BIOLOGICAL CONTROL 5, 251–261 (1995)

Rapid Formation of Few Polyhedra Mutants of *Lymantria dispar* Multinucleocapsid Nuclear Polyhedrosis Virus during Serial Passage in Cell Culture

JAMES M. SLAVICEK,[1] NANCY HAYES-PLAZOLLES, AND MARY ELLEN KELLY

USDA Forest Service, Northeastern Forest Experiment Station, Forestry Sciences Laboratory, 359 Main Road, Delaware, Ohio 43015

Received May 4, 1994; accepted November 3, 1994

Four genotypic variants of *Lymantria dispar* multinucleocapsid nuclear polyhedrosis virus (LdMNPV) were used to investigate the generation of few polyhedra (FP) mutants during viral propagation in the *L. dispar* 652Y cell line. Titers of budded virus, the percentage of infected cells producing polyhedra, the amount of polyhedra produced per cell, the proportion of virus exhibiting an FP phenotype, virion occlusion characteristics, and changes in genomic DNA digestion profiles were investigated through five serial passages. LdMNPV FP mutants arose and became predominate very quickly during serial passage. Greater than 92% of the virus present after the second passage exhibited an FP phenotype in three of the four LdMNPV isolates studied. The amount of budded virus produced by the isolates was from approximately 150- to 250-fold greater at the third passage compared to the amount produced at the first passage. The percentage of infected cells producing polyhedra decreased by an average of 35.6% during serial passage. The number of polyhedra produced per cell decreased between 2.3- and 5.6-fold from the first to the third passage. Restriction endonuclease digestion profiles of the genomic DNA of the FP mutants were analyzed to determine whether genotypic changes occurred that could be correlated with the appearance of the FP phenotype. The genomic DNA digestion profiles of FP mutants in three of the four isolates matched the profiles of the original wild-type isolates. A DNA insertion of 0.9 kb was found in the fourth isolate; however, it was shown not to be the basis for the FP phenotype. These results indicate that FP mutants arise rapidly during serial propagation of LdMNPV in cell culture. In contrast to other baculoviruses, the LdMNPV FP mutants studied in this investigation appear to lack DNA insertions of readily detectable length that could be correlated with the appearance of the FP phenotype. © 1995 Academic Press, Inc.

KEY WORDS: ***Lymantria dispar* multinucleocapsid nuclear polyhedrosis virus, *Lymantria dispar*, gypsy moth, cell culture, few polyhedra mutants, biocontrol.**

[1] To whom correspondence should be addressed. Fax: (614) 363-0182.

INTRODUCTION

During propagation of baculoviruses in cell culture a class of viruses with an altered plaque phenotype arises at high frequency which is termed few polyhedra (FP) mutants. These mutants have been identified in the *Autographa californica* multicapsid nuclear polyhedrosis virus (AcMNPV, Hink and Strauss, 1976), *Trichoplusia ni* MNPV (TnMNPV, MacKinnon *et al.*, 1974; Potter *et al.*, 1976), *Galleria mellonella* MNPV (GmMNPV, Fraser and Hink, 1982), and the *Lymantria dispar* MNPV (LdMNPV, Slavicek *et al.*, 1992). FP mutants form fewer polyhedra per cell (approximately 4–10), occlude fewer virions in polyhedra, synthesize polyhedra with lower potency, and synthesize greater amounts of budded virus when compared to wild-type (many polyhedra, MP) virus. Enhanced production of budded virus results in the conversion of the virus population from one exhibiting an MP phenotype to one with an FP phenotype during serial passage.

DNA insertions are usually found in association with the appearance of an FP phenotype during passage in cell culture. These insertions often range from approximately 0.4 to 2.8 kilobases (kb) and occur predominately within the 36 to 37 map unit region of the AcMNPV and GmMNPV genomes (Fraser *et al.*, 1983; Kumar and Miller, 1987; and Cary *et al.*, 1989). Analysis of this genomic region has identified a gene coding for a 25-Kda protein that is necessary for the MP phenotype (Beames and Summers, 1988). Mutants with a partial FP phenotype can arise as a consequence of amino acid changes in the polyhedrin gene. A viral mutant that produces a few large cuboidal polyhedra (Brown *et al.*, 1980) was found to contain a single amino acid change in the polyhedrin gene (Carstens *et al.*, 1986).

The formation of FP baculovirus mutants during

1049-9644/95 $6.00

propagation in cell culture is a significant impediment to development of cell-culture-based virus production systems. Baculovirus production systems based on cell culture would provide an alternative to synthesis of virus *in vivo* and may enhance the availability of these viruses for biocontrol purposes. In the course of working with LdMNPV in the *L. dispar* 652Y cell line, we noted the virus' high propensity for exhibiting an FP phenotype after only a few passages in cell culture. This study was initiated to investigate the generation of LdMNPV FP mutants during propagation in cell culture and to determine whether the apparent FP phenotype was genetically induced. Our results indicate that LdMNPV FP mutants quickly arise and become predominate in the viral population during serial passage in cell culture, and that these FP mutants do not contain readily detectable DNA insertions that could be correlated with the appearance of the FP phenotype.

MATERIALS AND METHODS

Insects, Generation of Viral Isolates, and Identification of Genotypic Variants

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, Massachusetts, and reared to the fourth instar on the gypsy moth diet (Bell *et al.*, 1981). *L. dispar* MNPV isolate lines were generated essentially as described by Smith and Crook (1988). Approximately 1×10^4 LdMNPV polyhedra obtained from a production lot of Gypchek (the Environmental Protection Agency registered Forest Service formulation of a mixture of LdMNPV genotypes used for biocontrol of the gypsy moth, USDA, Forest Service, 51 Mill Pond Road, Hamden, CT) were applied to a 10-cm disc of the diet in a rearing container. One hundred fourth instar larvae were added to the container, allowed to feed *ad libitum* for 24 h, and then placed on fresh diet. The larvae were checked daily and dead larvae were placed in individual containers and frozen until the polyhedra were isolated.

Viral DNA used for restriction endonuclease digestion analysis was isolated from occluded virions present in polyhedra from individual insects by a procedure adapted from Loh *et al.* (1981). The polyhedra were suspended in 0.14 *M* NaCl, 0.03 *M* KCl, 0.01 *M* Na_2HPO_4, and 2.0 m*M* KH_2PO_4 (pH 7.4) containing 1% sodium dodecyl sulfate, and ethylene diamine tetraacetic acid (EDTA, pH 8) was added to a final concentration of 0.08 *M*. The protein was digested with proteinase K (Gibco BRL, Gaithersburg, MD) at a concentration of 300 μg/ml for 1 h at 37°C. Na_2CO_3 (pH 10.5) was then added to a final concentration of 0.1 *M*, the solution was incubated for 1 h at 37°C, and then extracted with 1 vol of Tris-buffered phenol and 2 vol of chloroform:isoamyl alcohol (24:1). Viral DNA was then dialyzed against 10 m*M* Tris containing 1 m*M* EDTA, pH 8.0 (TE). The DNA was digested with the restriction enzymes *Bgl*II, *Hin*dIII, *Eco*RI, or *Bam*HI, and the fragments were separated by agarose gel electrophoresis.

