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Properties of Two *Lymantria dispar* Nuclear Polyhedrosis Virus Isolates Obtained from the Microbial Pesticide Gypchek

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Two *Lymantria dispar* nuclear polyhedrosis virus isolates, 5-6 and A2-1, differing in the phenotypic characteristic of the number of viral occlusions in infected cells, were obtained from a production lot of the microbial pesticide Gypchek and several of their replication properties were investigated and compared. Budded virus titer produced in cell culture, polyhedral inclusion body production in cell culture and *in vivo*, the number of virions present within occlusion body cross sections, and potency determinations suggest that isolate 5-6 is a few polyhedra plaque variant and that A2-1 is a many polyhedra wild-type isolate.

KEY WORDS: ***Lymantria dispar;* nuclear polyhedrosis virus; plaque variant; few polyhedra mutant.**

INTRODUCTION

The gypsy moth, *Lymantria dispar,* a serious forest insect pest in the northeastern United States, is rapidly spreading both south and west. To augment current control techniques, and as an alternative to chemical pesticides, the U.S. Forest Service is encouraging the development of biological control agents for use against the pest (17). Much of the research is focused on the *L. dispar* multinucleocapsid nuclear polyhedrosis virus (LdMNPV) and its development as the microbial pesticide Gypchek.

LdMNPV exists in two forms: a budded virus and a virus occluded in polyhedra. The viral application form of choice is the polyhedra, due to its environmental stability (17). Efforts are being directed toward the development of an *in vitro* polyhedra production methodology as an alternative to production *in vivo* (12, 28, 29). However, passage of baculoviruses in cell culture generates mutants with an altered plaque phenotype, and this mutation occurs at a high frequency. These variants produce few polyhedra within infected cells in culture, and are termed few polyhedra (FP) mutants (8, 11, 20). FP mutants are mostly deficient in the packaging of virions into polyhedra. Consequently, these polyhedra exhibit an extremely low potency against their host. In addition, FP mutants exhibit higher budded virus titers compared to wild-type strains. Consequently, after several rounds of replication the FP mutant becomes the prominent virus type in a mixture of FP and wild-type (many polyhedra, MP) viruses (18, 19). Therefore, the generation of FP mutants during cell culture passage would impede the use of this methodology for polyhedra production. Efforts to produce *Autographa californica* multinucleocapsid nuclear polyhedrosis virus (AcMNPV) polyhedra in *Spodoptera frugiperda* cells through a continuous cell culture process suggest that this is indeed the case (12). After a run time of 32 days, Kompier *et al.* (12) found that the production of AcMNPV polyhedra per infected cell decreased significantly. In addition, the polyhedra were found to contain very few occluded virions. These characteristics are typical of those exhibited by FP viral mutants.

The FP phenotype usually results as a consequence of DNA insertions into the viral genome (2, 4, 9). Typically, these insertions are in the same approximate genomic location, suggesting the presence of preferential insertion sites (7, 9, 13). The disruption of a single gene has been shown to be sufficient to generate the FP phenotype in AcMNPV (1). It is likely that generation of FP mutants in LdMNPV and in AcMNPV occurs in the same way, since the genomes of these viruses are similar (5; for a review of AcMNPV, 7, 23, 25, 26). The isolation of a LdMNPV FP mutant and the elucidation of the basis of the FP phenotype are prerequisite to efforts directed to prevent the genesis of this mutation during cell culture passage.

We obtained two LdMNPV isolates from microbial pesticide Gypchek and studied their replication char-

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acteristics. Properties investigated include the production of budded virus in cell culture, the production of polyhedra in cell culture and *in vivo,* the number of virions present within cross sections of polyhedra produced *in vivo,* and the potency of these isolates against the gypsy moth. In this report results are presented that indicate that isolate 5-6 is a FP plaque variant, and isolate A2-1 is a MP wild-type virus.

MATERIALS AND METHODS

Cells and virus. The *L. dispar* 652Y ovarian cell line (10) was used for *in vitro* studies. The cells were propagated in modified Goodwin's IPL-52B medium (Hazelton) with 10% heat-inactivated fetal bovine serum (HyClone) and 6.25 m*M* glutamine. The cells were seeded into tissue culture flasks (Corning) prior to infection with virus. Two LdMNPV isolates, both derived from Gypchek (the EPA registered Forest Service formulation of a mixture of LdMNPV genotypes used for biocontrol of the gypsy moth), were used in this study. Isolate 5-6 was passed at least 20 times in cell culture prior to three rounds of plaque purification, and isolate A2-1 was purified by the *in vivo* method of Smith and Crook (24) in fourth instar *L. dispar* larvae.

