used to infect gypsy moth larvae *per os* to establish an *in vivo* line of virus. To select against FP mutants that may have arisen during *per os* propagation, isolates A21-MPV and A21 were passaged *per os* in gypsy moth larvae two times prior to the infection used to begin the passage study. Investigations by Fraser and Hink (1982) have shown that FP mutants are eliminated from mixtures of MP and FP viruses through serial *per os* passage.

Isolate A21 exhibited a significant increase (ANOVA, Fisher's PLSD, $P < 0.05$) in BV production of approximately 160-fold from passage 1 to passage 2 (Fig. 1A). The $TCID_{50}$ decreased slightly from passages 3 through 5; however, the decrease was not significant (linear regression analysis, $P < 0.05$). In contrast, A21-MPV exhibited essentially no change in titer over five passages (linear regression analysis, $P < 0.05$). The BV titer of A21-MPV was significantly greater than the titer of A21 at passage 1, but for the remaining passages A21 exhibited a significantly greater (ANOVA, Fisher's PLSD, $P < 0.05$) BV titer than A21-MPV.

Isolate A21 exhibited a significant decrease (polyno-

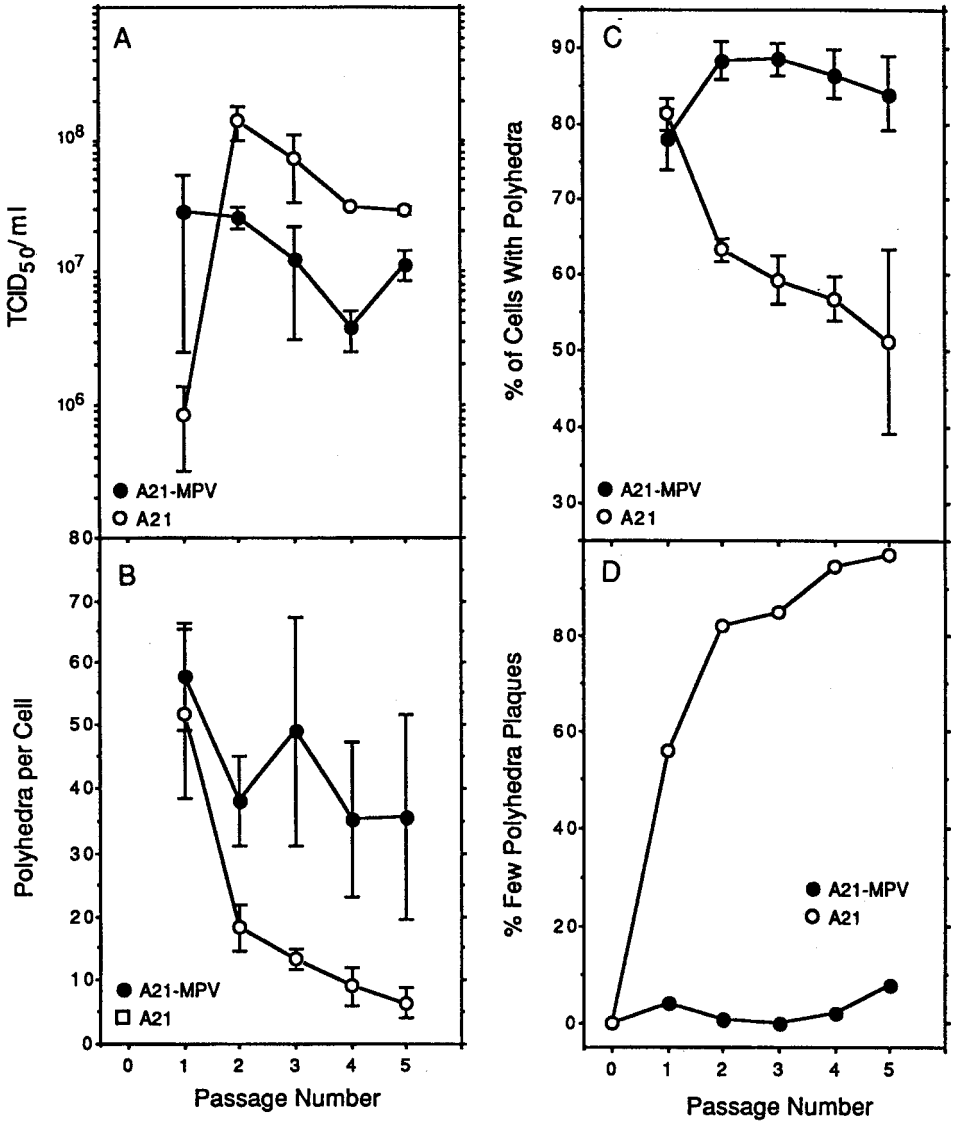

FIG. 1. (A) Budded virus production during serial passage in Ld652Y cells. The $TCID_{50}$ values were determined after each serial passage and are expressed as the $TCID_{50}$/ml of cell culture medium. (B) Polyhedra synthesis during serial passage in Ld652Y cells. The number of polyhedra produced per cell within cells that synthesized polyhedra was determined after each serial passage. (C) Percentage of cells that contain polyhedra during serial passage in Ld652Y cells. The percentage of infected cells that produced polyhedra was determined after each serial passage. The values shown in A–C are the averages of three determinations. One standard deviation is shown. (D) Percentage of A21 and A21-MPV viral plaques exhibiting a FP phenotype during serial passage in Ld652Y cells. Plaques were generated using BV after serial passage and were used to inoculate Ld652Y cells in 96-well plates. One week after inoculation, the phenotype of the infection was scored as FP or MP based on a visual inspection of the number of polyhedra present in cells. Infections from between 58 and 96 plaques were analyzed for each determination.