Serial Passage of Viral Isolates, Determination of the Percentage of Cells Containing Polyhedra, and the Number of Polyhedra Produced per Cell

Fourth instar larvae were infected *per os* by placing them on a diet containing surface-applied polyhedra (2.6×10^5 per mm^2 of diet surface). Larvae were bled on Day 6 postinfection (p.i.), and the hemolymph was spun in an Eppendorf table-top centrifuge at 14,000 RPM for 10 min to remove large debris. One ml of the hemolymph was then added to 9 ml of a complete medium (Goodwin's IPL-52B medium, JRH Biosciences, Lenexa, KS; with 10% heat-inactivated fetal bovine serum, Atlanta Biologicals, Norcross, GA, and 6.25 m*M* glutamine, Gibco BRL) and passed through a series of syringe filters (5, 1.2, and 0.45 μm) for further purification. The medium and hemolymph mixture was titrated by end-point dilution assay and used as inoculum for three separate infections at 0.2 50% tissue culture infectious dose ($TCID_{50}$) units per 652Y cell in T25 flasks containing 1×10^6 cells. The cells were infected for 1 h at room temperature. At the end of the infection period, the inoculum was removed, the cells were rinsed four times with 10 ml of the complete medium, and 5 ml of the medium was applied after the final rinse. This infection constitutes passage 1 of virus in cell culture.

The passage 1 material was harvested on Day 7 p.i. 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. The polyhedra were pelleted by centrifugation at 550*g* for 5 min. The supernatant containing the budded virus was removed and used to determine budded virus $TCID_{50}$, for plaque assay to determine the percentage of virus that exhibited an MP or FP phenotype, and for inoculum for passage 2. The polyhedra pellet was resuspended in 5 ml of the complete medium and sonicated for 30 s, and the number of polyhedra was determined using a hemacytometer. This number and the number of cells containing 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) and were examined by transmission electron microscopy (TEM) as described below.

Budded virus from passage 1 was used as inoculum to generate passage 2. Three separate infections, at 0.2

$TCID_{50}$ units per cell in T25 flasks seeded with 1×10^6 cells, were done with each isolate at each serial passage. $TCID_{50}$ determinations were performed 7 days p.i. as described below. The percentage of cells containing polyhedra and the number of polyhedra per cell were determined 7 days p.i. as described above. Serial passages 3 through 5 were performed as described for passage 2.

Viral isolates A21, B21, 122, and 163 were plaqued on 652Y cells as described below. Plaques from each isolate that were well separated were picked and used to infect 652Y cells in P96 wells. A viral line of each isolate (A21-16, B21-13, 122-6, and 163-9) that exhibited MP phenotypes were serially passaged up to eight times in T25 flasks seeded with 1×10^6 cells infected at 0.2 $TCID_{50}$ units per cell. $TCID_{50}$ determinations were performed 7 days p.i. as described below, and the number of polyhedra present per flask was determined by counting polyhedra in a hemacytometer. The proportion of virus exhibiting an FP or MP phenotype was determined after plaque assay as described below.

$TCID_{50}$ Determinations

Viral titers from cell culture media were determined by end-point dilution assay. 652Y cells (1×10^4 per well) were seeded in P96 plates, allowed to attach, and infected with virus diluted from 10^{-1} through 10^{-11}, 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.

Determination of the Proportion of Virus after Serial Passage That Exhibits an FP or MP Phenotype

Viral isolates were plaqued on 652Y cells in 60-mm dishes seeded at 2.0×10^6 cells/plate after each serial passage. After a 1-h infection time, the inoculum was removed and replaced with cell medium containing 0.8% agarose and the plates were incubated at 27°C for 2 weeks. Since it was difficult to differentiate FP from MP plaques, between 50 and 146 well separated plaques of each isolate were picked and used as inoculum to infect 652Y cells in P96 plates. After 1 week at 27°C the infections were inspected and scored as either exhibiting an FP phenotype if the cells contained from approximately 1 to 10 polyhedra or an MP phenotype if the majority of cells appeared opaque due to the presence of approximately 50+ 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 plaqued using hemolymph containing budded virus from infected larvae. Seven days p.i., 100 μl of hemolymph was added to 9.90 ml medium and passed through the filter series as previously described. Additional dilutions were prepared, and cells were infected as described above. After infection, the inoculum was removed and each dish was rinsed with 5 ml of the medium before the final 3 ml of overlay containing agarose was applied. After a 2-wk incubation period, plaques were isolated and treated as described above.

Analysis of Restriction Fragment Length Polymorphism of Viral Genomic DNA

Viral DNA was isolated after each serial passage and analyzed for restriction fragment length polymorphisms (RFLP). Budded virus was pelleted from media harvested from infected cells by centrifugation at 112,700*g* for 45 min at 4°C, and the pellet was resuspended in 10 m*M* Tris–Cl (pH 8.0), 1 m*M* EDTA (pH 8.0) overnight at 4°C. DNA was isolated by adding 1 vol of the extraction buffer (20 m*M* Tris, pH 7.5, 120 m*M* NaCl, 20 m*M* EDTA, pH 8, 1% SDS, and 0.04 mg/ml proteinase K) and incubating at 50°C for 1 h. The sample was then adjusted to 1% Sarkosyl (Sigma, St. Louis, MO) and incubated for an additional hour at 50°C. The solution was extracted with 1 volume of phenol saturated with 0.01 *M* Tris-0.001 *M* EDTA, pH 7.5 and two volumes of chloroform:isoamyl alcohol (24:1) and the nucleic acid was precipitated with ethanol. The viral DNA was digested with *Bgl*II, and the fragments were separated by agarose gel electrophoresis and visualized after staining with ethidium bromide.

Viral lines of each isolate were generated by plaque purification of budded virus from passage 5. Viral plaques were isolated and used as inoculum for 652Y cells in P96 wells. Viral isolates from infections that exhibited an FP phenotype (all isolates) and MP phenotype (isolate 122) were further analyzed for RFLPs as described above.

Transmission Electron Microscopy

Polyhedra produced by the isolates at passage 1 and passage 5 were examined for the presence of viral nucleocapsids by TEM. The polyhedra were prepared and sectioned for electron microscopic analysis as described by 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 per polyhedra cross section.

Bioassays

The relative biological activities of LdMNPV isolates were determined by *in vivo* bioassay using newly molted fourth-instar larvae from a standard laboratory strain of the gypsy moth (New Jersey F_{36}). Polyhedra produced by isolates A21-E, B21-A, 122-B, 163-B, A21-1B, B21-2B, 122-1E, and 163-1A were suspended in the larval diet at a concentration of 1×10^5 or 1×10^6 polyhedra per milliliter of diet. Three groups of 25 larvae were placed on virus-treated diet for 24 h for each viral isolate and allowed to feed *ad libitum*. The larvae were given untreated diet *ad libitum* for the remaining 12 days of the bioassay. One group of 25 larvae (con-

trols) was fed untreated diet for 14 days. The larvae were reared in a growth chamber at 26 ± 2°C under a 16/8 h light/dark photoperiod and observed daily for mortality. Dead larvae were removed and examined microscopically to confirm death due to virus.