Infections and budded virus titrations. T25 flasks were seeded with 1×10^6 cells and infected with virus for 1 hr at a multiplicity of infection of 1 $TCID_{50}$ unit per cell. At the times indicated in the Results and Discussion sections, either the number of polyhedra produced was quantified (as described below) or the titer of nonoccluded virus determined. Viral titers from cell culture media were determined by end-point dilution assays first described by Reed and Muench (21), and adapted by Summers and Smith (27). Briefly, 652Y cells (2×10^4 per well) were seeded in P96 plates (Corning), allowed to attach, infected with virus diluted from 10^{-1} through 10^{-11}, and the plates incubated at 28°C. The plates were scored for infection 2 weeks after infection, and the viral titer was expressed as the $TCID_{50}$/ml of cell culture media.

Polyhedra quantifications. 652Y cells were seeded in T25 flasks and infected with the viral isolates as previously described. Five days after infection the percentage of cells containing polyhedra was determined by light microscopy, and the cells were harvested, sonicated, and the polyhedra collected by centrifugation and quantified by visual counting in a hemacytometer. Fourth instar *L. dispar* larvae were infected with isolate A2-1 per os at approximately 10× the LD_{95} dose. Fourth instar gypsy moth larvae were infected with isolate 5-6 by injection with cell culture media containing budded virus. Upon death the larvae were collected, homogenized, and the homogenate was passed through a buchner funnel. The remaining debris was extensively washed, and the flow through was combined with the primary eluent. The polyhedra were collected by low-speed centrifugation (550*g* for 5 min) and quantified as described above.

Electron microscopy and polyhedra virion quantification. Polyhedra were fixed in sodium cacodylate buffer, pH 7.2 (0.05 M sodium cacodylate, 0.5 mM HCl), containing 1% glutaraldehyde at 4°C for 20 min. A2-1 polyhedra were then fixed in sodium cacodylate buffer with 3% glutaraldehyde for 1 hr at 4°C, and 5-6 polyhedra in the same buffer for 2.5 hr. The samples were washed 3× in sodium cacodylate buffer over a 1-hr (A2-1) or a 2-hr period (5-6). The samples were postfixed in sodium cacodylate buffer containing 2% osmium tetroxide for 2.5 hr at ambient temperature, and then washed 3× in sodium cacodylate buffer over a 1-hr period. Molten agar was added to the samples (final concentration 2%) and allowed to gel; the samples were cut into 1-mm^2 blocks and incubated overnight in 35% ethanol. The samples were dehydrated through incubation in an ethanol series (35, 50, and 70% with 2% uranylacetate, 85, 95, 100%-3×) for 5–7 min at each step, except for the 70% step which was for 1 hr. The samples were then washed 3× (10 min each wash) in propylene oxide. A2-1 was infiltrated over a 24-hr period with propylene oxide:Poly/Bed 812 (25.55 g poly bed, 13.5 g dodecenylsuccinic anhydride, 10.9 g nadic methylanhydride, Polysciences) at 2:1, 1:1, and 1:2 ratios. 5-6 was infiltrated with propylene oxide:Poly/Bed 812 at 4:1, 2:1, 1:1, 1:2, and 1:4 ratios over a 46-hr period. After infiltration the polyethylene capsules were flushed with freon and filled with Poly/Bed 812 containing 2% DMP 30 (Polysciences), incubated at 35°C for approximately 15 hr, 45°C for 8 hr and finally at 60°C overnight. The samples were sectioned with a diamond knife on a Reichert-Jung Ultracut E43 microtome, the sections stained for 20 min in 5% uranylacetate in methanol, poststained in lead nitrate (2.7% lead citrate, 3.5% sodium citrate, 0.16 N NaOH), and viewed with a Hitachi model HU 11E-1 transmission electron microscope. Polyhedra cross sections were photographed, and the number of virions present within cross sections were quantified by counting.

Potency determinations. Potencies of isolates A2-1 and 5-6 relative to a standard Gypchek preparation (LDP226, Hamden standard) were determined using an *in vivo* bioassay modified after a procedure first described by Lewis and Rollinson (14). Polyhedra pastes of isolates A2-1 and 5-6 were suspended in 10 ml of sterile 0.05 M Tris–HCl buffer, pH 7.2, and briefly sonicated to disperse polyhedra. A 100-mg lyophilized sample of standard Gypchek was similarly suspended and vigorously ground in a glass tissue homogenizer for 3 min. Five serial 10-fold dilutions of each of the stock virus suspensions were prepared at Tris buffer. Dilutions were prepared such that final polyhedra concentrations in diet (3) fed to test insects ranged be-

tween 1×10^2 and 1×10^7/ml. One milliliter of a dilution was added to 99 ml of tempered (52°C) diet and vigorously mixed with a variable-speed stirrer (5000 rpm) for 30 sec. Virus-treated diet was cooled and cut into 1.25-cm^3 cubes.