mial regression $R = 0.92$, $R2 = 0.85$, $P < 0.0001$) in polyhedra production during serial passage (Fig. 1B). Approximately eightfold fewer polyhedra were produced by A21 at passage 5 compared to that at passage 1. In contrast, A21-MPV exhibited no significant change (polynomial regression analysis, $P < 0.05$) in the number of polyhedra produced per cell over five serial passages (Fig. 1B). A21 and A21-MPV produced essentially the same number of polyhedra at passage 1. At passages 2 through 5, isolate A21-MPV produced significantly more polyhedra per cell than isolate A21 (ANOVA, Fisher's PLSD, $P < 0.05$).

Isolate A21 exhibited a significant decrease (polynomial regression, $R = 0.88$, $R2 = 0.78$, $P = 0.0001$) in the percentage of infected cells that produced polyhedra during serial passage (Fig. 1C). In contrast, significantly more (ANOVA, Fisher's PLSD, $P < 0.05$) cells infected with isolate A21-MPV at passages 2 through 4 produced polyhedra compared to that at passage 1. At passage 1 no significant difference was found in the percentage of cells that produced polyhedra when infected with A21 or A21-MPV (ANOVA, Fisher's PLSD, $P < 0.05$). At passages 2 through 5, cells infected with A21-MPV exhibited a significantly greater (ANOVA,

Fisher's PLSD, $P < 0.05$) percentage with polyhedra compared to cells infected with A21.

Budded virus generated by isolates A21 and A21-MPV after each serial passage was plaque purified to determine the percentage of virus exhibiting a FP or MP phenotype. Since it was difficult to distinguish the FP from MP phenotype with viral plaques, plaques were used to inoculate Ld652Y cells in P96 wells, and the infections were scored as exhibiting a FP or MP phenotype, thereby confirming the polyhedron formation phenotype. All plaques generated from virus obtained from insect hemolymph (passage 0) exhibited a MP phenotype using this assay (Fig. 1D). After the first passage of A21 approximately 55% of the virus exhibited a FP phenotype. This result is in close agreement with previous studies that found FP mutants present after a single passage of both *in vivo* maintained and plaque-purified Ld*M*NPV in Ld652Y cells (Slavicek *et al.,* 1995). After passage 5 approximately 95% of A21 virus exhibited a FP phenotype. In contrast, FP mutants of A21-MPV were found at a much lower frequency during serial passage (Fig. 1D). After the fifth passage, approximately 8 % of A21-MPV exhibited a FP phenotype.