RESULTS

Generation of LdMNPV Isolates in Vivo

The *in vivo* method of Smith and Crook (1988) was used in an effort to generate viral isolates consisting of a single genotype. Polyhedra from individual insect cadavers were isolated and used to infect fourth instar gypsy moth larvae *per os,* and used for isolation of viral DNA to assess genotypic purity. One or two additional passages in fourth instar larvae were performed with some of the viral isolates in attempts to increase genotypic homogeneity. Observed larval mortality ranged from 1.4 to 26.0% in the first infection and from 1.4 to 5.8% in the last infection.

Genomic DNA restriction endonuclease (REN) patterns of LdMNPV viral isolates were generated by digestion with *Eco*RI, *Hin*dIII, *Eco*RV, and *Bgl*II. These patterns were analyzed to assess the isolates' genotypic homogeneity and compared to identify restriction fragment length polymorphisms (data not shown). Analysis of 21 viral isolates identified 12 distinct *Bgl*II digestion patterns (data not shown). Four of these isolates, designated A21, B21, 122, and 163, were defined as genotypic variants on the basis of unique genomic REN digestion patterns (Fig. 1) and were used in this study. Submolar DNA fragments were not evident in REN digestion profiles of these isolates, indicating that each was composed of essentially a single genotype. Digestion of the genomic DNA of isolates A21, B21, 122, and 163 with *Bgl*II generated 20 (A21) or 19 (all others) fragments, 15 of which (24.4, 23.1, 16.0, 11.4, 10.5, 9.4, 8.2, 8.0, 6.2, 5.0, 3.9, 3.7, 3.2, 1.1, and 0.7 kb) were common to all isolates. Four fragments were present in 2 or 3 isolates. A 9.2-kb *Bgl*II fragment was present in isolates B21, 122, and 163; isolates A21, B21, and 163 contained a 7.1-kb fragment; a fragment of 6.9 kb was specific to isolates B21 and 122; and only isolates A21 and 122 contained a 3.6-kb fragment. *Bgl*II genomic digestion fragments of 9.5, 5.5, and 1.6 kb were specific to isolate A21, a second 3.7-kb fragment was specific to B21, a 6.8-kb fragment was found only in isolate 122, and fragments of 7.2 and 3.5 kb were specific to 163 (Fig. 1). The genomic sizes of isolates A21, B21, 122, and 163 were calculated to be approximately 162.1, 161.8, 161.4, and 161.9 kb, respectively, through summation of *Bgl*II fragment lengths.

Serial Passage of Viral Isolates

The formation of FP mutants during serial passage in *L. dispar* 652Y cells was investigated through analysis of budded virus production, the percentage of cells containing polyhedra, the number of polyhedra produced per cell, and the proportion of virus exhibiting the FP phenotype. These studies were performed with four LdMNPV isolates (A21, B21, 122, and 163) to assure that the observations were representative of wild-type LdMNPV as opposed to a viral variant. Initially we attempted this investigation with plaque-purified viral lines. However, cells containing few polyhedra were evident in the first postplaque infection performed to produce viral stocks. In a subsequent *in vitro* passage of this material the majority of cells appeared to be infected by FP mutants since the cells produced few polyhedra (data not shown). Because a homogeneous MP viral stock was needed to initiate the passage study it was necessary to use budded virus obtained from infected larvae. Previous investigations have shown that serial *per os* passage at LC_{50} polyhedra concentrations of *in vitro*-generated polyhedra was sufficient to eliminate FP mutants from mixtures of MP and FP variants (Fraser and Hink, 1982). In order to select against FP mutants that may have arisen during *per os* propagation, the LdMNPV isolates used in this study were passaged *per os* in larvae two or three times at polyhedra concentrations that resulted in less than 25% mortality prior to the infection used to begin the passage study.

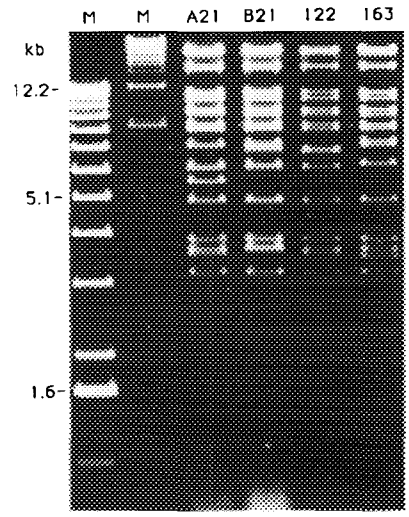


FIG. 1. Genomic DNA digests of LdMNPV isolates A21, B21, 122, and 163. Viral genomic DNA was digested with the restriction endonuclease *Bgl*II, the fragments were separated by agarose gel electrophoresis, and the gel was stained with ethidium bromide and photographed. The lanes marked M contain DNA size markers (from BRL), and the size (in kb) of selected markers are indicated to the left.

Determination of Budded Virus Titer, Percentage of Cells Containing Polyhedra, and the Number of Polyhedra per Cell during Serial Passage

The amount of budded virus produced by the isolates during serial passage was determined by $TCID_{50}$ analy-

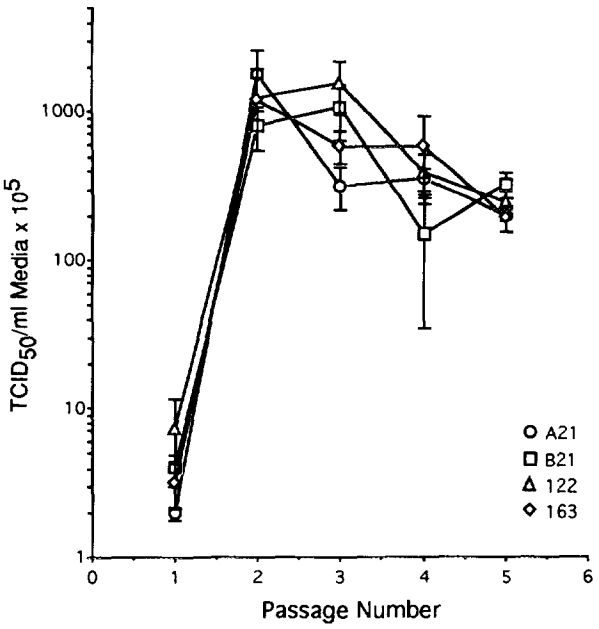


FIG. 2. Budded virus production during serial passage. The $TCID_{50}$ values were determined for LdMNPV isolates A21, B21, 122, and 163 after each serial passage. The values are the averages of three determinations. One standard deviation is shown for each value.