Each virus dosage was presented to five groups of 10 newly molted second instar larvae from a standard laboratory strain of the gypsy moth (New Jersey, F_{34}). Each group of 10 larvae was confined to a 100 × 15-mm plastic petri dish and given two 1.25-cm^3 virus-treated diet cubes on which to feed for 48 hr. Larvae were given untreated diet *ad libitum* for the remaining 12 days of the bioassay. Five groups of 10 larvae (controls) were fed untreated diet for 14 days. Larvae were reared in a growth chamber at 24 ± 2° under a 16/8-hr light/dark photoperiod and observed daily for mortality. Dead larvae were removed and examined microscopically to confirm NPV deaths.

Mortality data were examined by Probit analysis (6) using a POLO-PC program (LeOra Software, Berkeley, CA) (22). Potencies of isolates A2-1 and 5-6 relative to standard Gypchek were estimated by comparing LC_{50} values.

RESULTS AND DISCUSSION

The purity of isolates 5-6 and A2-1 was assessed by genomic restriction endonuclease analysis. The viral genomes were digested with the restriction enzymes *Hin*dIII, *Eco*RI, and *Bam*HI, and the fragments were separated by gel electrophoresis and stained with ethidium bromide. After three rounds of plaque purification of isolate 5-6, and three rounds of purification of isolate A2-1 *in vivo*, no submolar DNA fragments were detected (Fig. 1). In addition to the phenotypic differences exhibited by the isolates described below, genotypic differences between the isolates were also identified. The restriction fragment profiles of 5-6 and A2-1 were mostly similar with differences existing in the sizes of some of the fragments. For example, in the *Bam*HI digests of isolates 5-6 and A2-1 (Fig. 1, lanes 3 and 6, respectively) fragments of approximately 47, 37,