The number of virions present within polyhedra produced by A21 and A21-MPV during serial passage was determined by counting virions present in polyhedra cross sections. Polyhedra were sectioned randomly with respect to the cutting plane, to generate representative cross sections. Most cross sections of polyhedra produced at passage 1 by isolates A21 and A21-MPV were found to contain numerous viral nucleocapsids (data not shown). Polyhedra produced by A21 exhibited a significant decrease (linear regression, $R = 0.48$, $R2 = 0.23$, $P < 0.0001$) in virion occlusion during serial passage (Fig. 2). Polyhedra generated at passage 5 contained approximately ninefold fewer viral nucleocapsids compared to polyhedra generated at the first passage. In contrast, polyhedra produced by A21-MPV were found to contain similar amounts of viral nucleocapsids during serial passage (linear regression analysis, $P > 0.05$).

Serial passage of plaque-purified A21-MPV and LdMNPV wild-type isolates. The stability of the MP phenotype of plaque-purified A21-MPV was compared to the stability of plaque-purified lines of Ld*M*NPV isolates A21, B21, 122, and 163. Ld*M*NPV isolates A21, B21, 122, and 163 are genotypic variants that have been previously described (Slavicek *et al.,* 1995). A21-MPV and two plaque-purified lines from isolates A21 (A21-15 and A21-17), B21 (B21-12 and B21-14), 122 (122-7 and 122-8), and 163 (163-10 and 163-11) were serially passaged up to 14 times in Ld652Y cells. The amount of BV produced and the number of polyhedra generated per flask were determined for each isolate until the majority of virus present exhibited a FP phenotype (based on the presence of few polyhedra per cell and a decrease in the number of polyhedra produced per flask).

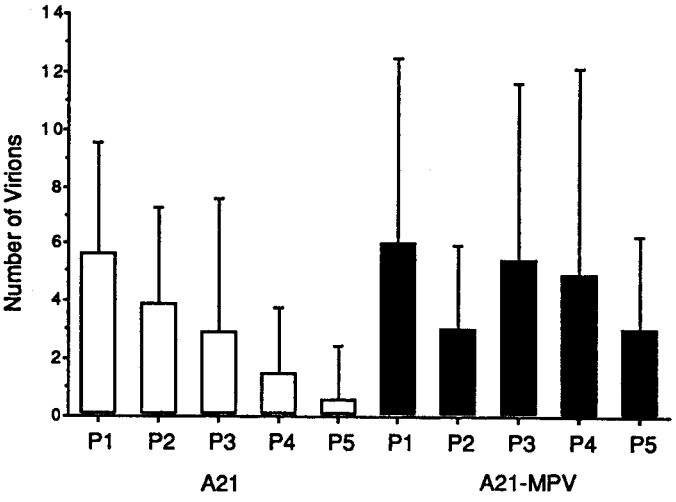

FIG. 2. Virion occlusion within polyhedra. The number of viral nucleocapsids present within cross sections was quantitated for each isolate and expressed as the number of viral nucleocapsids per square micrometer of polyhedra cross-section surface area. The values are the averages of from 25 to 50 polyhedra cross sections at each serial passage. One standard deviation is shown.

The BV $TCID_{50}$ produced by the viral lines of isolates A21, B21, 122, and 163 increased approximately 10- to 100-fold from passage 1 to passage 3 (Table 1). In contrast, the amount of BV produced by A21-MPV remained essentially constant through 11 serial passages and increased at passages 12 and 13 (Table 1). Polyhedra production by viral lines of isolates A21, B21, 122, and 163 was found to decrease during serial passage (Table 2). A decrease of approximately 2- to 10-fold was found with isolates A21-17, B21-12, B21-14, 122-7, and 122-8 after from 2 to 5 serial passages. Isolates A21-15 and 163-11 exhibited a similar degree of decrease in polyhedron production after eight serial passages. A21-MPV, in contrast to all other isolates, produced wild-type amounts of polyhedra through approximately 12 passages. The number of polyhedra produced by A21-MPV was found to decrease at passages 13 and 14 (Table 2).