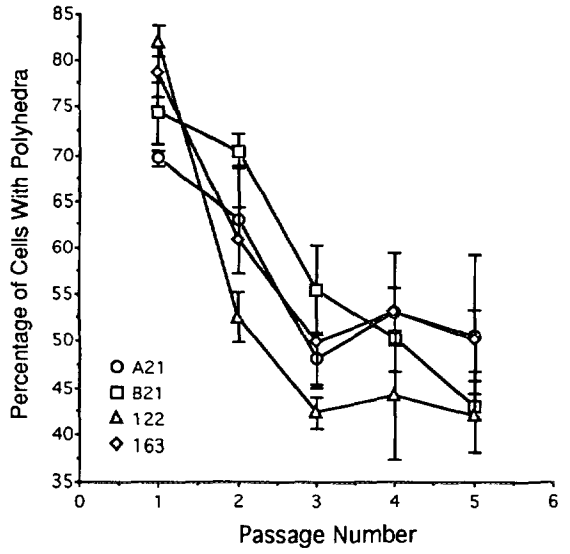


FIG. 3. Percentage of cells that contain polyhedra during serial passage. The percentage of infected cells that produced polyhedra was determined for LdMNPV isolates A21, B21, 122, and 163 after each serial passage. The values are the averages of three determinations. One standard deviation is shown.

sis. The isolates exhibited the lowest budded virus titer at passage 1 compared to passages 2 through 5 (Fig. 2). At passage 2 all isolates exhibited a significant increase in $TCID_{50}$ compared to passage 1 (ANOVA, Fisher's PLSD, $P < 0.05$), ranging from 164-fold for isolate 122 to 900-fold for isolate A21. Comparisons between isolate $TCID_{50}$ values at each passage revealed no significant differences (ANOVA, Fisher's PLSD). During passages 2 through 5 the amount of budded virus produced by the isolates ranged from 1.25×10^8 to 2.39×10^7 $TCID_{50}$ units per milliliter of medium. All isolates exhibited the highest $TCID_{50}$ values at passage 2, and then decreased through passage 5; however, this decrease was found not to be significant (linear regression analysis, $P < 0.05$).

LdMNPV isolates A21, B21, 122, and 163 exhibited a significant decrease in the percentage of infected cells that contained polyhedra during serial passage (Fig. 3). Polynomial regression analysis indicated that the decrease in this characteristic was best described by a second-order equation (A21, $R = 0.70$, $R^2 = 0.49$, $P = 0.02$; B21, $R = 0.94$, $R^2 = 0.87$, $P = 0.0001$; 122, $R = 0.92$, $R^2 = 0.85$, $P = 0.0001$; 163, $R = 0.85$, $R^2 = 0.72$, $P = 0.0005$). The isolates exhibited decreases ranging from 28 (isolate A21) to 49% (isolate 122) from passage 1 to passage 5 (Fig. 3). The greatest decrease in the percentage of cells with polyhedra occurred from passages 1 to 3. Comparisons between the isolates in the percentage of cells containing polyhedra at each passage revealed significant differences only between isolates B21 and 122 at passages 1, 2, and 3 (ANOVA, Fisher's PLSD, $P < 0.05$).

The number of polyhedra produced per cell within cells containing polyhedra was determined during serial passage (Fig. 4). All isolates exhibited a significant decrease in polyhedra production as a function of passage (polynomial regression, A21, $R = 0.86$, $R^2 = 0.74$, $P = 0.0003$;

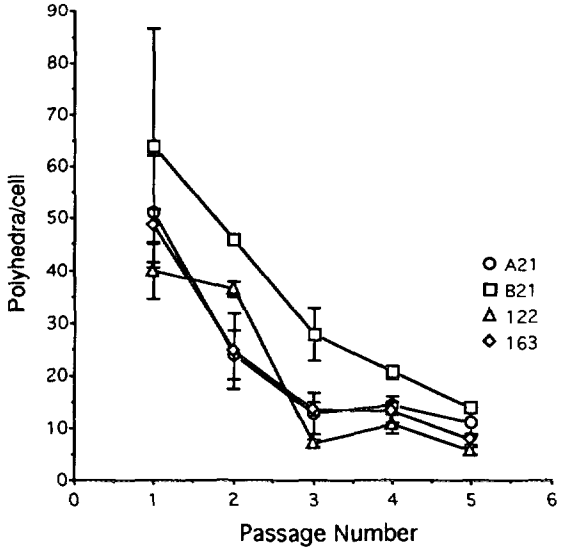


FIG. 4. Polyhedra production during serial passage. The number of polyhedra produced per cell within cells that produced polyhedra was determined for LdMNPV isolates A21, B21, 122, and 163 after each serial passage. The values shown are the averages of three determinations. One standard deviation is shown.

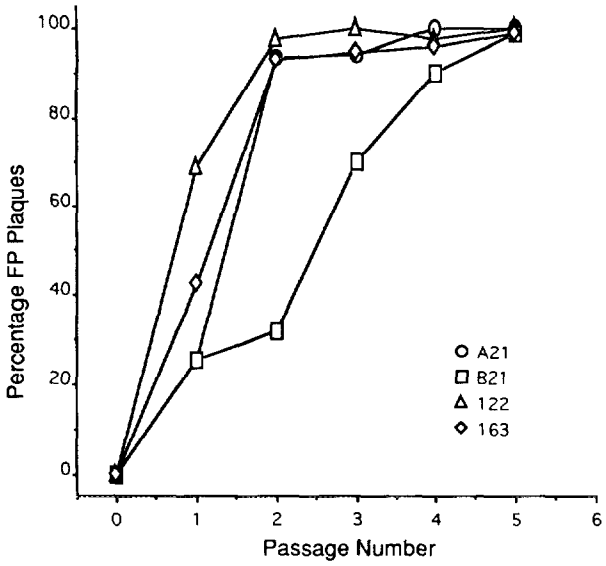


FIG. 5. Percentage of LdMNPV isolate viral plaques exhibiting an FP phenotype during serial passage in 652Y cells. Plaques were generated using budded virus of each viral isolate after serial passage and used to inoculate 652Y cells in P96 well plates. Two weeks after inoculation the phenotype of the infection was scored as FP or MP based on a visual inspection of the number of polyhedra present.

B21, $R = 0.78$, $R^2 = 0.61$, $P = 0.004$; 122, $R = 0.90$, $R^2 = 0.81$, $P = 0.0001$; 163, $R = 0.93$, $R^2 = 0.86$, $P = 0.0001$). Polyhedra production by isolates A21 and 163 decreased approximately 50% from the first to the second passage. In contrast, isolates B21 and 122 exhibited the greatest decrease, 39 and 80%, respectively, between passages 2 and 3. Overall, the isolates had total decreases in polyhedra per cell ranging from 4.6- to 7.0-fold from passage 1 to passage 5. Isolates A21, B21, 122, and 163 exhibited no significant differences in the amounts of polyhedra per cell at passage 1 (ANOVA, Fisher's PLSD, $P < 0.05$). Isolate B21 exhibited significantly more polyhedra per cell than isolates A21 and 163 at passage 2, isolates A21, 122, and 163 at passages 3 and 4, and isolates 122 and 163 at passage 5 (ANOVA, Fisher's PLSD, $P < 0.05$).