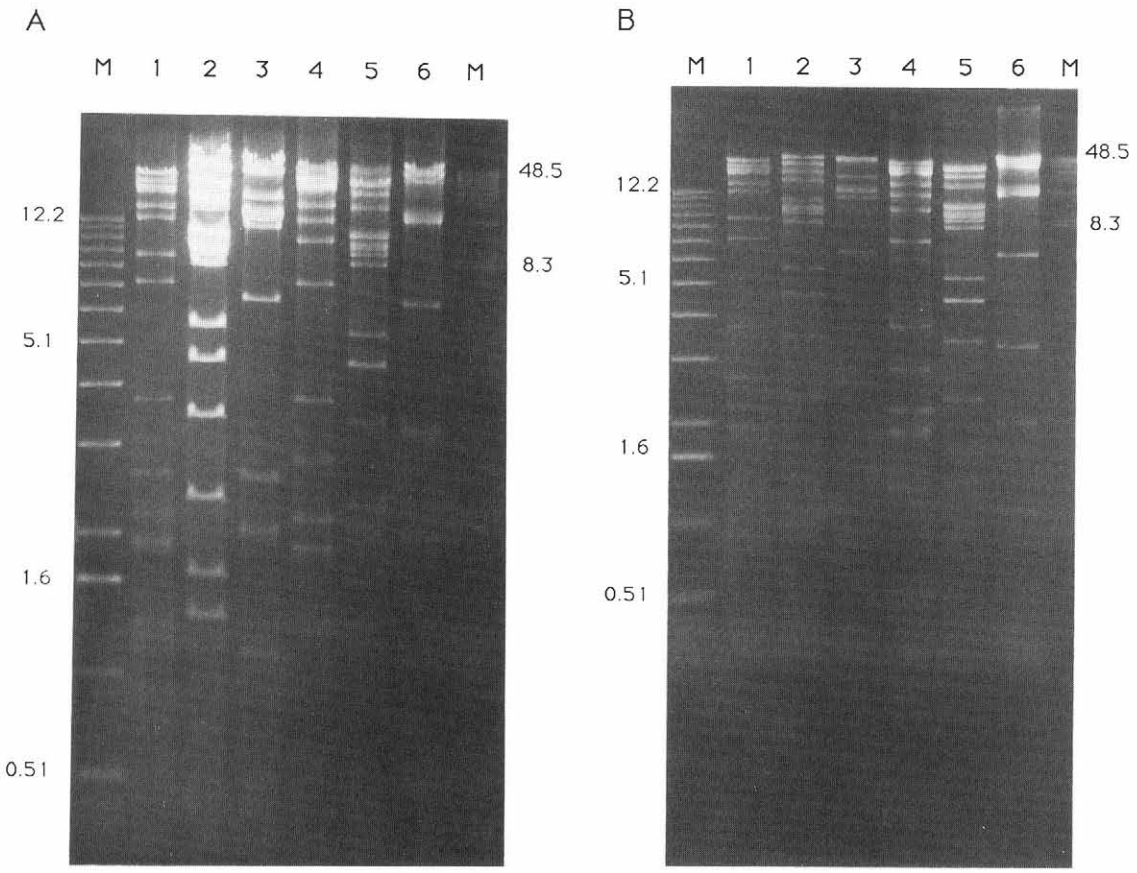


FIG. 1. Genomic DNA digests of LdMNPV isolates 5-6 and A2-1. Viral genomic DNA was digested with the restriction endonucleases *Hin*dIII (lanes 1 and 4), *Eco*RI (lanes 2 and 5), and *Bam*HI (lanes 3 and 6). The fragments were separated by agarose gel electrophoresis and stained with ethidium bromide, and the gels were photographed. Lanes 1–3 and 4–6 contain genomic fragments from isolates 5-6 and A2-1, respectively. DNA size markers (from BRL), along with sizes (in kilobase pairs) of a few of the fragments, are in the lanes marked M (parts A and B). Part B is the same as part A except that less DNA was loaded in each lane to improve the resolution of the higher molecular weight DNA fragments.

13.7, 12.9, 12.7, 6.4, 2.7, 2, and 1.2 kb were present in both digests. Fragments of 16.5, 11.5, and 2.7 kb and fragments of 28 and 3.5 kb were specific to isolates 5-6 and A2-1, respectively. Investigations are in progress to ascertain if heterogeneities in restriction endonuclease sites are correlatable with the basis of phenotypic characteristics specific to isolate 5-6.

The serial passage of baculoviruses in cell culture often leads to the genesis of FP mutants. To avoid a mixture of FP and MP viral phenotypes of isolate A2-1, we used nonoccluded virus (NOV) isolated from larvae infected per os with A2-1 polyhedra to determine NOV production in 652Y cells. Isolate 5-6 NOV virus was difficult to obtain from larvae infected per os with polyhedra, probably as a consequence of a very low infection rate. Therefore, for isolate 5-6, NOV generated in cell culture was used as the inoculum for NOV production analysis. At all times after infection isolate 5-6 produced a greater NOV titer compared to A2-1 (Fig. 2). The titer of A2-1 reached a peak of 3.6×10^5 $TCID_{50}$ units/ml media compared to 3.8×10^6 $TCID_{50}$ units/ml media for 5-6, a 10-fold difference. This analysis was repeated using A2-1 NOV produced in cell culture (the product of first passage) as the inoculum. Similar to the results shown in Fig. 2, the titer of A2-1 was less than that of isolate 5-6 at all times after infection (data not shown), and exhibited a peak of 2.2×10^5 $TCID_{50}$ units/ml media. In addition, the titer of 5-6 is greater than the values reported for three other MP LdMNPV isolates produced in 652Y cells (17). The high NOV titer of isolate 5-6 is a trait exhibited by AcMNPV FP mutants (18, 19).

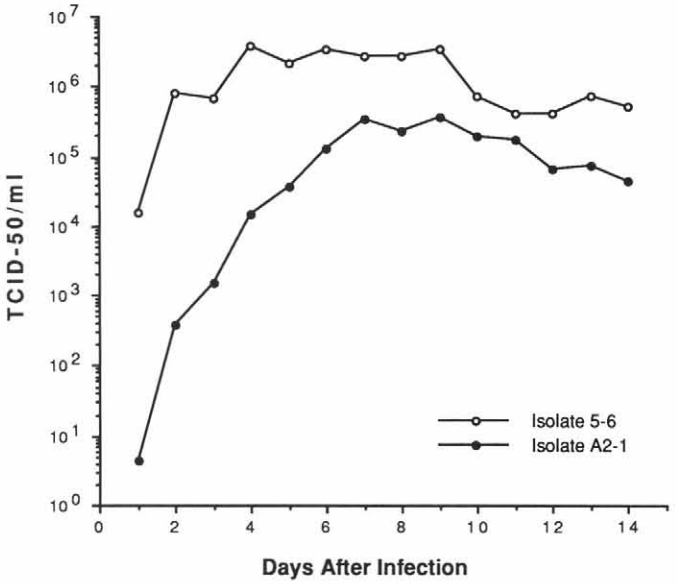


FIG. 2. Nonoccluded virus production in 652Y cells. Cells in culture were infected at 1 $TCID_{50}$ unit per cell, and at 1–14 days after infection the cell media was harvested and the titer of NOV determined by end-point dilution assay. Each value is the average of four determinations.