The proportion of virus exhibiting a FP phenotype was determined for all the isolates after the 1st passage and at the passage in which the majority of infected cells produced few polyhedra (Table 3). After the 1st passage, from 5 to 70% of B21-12, 122-7, 122-8, and 163-10 virus exhibited a FP phenotype. From 57 to 98% of isolates A21-17, B21-12, B21-14, 122-7, and 122-8 present after the 3rd passage exhibited a FP phenotype. After passage 8, 86 and 92% of 163-11 and A21-15 virus, respectively, exhibited a FP phenotype. In contrast, A21-MPV maintained a strong MP phenotype through 12 serial passages. At passages 13 and 14, 32 and 70% of A21-MPV virus, respectively, was found to exhibit a FP phenotype.

TABLE 1

Budded Virus Production during Serial Passage of Plaque-Purified Viral Lines of Isolates A21, B21, 122, and 163 and Isolate A21-MPV

	$TCID_{50}$/ml of cell culture medium × 10^6 Passage No.												
Viral isolate	1	2	3	4	5	6	7	8	9	10	11	12	13
A21-MPV	4.6	13.0	3.7	4.6	7.4	3.2	3.7	1.5	4.6	4.6	3.2	13.7	31.6
A21-15	2.2	13.0	45.0	19.7	30.0	6.1							
A21-17	0.3	4.1	31.0	24.2	25.0								
B21-12	2.7	1.3	15.0	15.8	7.4								
B21-14	4.6	0.5	21.0	31.6	43.0								
122-7	0.3	7.5	43.0	46.4									
122-8	0.6	0.8	19.0	15.8									
163-10	3.2	31.0	21.0	3.2									
163-11	2.5	5.0	17.0	2.2	49.0	15.0	3.9	2.0					

Genotypic analysis of A21 and A21-MPV. The genomes of A21 and A21-MPV were digested with several restriction endonucleases and the profiles were compared in an effort to determine if A21-MPV was derived from A21 and to identify genotypic differences between the isolates. The genomic DNA restriction endonuclease digestion profiles of A21 and A21-MPV were identical after digestion with *Bgl*II, *Eco*RI, *Eco*RV, *Hin*dIII, *Bam*HI, and *Pst*I (data not shown).

Investigations on AcMNPV FP mutants have found a correlation between insertion of host DNA sequences into the viral genome and the appearance of the FP phenotype (Fraser *et al.,* 1983; Kumar and Miller, 1987; Cary *et al.,* 1989). DNA from budded virus isolated after each passage was digested with the restriction endonuclease *Bgl*II and the DNA digestion profiles were compared to genomic DNA digestion profiles of A21 and A21-MPV stocks used to initiate the passage studies. The digestion profiles of virus obtained after each passage matched the profile of the viral stock used to initiate the passage study (data not shown). The lack of detectable evidence of a DNA insertion that could be correlated with the FP phenotype of isolate A21 is in agreement with the results of an earlier study on the generation of Ld*M*NPV FP mutants (Slavicek *et al.,* 1995).

DISCUSSION

We have isolated and characterized an Ld*M*NPV isolate, A21-MPV, that exhibited greater stability of the MP phenotype during serial passage in Ld652Y cells, in contrast to all other viral isolates tested. Serial passage of baculoviruses in cell culture results in the formation of FP mutants at a high frequency (Hink and Strauss, 1976; MacKinnon *et al.,* 1974; Potter *et al.,* 1976; Fraser and Hink, 1982; Slavicek *et al.,* 1995). Ld*M*NPV FP mutants exhibit the traits of formation of few polyhedra per cell, a decrease in the percentage of infected cells that form polyhedra, synthesis of increased amounts of BV, and synthesis of polyhedra with very few viral nucleocapsids in comparison to wild-type virus (Slavicek *et al.,* 1995). Under identical conditions in parallel serial passages the synthesis of BV increased, and polyhedra production, the percentage of infected cells that produced polyhedra, and virion occlusion all decreased during serial passage of Ld*M*NPV isolate A21, suggesting that FP mutants had

TABLE 2

Polyhedra Production during Serial Passage of Plaque-Purified Viral Lines of Isolates A21, B21, 122, and 163 and Isolate A21-MPV