Determination of the Proportion of Virus Exhibiting an FP Phenotype during Serial Passage

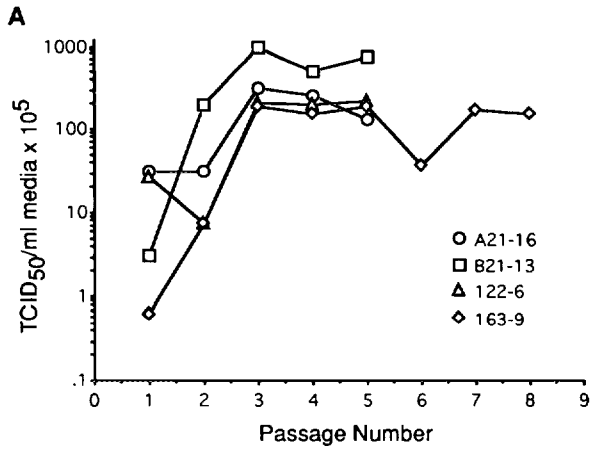
Budded virus generated by the isolates after each serial passage was analyzed to determine the proportion of virus that exhibited the FP phenotype. All P96 well infections exhibited an MP phenotype when infected with plaques generated from virus obtained from insect hemolymph with all four viral isolates (Fig. 5). In contrast, after the first passage 25.9, 25.5, 69.2, and 42.6% of virus from isolates A21, B21, 122, and 163, respectively, exhibited an FP phenotype (Fig. 5). After the second passage, 93.3, 97.8, and 92.7%, respectively, of the virus generated by isolates A21, 122, and 163 exhibited an FP phenotype (Fig. 5). Isolate B21 exhibited a similar proportion of virus with an FP phenotype after passage 4.

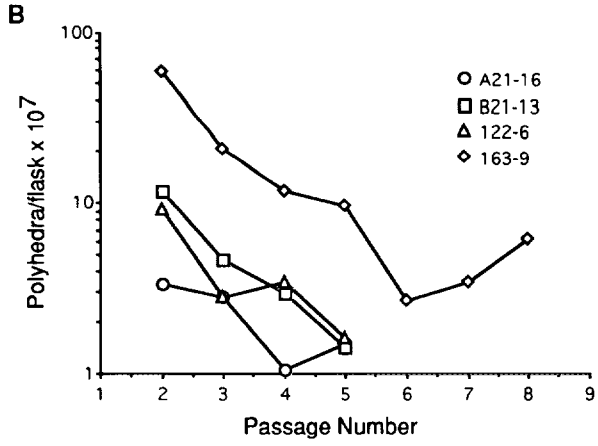
Serial Passage of Plaque-Purified Virus

To determine whether the formation of FP mutants with plaque-purified virus is similar to virus maintained *in vivo* the formation of FP mutants during serial passage with a plaque-purified viral line of each isolate was investigated. The amount of polyhedra and budded virus produced during serial passage was determined until the culture appeared to contain predominantly FP mutants. At the first and final passages the proportion of virus exhibiting an FP phenotype was determined. All four plaque-purified viral lines exhibited increases in budded virus production (Fig. 6A). The increases in $TCID_{50}$ values ranged from 10- to 300-fold from passage 1 to passage 3. The number of polyhedra produced by the isolates decreased during serial passage (Fig. 6B). The reduction ranged from approximately 2- to 8-fold from passage 1 to passage 4. The FP mutants were found after the first passage of isolates A21-16 and B21-13 (Fig. 6C). Greater than 80% of the virus present after the third passage of isolates A21-16, B21-13, and 122-6 exhibited an FP phenotype.

Analysis of Virion Occlusion into Polyhedra during Serial Passage

The relative number of virions present within polyhedra produced by the isolates at passage 1 and passage 5 was examined 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 the isolates at the first passage contained numerous virions. In contrast, polyhedra generated at the fifth passage contained few virions (data not shown). All isolates exhibited a significant (ANOVA, Fisher's PLSD, $P < 0.05$) reduction in the number of virions/μm^2 of polyhedra cross-section area in polyhedra produced at passage 5 compared to those generated at passage 1 (Fig. 7A). The decrease ranged from approximately 4-fold, for isolate B21, to 14-fold, for isolate 122. Polyhedra generated at passage 1 exhibited a range from 0 to approximately 18 virions/μm^2 of polyhedra cross-section area. At the fifth passage, the majority (from 72 to 88%) of polyhedra cross sections contained from 0 to 1 virion/μm^2 of polyhedra cross-section area. All four LdMNPV isolates exhibited similar ranges of virion densities in polyhedra at the first and fifth passages. The distribution patterns found with isolate A21 at the first and fifth passages are shown in Fig. 7B.





C

	Percentage FP Plaques		
	Passage Number		
Viral Isolate	1	3	8
A21-16	14.0	95.6	
B21-13	10.0	80.2	
122-6	0.0	84.8	
163-9	0.0		97.9

FIG. 6. Serial passage of plaque-purified viral lines. The amount of polyhedra produced per flask (A), the amount of budded virus produced (B), and the percentage of viral plaques exhibiting an FP phenotype (C) were determined for plaque-purified isolates A21-16, B21-13, 122-6, and 163-9 during serial passage.

Determination of Biological Activity of LdMNPV Isolates

In order to confirm that the FP phenotype of LdMNPV FP mutants is a consequence of a genetic mutation as opposed to a cell-culture- or cell-line-specific effect, the biological activity of polyhedra generated *in vivo* by FP and MP viral lines derived from isolates A21, B21, 122, and 163 was investigated. Viral lines used for potency determination originated in the studies on analysis of the proportion of virus exhibiting an FP phenotype described above. Plaque-purified viral lines A21-1B, B21-2B, 122-1E, and 163-1A were selected for study, and all exhibited the traits of FP mutants. For comparison, plaque-purified lines of the isolates (A21-E, B21-A, 122-B, and 163-B) exhibiting an MP phenotype were also bioassayed. Polyhedra for bioassay were generated in *L. dispar* larvae after infection via budded virus injection with the FP mutants. Larval infections with the MP isolates at a dose of 1×10^5 polyhedra/ml resulted in from 62.5 to 88.5% mortality, which is similar to previous determinations for wild-type viral strains (Table 1, Slavicek *et al.*, 1992). In contrast, infections with the isolates exhibiting an FP phenotype at a dose of 1×10^6 polyhedra/ml of diet generated 0 to 9.1% mortality (Table 1).

Analysis of Genomic Changes of the Isolates as a Function of their Serial Passage

*Bgl*II REN digestion patterns of viral genomic DNA generated during serial passage were analyzed to determine if genomic changes had occurred during serial passage that could be correlated with the appearance

A

	Virions per μm^2 of Polyhedra Cross Section Area	
Viral Isolate	Passage 1	Passage 5
A21	6.9 + 5.7 (a)	1.2 + 2.4 (b)
B21	7.5 + 3.9 (a)	1.8 + 4.0 (b)
122	5.6 + 4.0 (a)	0.4 + 0.8 (b)
163	6.0 + 3.3 (a)	0.9 + 1.4 (b)

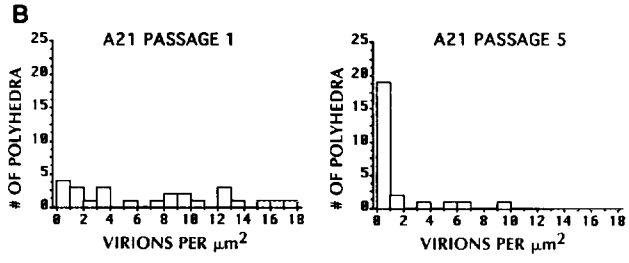


FIG. 7. Analysis of polyhedra generated during serial passage. (A) Determination of the number of virions/μm^2 of polyhedral cross-section area of polyhedra generated during passages 1 and 5 by isolates A21, B21, 122, and 163. The values are the averages of determinations from 25 polyhedral cross sections and are followed by the standard deviation of the mean. Rows followed by different letters in parentheses were found to be significantly different ($P > 0.05$, ANOVA, Fisher's PLSD). (B) The distribution in the number of polyhedra cross sections containing the respective number of virions/μm^2 of polyhedra cross-section area.