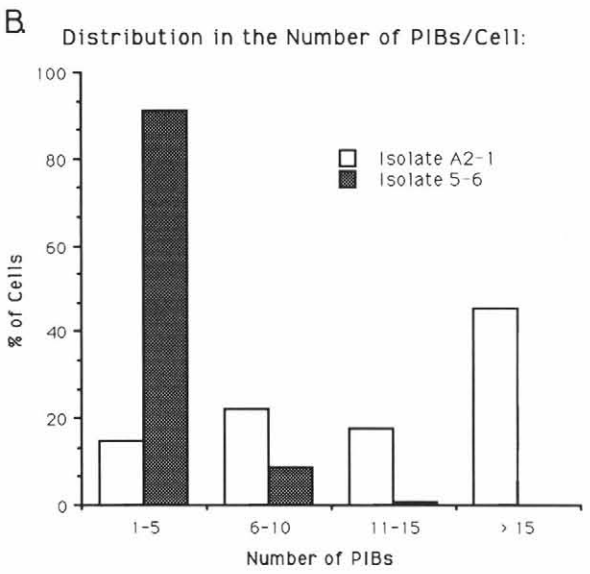
The number of polyhedra produced by the isolates was determined *in vitro* and *in vivo*. 652Y cells were infected at a multiplicity of 1 $TCID_{50}$ unit per cell, and 5 days later the percentage of cells containing polyhedra was determined through examination by light microscopy. The total number of polyhedra produced was quantified after the cells were disrupted by sonication. Thirty-eight percent of the cells infected with isolate A2-1 contained an average of 51.0 polyhedra/cell (Fig. 3A). In contrast, 63% of the cells infected with isolate 5-6 contained an average of 4.4 polyhedra/cell. The distribution in the number of polyhedra per cell was approximated by examination through light microscopy. 652Y cells were infected by each isolate at a multiplic-

A.

PIB Production in 652Y Cells:

Isolate	% of Cells With PIBs	PIBs/Cell
A2-1	38.0 + 1.0	51.0 ± 6.0
5-6	63.0 + 1.0	4.4 ± 0.9

B.



C.

PIB Production in Larvae:

Isolate	PIBs/Larvae
A2-1	2.1×10^9
5-6	8.6×10^7

FIG. 3. Polyhedra production in 652Y cells and in *L. dispar* larvae. (A) The number of polyhedra produced by isolates A2-1 and 5-6 in 652Y cells 5 days after infection was determined. The number of polyhedra/cell is the average (with standard deviation) of three determinations, each performed with 2×10^6 to 3×10^6 cells. (B) The distribution in the number of polyhedra/cell was estimated through light microscopy. The number of polyhedra in at least 200 cells was counted for this analysis. (C) Polyhedra production in fourth instar *L. dispar* larvae was determined. The values for isolates 5-6 and A2-1 are the averages of two and three determinations, respectively. From 260 to 500 larvae were used per determination.