	Polyhedra per flask × 10^7 Passage No.												
Viral isolate	2	3	4	5	6	7	8	9	10	11	12	13	14
A21-MPV	17.3	15.0	23.8	20.0	10.0	25.0	31.0	15.0	14.4	8.8	7.3	2.7	3.9
A21-15	13.6	4.8	8.9	8.0	5.9	5.4	5.3						
A21-17	7.2	1.7	1.4	2.2									
B21-12	4.0	2.1	4.0	1.8									
B21-14	8.1	2.0	4.4	2.1									
122-7	14.0	2.7	3.8	1.4									
122-8	14.3	2.4	3.3	2.0									
163-10	3.3	0.9	4.3	1.2									
163-11	16.5	19.0	16.0	8.3	3.3	2.0	1.0						

TABLE 3

The Percentage of Plaques Exhibiting a FP Phenotype during Serial Passage of Plaque-Purified Viral Lines of Isolates A21, B21, 122, and 163 and Isolate A21-MPV

	Percentage of FP plaques[a] Passage No.							
Viral isolate	1	2	3	5	6	8	13	14
A21-MPV	0						32	70
A21-15	0				44	92		
A21-17	0	95						
B21-12	5	57	96					
B21-14	0	75						
122-7	5	78						
122-8	30	98						
163-10	70							
163-11	0					86		

[a] Infections from between 50 and 146 plaques were analyzed for each determination.

formed and became predominate. The presence of FP mutants was confirmed through analysis of viral plaques. These results are in agreement with our previous findings, in which isolate A21 and other wild-type viral strains mutated to FP plaque variants during serial passage in Ld652Y cells (Slavicek *et al.,* 1995). The enhanced stability of A21-MPV was confirmed by the finding that after five serial passages less than 8% of A21-MPV virus exhibited a FP phenotype in contrast to greater than 95% of A21 virus. A21-MPV also exhibited greater stability of the MP phenotype compared to serially passaged plaque-purified Ld*M*NPV viral lines. The biological activity of isolate A21-MPV was found to be the same as isolate A21 (Slavicek and Mercer, 1995).

Isolate A21-MPV was isolated from a FP culture of isolate A21. Comparisons of A21-MPV and A21 genomic DNA restriction endonuclease digestion profiles generated with several enzymes did not reveal restriction fragment length polymorphisms. These results suggest that A21-MPV is derived from A21 and that the genomic change that confers enhanced stability of the MP phenotype is likely a point mutation or short DNA insertion or deletion. Ac*M*NPV FP mutants often arise as a consequence of detectable DNA insertions or deletions in the genomic region from 36 to 37 map units (Fraser *et al.,* 1983; Kumar and Miller, 1987; Cary *et al.,* 1989). A 25K protein gene that is necessary for the MP phenotype in Ac*M*NPV and Gm*M*NPV has been identified in this region. DNA insertions into this gene often occur in the same location, which suggests the presence of a preferential insertion site (Beames and Summers, 1988, 1989, 1990; Wang *et al.,* 1989). Wang *et al.* (1989) identified a spontaneous revertant in which the DNA insertion excised from the Gm*M*NPV genome and restored the expression of the 25K protein and the MP phenotype. It is possible that imprecise excision of a DNA insertion occurred in an Ld*M*NPV FP mutant that resulted in an alteration of nucleotides at a preferential DNA insertion site. The elimination of a preferential DNA insertion site in a putative 25K protein homologue gene in Ld*M*NPV could generate the enhanced MP phenotype stability of isolate A21-MPV.

The enhanced stability of the MP phenotype exhibited by A21-MPV could provide improvements in the economics of cell culture production of Ld*M*NPV in comparison to production of wild-type virus. In addition, elucidation of the molecular basis for the enhanced stability of the MP phenotype of A21-MPV may lead to development of more stable strains of Ld*M*NPV and other baculoviruses. The molecular basis for the enhanced stability of the MP phenotype of A21-MPV is currently under investigation. Isolate A21-MPV has been patented (No. 5,420,031) and is available for research purposes.

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