TABLE 1

Biological Activities of Wild-Type and Few Polyhedra Mutants of Viral Isolates A21, B21, 122, and 163 Produced in *L. dispar* larvae[a]

Wild-type isolates	% Mortality 1×10^5 polyhedra/ml[b]	Few polyhedra mutants	% Mortality 1×10^6 polyhedra/ml[b]
A21-E	84.6	A21-1B	4.0
B21-A	62.5	B21-2B	3.8
122-B	88.5	122-1E	9.1
163-B	77.0	163-1A	0.0

[a] Bioassays were performed using from 24 to 33 fourth instar *L. dispar* larvae per determination.

[b] Larvae were placed on artificial diet containing either 1×10^5 or 1×10^6 polyhedra per milliliter of diet.

of the FP phenotype. The genomic DNA REN digestion patterns of isolates A21, B21, and 163 at passages 1 through 5 (lanes P1–P5, Fig. 8) were found to match the REN digestion patterns of genomic DNA of the original wild-type viral isolates used in this study (lanes WT, Fig. 8). In contrast, the REN digestion profile of isolate 122 exhibited a change during serial passage. A 16.0-kb fragment disappeared during serial passage, while fragments of 12.2 and 4.7 kb exhibited a concomitant increase (Fig. 8). These results suggest that a DNA segment of approximately 0.9 kb, with a *Bgl*II restriction site, was inserted into the viral genome in the region defined by the *Bgl*II 16-kb fragment. Submolar DNA fragments of 5.5 and 5.9 kb were detected in the digests of isolate B21, and fragments of 4.7, 5.5, 5.9, and 12.2 kb were found in the digests of isolate 163. However, since these fragments were submolar and the majority of virus present at the second through fifth passages exhibits an FP phenotype (Fig. 5), the minor genotype components present in isolates B21 and 163 do not appear to be the basis for the observed FP phenotype.

After the fifth passage the isolates were composed of a mixture of FP and MP viruses that appeared to lack either detectable genomic DNA insertions, with the exception of 122, or deletions. In order to investigate more throughly the genomic changes in FP mutants, plaques obtained from the fifth passage exhibiting an FP phenotype were used for further analysis. Genomic *Bgl*II REN digestion profiles of 10 FP viral lines of isolate A21 matched the profile obtained with passage 5 virus (Fig. 9), as well as with the original wild-type A21 isolate (Figs. 1 and 8). In order to investigate further the possible genomic differences between FP and MP viruses DNA, REN digestion patterns of isolate A21 (MP) and viral line A21-2 (FP), generated with restriction endonucleases *Bam*HI, *Hin*dIII, *Eco*RI, *Eco*RV, *Cla*I, *Pst*I, and *Nde*I, were compared. No differences in the genomic REN digestion patterns of isolates A21-2 and A21 were found in any of the digests (data not shown). Four of 10 plaque lines of isolate B21 exhibited a difference in the length of one *Bgl*II fragment in comparison to passage 5 virus (Fig. 9). In place of the 6.9-kb fragment present in the original viral line, a fragment of 6.8 kb was found in plaque lines B21-2, -3, -5, and -6. Since this change was not present in all B21 FP viruses, it does not appear to be the basis for the FP phenotype. All 10 plaque lines of isolate 122 were found to lack the 16-kb fragment present in the original 122 isolate (Figs. 1 and 8) and contain fragments of 12.2 and 4.7 kb that were absent in the original isolate (Fig. 9). The digestion profiles of 9 of the 10 plaque lines of isolate 163 matched both passage 5 virus and the original 163 isolate (Figs. 1, 8, and 9). Plaque line 163-1 exhibited a loss of the 16-kb fragment and the appearance of 12.2- and 4.7-kb fragments, as was found in isolate 122. However, in contrast to isolate 122, not all of the FP viruses contained the 0.9-kb insertion, suggesting that the insertion may not be causing the FP phenotype.

In order to clarify further the possible correlation between the 0.9-kb insertion in isolate 122 and the FP phenotype, viral DNA from five 122 plaque lines exhibiting an MP phenotype was digested by *Bgl*II and the digestion profiles were analyzed. Genomic DNA REN digestion profiles were obtained that matched the profile of the original isolate (i.e., contained a 16-kb fragment), and others matched the profiles of the FP mutants (i.e., contained a 12.2- and 4.7-kb fragment in place of a 16-kb fragment). Representative profiles of these MP isolates are shown in Fig. 10, lanes 1 and 2. Analysis of additional FP plaque lines of isolate 122 revealed the existence of genomic profiles that matched the original isolate (Fig. 10, lane 3).

DISCUSSION

Investigations of the formation of baculovirus FP mutant will further our understanding of the processes of polyhedron formation and virion occlusion. These studies may also lead to the development of improved means of baculovirus production in cell culture as an alternative to *in vivo* systems, thus promoting the availability of baculoviruses for use as viral insecticides. In working with the LdMNPV in the *L. dispar* 652Y cell line, we observed the rapid appearance of viruses exhibiting an FP phenotype. In this study we sought to characterize the generation of LdMNPV FP mutants during viral propagation in the 652Y cell line, and to determine if the apparent FP phenotype was genetically based.

The formation of LdMNPV FP mutants was investigated using four LdMNPV genotypic variants to ensure that the results would be representative of the LdMNPV wild-type virus as opposed to a viral variant. LdMNPV isolates A21, B21, 122, and 163 were purified from the microbial pesticide Gypchek (a mixture of