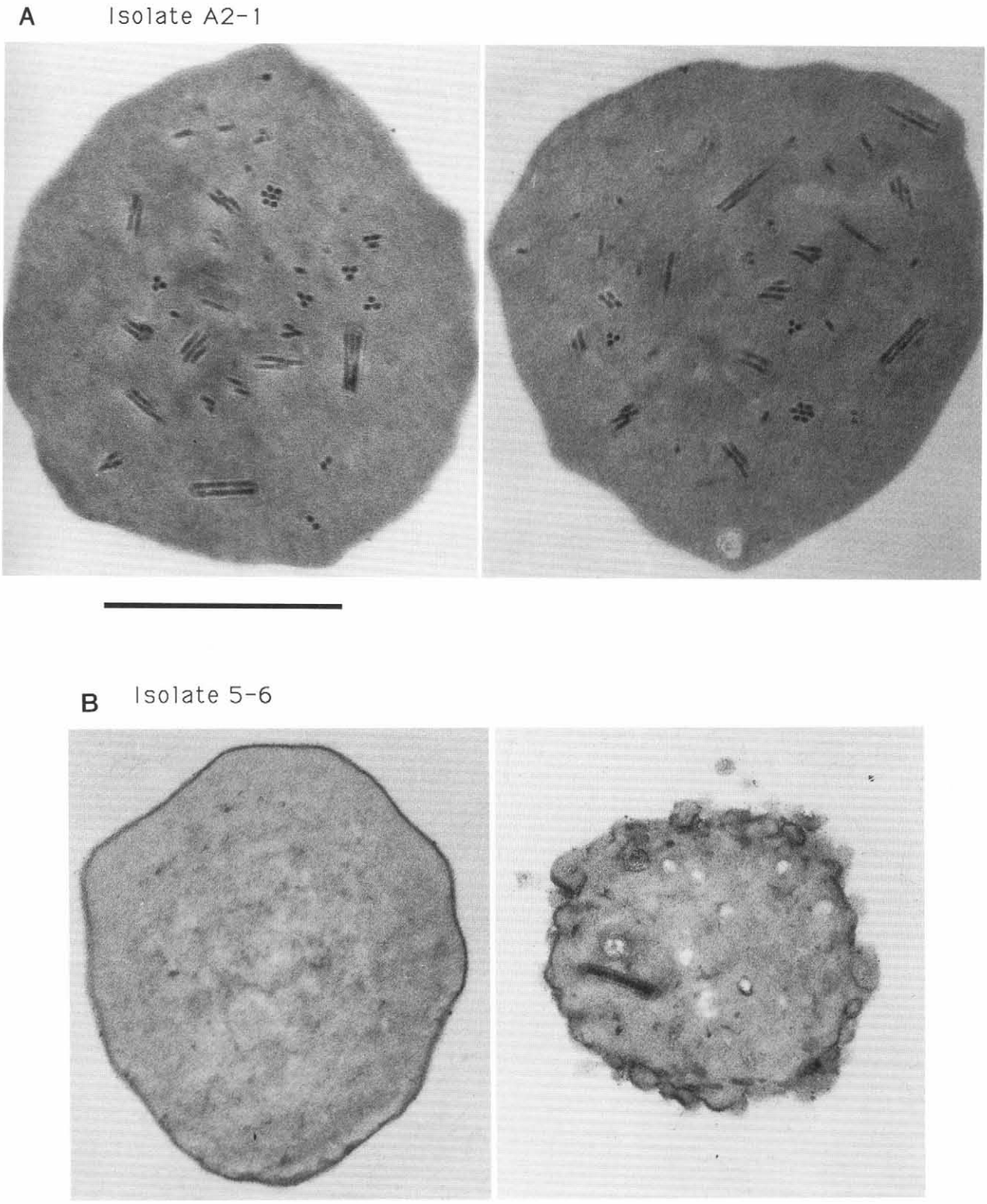


FIG. 4. Electron micrographs of polyhedra cross sections. Cross sections of two representative polyhedra produced in fourth instar gypsy moth larvae from isolates A2-1 and 5-6 are shown in parts A and B, respectively. Most cross sections of isolate 5-6 polyhedra were devoid of virions; however, a virion is evident in one of the isolate 5-6 cross sections (scale line = 1 μM).

ity of 1 $TCID_{50}$ unit per cell, and at least 200 cells containing polyhedra from each isolate were examined for this analysis. With isolate A2-1, 15, 22, 17.5, and 45.5% of the cells contained approximately 1–5, 6–10, 11–15, and >15 polyhedra/cell, respectively (Fig. 3B). In contrast, 91, 17, and 2% of the cells infected with isolate 5-6 contained approximately 1–5, 6–10, and 11–15 polyhedra/cell, respectively. Polyhedra production in *L. dispar* fourth instar larvae was also investigated. Larvae were infected with isolate A2-1 per os and with isolate 5-6 by injection of NOV in cell culture media into the larval hemocoel. Polyhedra were isolated after larval death and quantified. Larvae infected with isolate A2-1 yielded an average of 2.1×10^9 polyhedra/larva (Fig. 3C). In contrast, larvae infected with isolate 5-6 produced an average of 8.6×10^7 polyhedra/larva. In 652Y cells and *in vivo* isolate 5-6 produced approximately 10× and 20× less polyhedra, respectively, compared to isolate A2-1. In addition, most 652Y cells infected with isolate 5-6 contained only 1–5 polyhedra/cell. The few polyhedra produced by isolate 5-6 is characteristic of FP plaque mutants (8, 11, 20).

The relative number of virions present within polyhedra generated by isolates A2-1 and 5-6 in fourth instar *L. dispar* larvae was investigated through electron microscopic examination of sectioned polyhedra. Each beam section contained approximately 120 polyhedra that were sectioned randomly with respect to the cutting plane thereby generating representative cross sections from all areas of the polyhedra. Numerous virions are evident in electron micrographs of cross sections of polyhedra produced by isolate A2-1, and all cross sections contained virions (Fig. 4A). In contrast, few virions are present in cross sections of polyhedra generated by isolate 5-6 (Fig. 4B). The number of virions observed in polyhedra cross sections ranged from 8 to 76 for isolate A2-1, and from 0 to 2 for isolate 5-6. Virions were not evident in most isolate 5-6 polyhedra cross sections. The number of virions present within polyhedra cross sections was quantified and expressed as the number of virions per square micrometer of polyhedra surface area (Table 1). An average of 12.2 virions/μm^2 were present in cross sections of isolate A2-1 polyhedra, in contrast to a value of an average of 0.13 virions/μm^2 in isolate 5-6 polyhedra, a difference of approximately 94-fold. When these values are extrapolated to make a volumetric comparison, polyhedra produced by isolate A2-1 contains approximately 900-fold more virions/μm^3 than isolate 5-6.

An *in vivo* bioassay of isolates A2-1 and 5-6 revealed that they were distinctly different with regard to potency (Table 2). The LC_{50} of A2-1 for second instar gypsy moth larvae was 9.90×10^3 polyhedra/ml diet. Isolate 5-6 killed no larvae, even at the highest dosage (7.4×10^6 polyhedra/ml diet). Though an LC_{50} value for 5-6 could not be calculated from the data, it is clearly in excess of 7.4×10^6 polyhedra/ml diet; a dosage of gypsy moth NPV that normally elicits >95% mortality in test larvae. These results reflect the difference in virions/polyhedra between the FP variant 5-6 and the wild-type A2-1. Isolate A2-1 was indistinguishable from LDP 226 (standard) on the basis of LC_{50} values. However, the slope of the A2-1 dose–response curve (2.68 ± 0.35) was significantly steeper ($P < 0.05$) than that of LDP 226 (1.79 ± 0.23). This is, in part, a reflection of the genetic purity of A2-1 relative to the diverse genetic nature of LDP 226. Of greater practical significance is the twofold lower LC_{90} value seen for A2-1 compared to LDP 226. This suggests that either A2-1 or perhaps other plaque purified isolates from the Gypchek mixture of genotypes may have potency properties that would promote their candidacy as gypsy moth control agents.

The characteristics of a high NOV titer, production of few polyhedra *in vivo* and in cell culture, polyhedra containing few occluded virions, and an extremely low potency are consistent with the attributes of few polyhedra plaque mutants as exemplified by AcMNPV FP mutants. The characteristics exhibited by isolate A2-1, a lower NOV titer, production of many polyhedra *in vivo* and *in vitro,* polyhedra containing many virions, and a potency similar to Gypchek and other LdMNPV isolates indicate that this is a many polyhedra isolate. Further studies on isolate 5-6 to discern the molecular basis for the FP phenotype are in progress. Once the molecular basis for FP mutants generated by cell culture passage is understood, a means of preventing this type of mutation, possibly by the alteration of preferential genomic insertion sites, may be devised which

TABLE 1

Number of Virions within Cross Sections of A2-1 and 5-6 Polyhedra Produced in Fourth Instar Gypsy Moth Larvae

Isolate	Number of polyhedra cross sections examined	Average diameter of polyhedra cross sections	Total number of virions	Number of virions[a] per μm^2
A2-1	25	2.0 μm	941	12.2 ± 5.5
5-6	59	1.4 μm	14	0.13

[a] ±SD.

TABLE 2

Lethal Concentrations of A2-1, 5-6, and LDP 226 for Second Instar Gypsy Moth Larvae

Isolate	LC_{50} (95% FL)[a]	LC_{90} (95% FL)	Slope[b]	PR[c]
A2-1	9.90 (7.29–13.47)	29.67 (20.80–49.6)	2.68 ± 0.35	0.94
5-6	>7400	—	—	<0.0013
LDP 226	9.31 (6.46–13.50)	48.61 (30.37–97.42)	1.79 ± 0.23	—

[a] Polyhedra/ml diet × 10^3; FL, fiducial limits.
[b] ±SEM.
[c] Potency ratio = LC_{50} LDP 226/ LC_{50} isolate.

would facilitate development of LdMNPV *in vitro* production methodologies.