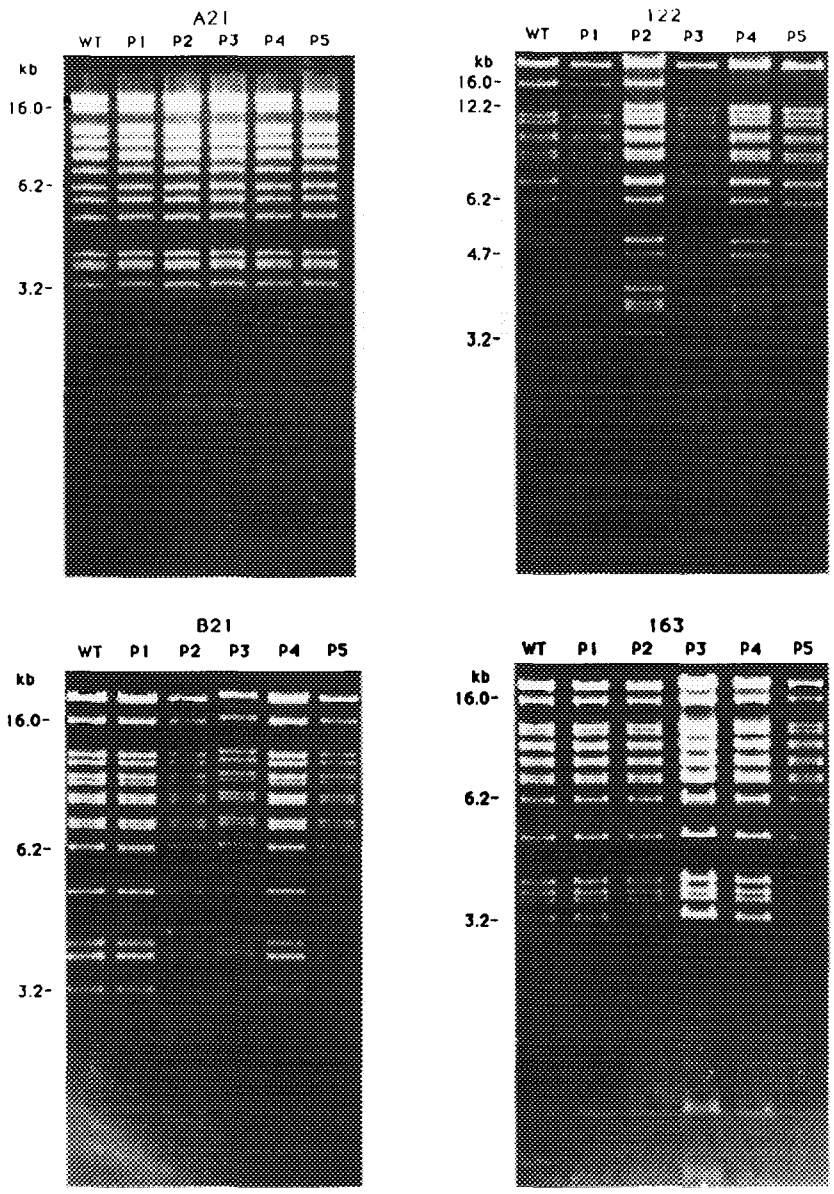


FIG. 8. Genomic DNA digestion profiles of isolates A21, B21, 122, and 163 during serial passage. Genomic DNA was isolated from budded virus produced after each serial passage and digested with *Bgl*II. DNA fragments were separated by agarose gel electrophoresis, and the gel was stained with ethidium bromide and photographed. The lanes marked WT contain the digestions of genomic DNA of the wild-type isolates obtained from occluded virions. Lanes marked P1–P5 contain digestions of genomic DNA obtained from budded virus after each serial passage. The sizes of selected DNA fragments are shown to the left.

many viral genotypes, unpublished data) and were shown to be genotypic variants through comparison of genomic fragment lengths after digestion with restriction endonucleases. These isolates were assumed to be genotypically similar based upon the finding that 15 of 19 (for isolates B21, 122, and 163) or 20 (A21) genomic *Bgl*II fragments were of identical length.

Increased budded virus production, decreased polyhedra formation, and decreased viral occlusion into polyhedra are traits exhibited by FP mutants (Hink and Strauss, 1976; MacKinnon *et al.*, 1974; Potter *et al.*, 1976; Fraser and Hink, 1982; and Slavicek *et al.*, 1992). These traits were observed during serial passage of viral isolates A21, B21, 122, and 163. In addition, a decrease in the percentage of infected cells that produced polyhedra during serial passage was found. This may be a trait specific to LdMNPV FP mutants or one not investigated in other baculovirus FP strains. FP mutants arose and became predominate during serial passage of LdMNPV very quickly. This was found to occur with virus maintained *in vivo* and plaque-purified viral lines, suggesting that the high frequency of

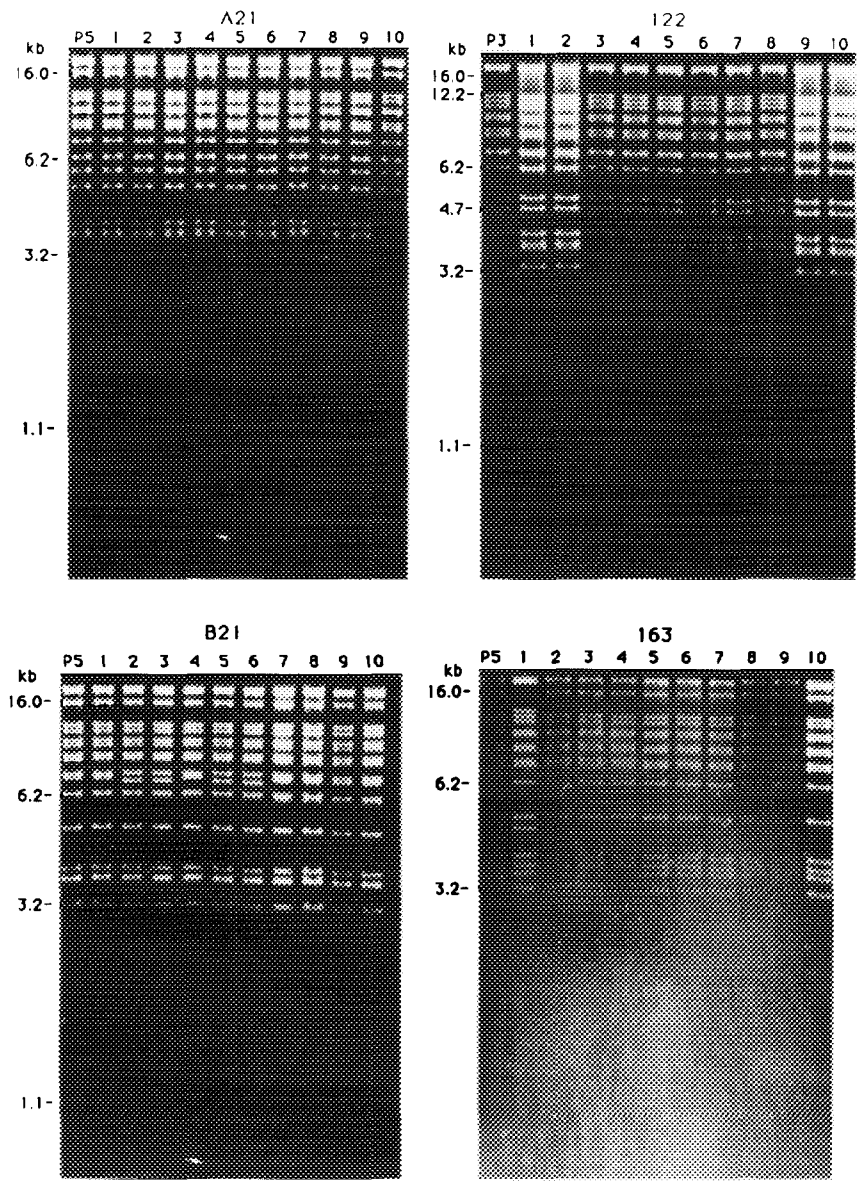


FIG. 9. Genomic DNA digestion profiles of 10 FP plaque-purified lines of isolates A21, B21, 122, and 163. Budded virus from serial passage 5 was used to generate plaque-purified viral lines of the isolates. Genomic DNA was obtained from budded virus, digested with *Bgl*II, and the DNA fragments were separated by agarose gel electrophoresis. The lanes marked P5 or P3 contain genomic DNA from passage 5 or passage 3 budded virus, respectively. Lanes marked 1–10 contain genomic DNA from 10 FP viral lines. The sizes of selected DNA fragments are shown to the left.

FP mutant formation observed during serial passage resulted from mutation and selection during cell culture propagation. The basis for the FP phenotype of LdMNPV FP mutants appears to be a genetic change since the FP phenotype, based on biological activity, was stable after passage of FP mutants *in vivo*.

AcMNPV FP mutants are often caused by insertion of DNA sequences into the viral genome. These insertions range predominately from approximately 0.4 to 2.8 kb in length in AcMNPV, and preferentially insert into the genomic region from 36 to 37 map units (Fraser *et al.*, 1983; Kumar and Miller, 1987; Cary *et al.*, 1989). LdMNPV genomic REN digestion profiles were analyzed to ascertain if DNA insertions could be correlated with the appearance of the FP phenotype. Analysis of REN digestion profiles of three of the four isolates used in this investigation failed to reveal evidence of DNA insertions in the predominate genotype present. Evidence of an insertion in isolate 122 was found; however,further analysis demonstrated that the insertion was not related to the appearance of the FP phenotype. Consequently, in contrast to AcMNPV FP mutants,

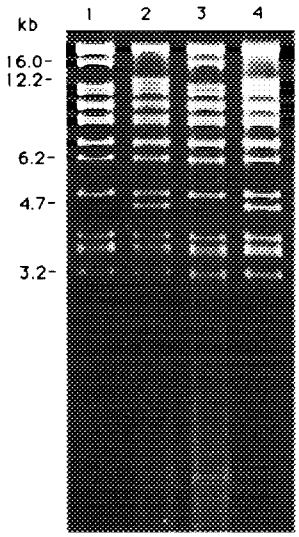


FIG. 10. Genomic DNA digestion profiles of isolate 122 MP and FP viral lines. Plaque-purified lines of isolate 122 that exhibited an MP or FP phenotype were generated. Genomic DNA was obtained from budded virus, digested with *Bgl*II, and the fragments were separated by agarose gel electrophoresis. Lanes 1 and 2 contain genomic DNA from viral lines exhibiting an MP phenotype, and lanes 3 and 4 contain genomic DNA from viral lines with an FP phenotype. The sizes of selected DNA fragments are shown to the left.

LdMNPV FP mutants are not generated through DNA insertions that are of sufficient size to allow detection by genomic REN digestion analysis. Further analysis of LdMNPV mutants is required to determine whether the genetic basis for the FP phenotype is small DNA insertions or deletions, or point mutations.

The rapid appearance of LdMNPV FP mutants during propagation in the 652Y cell line, along with the absence of readily detectable DNA insertions, may indicate that generation of FP mutants in LdMNPV is distinct from the mechanism of generating AcMNPV FP mutants. Alternatively, the mechanism of LdMNPV FP mutant formation could be the same as in AcMNPV; however, the genomic DNA insertions/deletions are not readily detectable. Studies are in progress to ascertain the molecular basis for FP mutant formation in the LdMNPV.

ACKNOWLEDGMENTS

We thank John Podgwaite for providing the LdMNPV Gypchek sample used in this study. This research was supported by funds provided by the U.S. Department of Agriculture, Forest Service, Northeastern Forest Experiment Station.

REFERENCES

Beames, B., and Summers, M. D. 1988. Location and nucleotide sequence of the 25K protein missing from baculovirus few polyhedra (FP) mutants. *Virology* **168,** 344–353.

Bell, R. A., Owens, C. D., and Shapiro, M. 1981. Development of mass rearing technology. In "The Gypsy Moth: Research Toward Integrated Pest Management" (C. C. Doane and M. L. McManus, Eds.), p. 608. Forest Service Tech. Bull. 1584, USDA, Washington, D.C.

Brown, M., Faulkner, P., Cochran, M. A., and Chung, K. L. 1980. Characterization of two morphology mutants of *Autographa californica* nuclear polyhedrosis virus with large cuboidal inclusion bodies. *J. Gen. Virol.* **50,** 309–316.

Carstens, E. B., Krebs, A., and Gallerneault, C. E. 1986. Identification of an amino acid essential to the normal assembly of *Autographa californica* nuclear polyhedrosis virus polyhedra. *J. Virol.* **58,** 684–688.

Cary, L. C., Goebel, M., Corsaro, B. G., Wang, H., Rosen, E., and Fraser, M. J. 1989. Transposon mutagenesis of baculoviruses: Analysis of *Trichoplusia ni* transposon IFP2 insertions within the FP-locus of nuclear polyhedrosis viruses. *Virology* **172,** 156–169.

Fraser, M. J., and Hink, W. F. 1982. The isolation and characterization of the MP and FP plaque variants of *Galleria mellonella* nuclear polyhedrosis virus. *Virology* **117,** 366–378.

Fraser, M. J., Smith, G. E., and Summers, M. D. 1983. Acquisition of host cell DNA sequences by baculoviruses: Relationship between host DNA insertions and FP mutants of *Autographa californica* and *Galleria mellonella* nuclear polyhedrosis viruses. *J. Virol.* **47,** 287–300.

Hink, W. F., and Strauss, E. 1976. Replication and passage of alfalfa looper nuclear polyhedrosis virus plaque variants in cloned cell cultures and larval stages of four host species. *J. Invertebr. Path.* **27,** 49–55.

Kumar, S., and Miller, L. K. 1987. Effects of serial passage of *Autographa californica* nuclear polyhedrosis virus in cell culture. *Virus Res.* **7,** 335–349.

Loh, L. C., Hamm, J. J., and Huang, E. 1981. *Spodoptera frugiperda* nuclear polyhedrosis virus genome: Physical maps for restriction endonucleases *Bam*HI and *Hin*dIII. *J. Virol.* **38,** 922–931.

MacKinnon, E. A., Henderson, J. F., Stoltz, D. B., and Faulkner, P. 1974. Morphogenesis of nuclear polyhedrosis virus under conditions of prolonged passage *in vitro*. *J. Ultrastruc. Res.* **49,** 419–435.

Potter, K. N., Faulkner, P., and MacKinnon, E. A. 1976. Strain selection during serial passage of *Trichoplusia ni* nuclear polyhedrosis virus. *J. Virol.* **18,** 1040–1050.

Slavicek, J. M., Podgwaite, J., and Lanner-Herrera, C. 1992. Properties of two *Lymantria dispar* nuclear polyhedrosis virus isolates obtained from the microbial pesticide Gypchek. *J. Invertebr. Path.* **59,** 142–148.

Smith, I. R. L., Crook, N. E. 1988. *In vivo* isolation of baculovirus genotypes. *Virology* **165,** 240–244.