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REFERENCES

1. Beames, B., and Summers, M. D. 1989. Location and nucleotide sequence of the 25K protein missing from baculovirus few polyhedra (FP) mutants. *Virology* **168**, 344–353.
2. Beames, B., and Summers, M. D. 1990. Sequence comparison of cellular and viral copies of host cell DNA insertions found in *Autographa californica* nuclear polyhedrosis virus. *Virology* **174**, 354–363.
3. 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, DC.
4. 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.
5. Doefler, W., and Bohm, P. 1986. The Molecular Biology of Baculoviruses. *Curr. Top. Microbiol. Immunol.* **131**, 1–168.
6. Finney, D. J. 1971. "Probit Analysis." Cambridge Univ. Press, Cambridge.
7. Fraser, M. J., Brusca, J. S., Smith, G. E., and Summers, M. D. 1985. Transposon-mediated mutagenesis of a baculovirus. *Virology* **145**, 356–361.
8. 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.
9. 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.
10. Goodwin, R. H., Tompkins, G. J., and McCawley, P. 1978. Gypsy moth cell lines divergent in viral susceptibility. *In Vitro* **14**, 485–494.
11. 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. Pathol.* **27**, 49–55.
12. Kompier, R., Tramper, J., and Vlak, J. M. 1988. A continuous process for the production of baculovirus using insect-cell cultures. *Biotechnol. Lett.* **10**, 849–854.
13. 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.
14. Lewis, F. B., and Rollinson, W. D. 1978. Effect of storage on the virulence of gypsy moth nucleopolyhedrosis virus inclusion bodies. *J. Econ. Entomol.* **71**, 719–722.
15. Lynn, D. E., Dougherty, E. M., McClintock, J. T., and Shapiro, M. 1989. Comparative replication of *Lymantria dispar* nuclear polyhedrosis virus strains in three continuous-culture cell lines. *Appl. Environ. Microbiol.* **55**, 1049–1051.
16. McClintock, J. T., and Dougherty, E. M. 1988. Restriction mapping of *Lymantria dispar* nuclear polyhedrosis virus DNA: localization of the polyhedrin gene and identification of four homologous regions. *J. Gen. Virol.* **69**, 2303–2312.
17. Podgwaite, J. D. 1985. Strategies for field use of baculoviruses. *In* "Viral Insecticides for Biological Control" (K. Maramorosch and K. E. Sherman, Eds.), pp. 775–797. Academic Press, New York.
18. 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.
19. Potter, K. N., Jaques, R. P., and Faulkner, P. 1978. Modification of *Trichoplusia ni* polyhedrosis virus passaged *in vivo*. *Intervirology* **9**, 76–85.
20. Ramoska, W. A., and Hink, W. F. 1974. Electron microscope examination of two plaque variants from a nuclear polyhedrosis virus of the alfalfa looper, *Autographa californica*. *J. Invertebr. Pathol.* **23**, 197–201.
21. Reed, L. J., and Muench, H. 1938. A simple method of estimating fifty percent endpoints. *Am. J. Hyg.* **27**, 493–497.
22. Russell, R. M., Robertson, J. L., and Savin, N. E. 1977. POLO: A new computer program for probit analysis. *Bull. Entomol. Soc. Am.* **23**, 209–213.
23. Slavicek, J. M. 1991. Temporal analysis and spatial mapping of *Lymantria dispar* nuclear polyhedrosis virus transcripts and *in vitro* translation polypeptides. *Virus Research* **20**, 223–236.
24. Smith, I. R. L., and Crook, N. E. 1987. *In vivo* isolation of baculovirus genotypes. *Virology* **165**, 240–244.
25. Smith, I. R. L., van Beek, N. A. M., Podgwaite, J. D., and Wood, H. A. 1988. Physical map and polyhedrin gene sequence of *Lymantria dispar* nuclear polyhedrosis virus. *Gene* **71**, 97–105.
26. Stiles, B., Burand, J. P., Meda, M., and Wood, H. A. 1983. Characterization of gypsy moth (*Lymantria dispar*) nuclear polyhedrosis virus. *Appl. Environ. Microbiol.* **46**, 297–303.
27. Summers, M. D., and Smith, G. E. 1987. "A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures." Texas Agric. Exp. Station Bulletin No. 1555.
28. Tramper, J., and Vlak, J. M. 1986. Some engineering and economic aspects of continuous cultivation of insect cells for the production of baculoviruses. *Ann. N. Y. Acad. Sci.* **469**, 279–288.
29. Wu, J., King, G., Daugulis, A. J., Faulkner, P., Bone, D. H., and Goosen, M. F. A. 1989. Engineering aspects of insect cell suspension culture: A review. *Appl. Microbiol. Biotechnol.* **32**, 249–255.