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# Impact of Deletion of the *Lymantria dispar* Nucleopolyhedrovirus *PEP* Gene on Viral Potency: Expression of the Green Fluorescent Protein Prevents Larval Liquefaction

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**The *Lymantria dispar* multicapsid nucleopolyhedrovirus (Ld*M*NPV) is an effective biological control agent of the gypsy moth, *L. dispar*, but is not in general use because the high cost of production limits availability. In an effort to generate a more cost efficient Ld*M*NPV biopesticide, two recombinant baculoviruses were generated to evaluate the impact of deleting the *polyhedral envelope protein* (*PEP*) gene on viral potency. One of these baculoviruses had the *PEP* gene inactivated by insertional mutagenesis with the *green fluorescent protein* (*GFP*) gene from *Aequorea victoria* (vGFP: PEP-); and the other contained a partial deletion of the *PEP* gene from the Ld*M*NPV viral genome (vPEP-). Both recombinant baculoviruses produced polyhedra that were lacking a polyhedral envelope and displayed an unusually pitted surface as analyzed by scanning and transmission electron microscopy. Polyhedra produced by vGFP:PEP- were considerably smaller than those produced by vPEP- or wild-type virus. Although vPEP- and vGFP:PEP- polyhedra exhibited abnormal phenotypes, bioassay results indicated that there was no significant difference in the potency of these polyhedral envelope-lacking polyhedra in comparison to wild-type polyhedra. In addition, expression of *GFP* prevented virus-induced larval liquefaction upon death, and the deletion of *PEP* prevented lysis of infected cells in culture.**

***Key Words:*** **baculovirus; *Lymantria dispar* nuclear polyhedrosis virus; *Lymantria dispar;* gypsy moth; *polyhedral envelope protein.***

## INTRODUCTION

Nucleopolyhedroviruses (NPVs) are members of the Baculoviridae that infect insects and other arthropods. All baculoviruses have two distinct morphological forms, producing both nonoccluded and occluded virus. Larvae become infected upon ingestion of the occluded form or "polyhedra" and the subsequent release of viral particles from the polyhedron in the alkaline midgut of the insect. Shortly after infection, nonoccluded virus is produced that infects different cell types within the larvae. Later during the infection cycle, polyhedra are produced in the infected cells. The viral life cycle is complete upon death of the larvae and release of the polyhedra to the environment (for review see Blissard and Rohrmann, 1990). In the *Autographa californica* multicapsid NPV (Ac*M*NPV), the prototype baculovirus, this release is mediated by two viral-encoded enzymes, cathepsin and chitinase, which together cause the larvae to disintegrate or "liquefy" (Hawtin *et al.*, 1997).

The polyhedra are protected from the environment by a polyhedral envelope (PE) composed mainly of carbohydrates (Minion *et al.*, 1979). The main protein component of the PE is the polyhedral envelope protein (PEP) (Gombart *et al.*, 1989; Russell and Rohrmann, 1990). Inactivation of the *PEP* gene in Ac*M*NPV results in polyhedra that lack a PE, are easily fractured, and rapidly release viral particles in an alkaline environment (Zuidema *et al.*, 1989). These polyhedra are six-fold more potent than the wild-type Ac*M*NPV polyhedra, probably due to the quick release of the viral particles in the alkaline midgut of the insect (Ignoffo *et al.*, 1995).

The *Lymantria dispar* multicapsid nucleopolyhedrovirus (Ld*M*NPV) is pathogenic to the gypsy moth, *Lymantria dispar* (L.). This insect is a serious defoliating pest in the northeastern United States that can feed on more than 480 species of trees. In years of high population densities, nearly complete defoliation of forest stands can occur, which often results in high levels of tree mortality. In 1981 and 1990 approximately 5 and 3 million hectares of forest were defoliated, respectively, by the gypsy moth. Because the Ld*M*NPV is specific for the gypsy moth, it is an attractive biological control agent that can be used as an alternative to the more commonly used nonspecific

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controls, *Bacillus thuringiensis* (Berliner) (Bt) and chemicals. Unfortunately, Ld*M*NPV is not widely used to control the gypsy moth due to the high cost of production of the virus *in vivo*. To encourage commercial scale production of the Ld*M*NPV, we have sought to decrease the cost of usage by enhancement of viral potency. Potency enhancement through deletion of the Ld*M*NPV *PEP* gene seemed plausible based on results observed in the Ac*M*NPV *PEP* minus virus (Ignoffo *et al.*, 1995). A homologue to the Ac*M*NPV *PEP* gene has been identified in the Ld*M*NPV (Bjornson and Rohrmann, 1992). The Ac*M*NPV and Ld*M*NPV PEPs are similar in size (322 vs 312 amino acids) and exhibit 27% amino acid identity. Because inactivation of the Ac*M*NPV *PEP* gene resulted in an increase in viral potency, we explored the possibility of generating a more potent Ld*M*NPV through inactivation of the Ld*M*NPV *PEP* gene.

## MATERIALS AND METHODS

### *Cells, Virus, and Insects*

*Lymantria dispar* 652Y (L*d*652Y) cells were grown as monolayers in Goodwin's IPL-52B medium supplemented with 6.25 mM glutamine and 10% fetal bovine serum. Cell cultures were inoculated with either Ld*M*NPV isolate A21-MPV, which produces wild-type polyhedra (Slavicek *et al.*, 1996), the vGFP:PEP- recombinant, or the vPEP-recombinant virus. *L. dispar* egg masses were obtained from the U.S. Department of Agriculture, Animal and Plant Health Inspection Service rearing facility at Otis Air Force Base, MA. Hatched larvae were reared on gypsy moth diet (Bell *et al.*, 1981).

### *Construction of PEP-Inactivated Viruses*

The 2.0-kb *Bam*HI fragment (140.0 to 142.0 kb on the viral genome) containing the entire *PEP* gene was subcloned into pBluescript (Stratagene, La Jolla, CA) to generate pDB171. This *PEP* gene was inactivated in pDB172 by deletion of a 0.46-kb *Sun*I/*Eco*RI fragment internal to the *PEP* gene and replacement of this fragment with a 1.1-kb cassette containing the *green fluorescent protein* (*GFP*) gene, from the bioluminescent jellyfish *Aequorea victoria* (Shimomura) (Prasher *et al.*, 1992), under control of the Ld*M*NPV *polyhedrin* (*polh*) promoter (Bjornson and Rohrmann, 1992). This cassette was created by annealing two pairs of phosphorylated complementary oligonucleotides (gggcccgggatccgggcgcgcgggcggtctccaataagta and, cgcccgggccctaggcccgcgcgcccgccagaggttattcataaaataag; tttttattcttttcgtaaagattttggaaaaatcaaatacaccgtaaaatg and, aaaagcatttctaaaaccttttagtttatgtggcattttacctag) together and then ligating the annealed pairs to reconstruct the 89-bp of sequence upstream of the *polh* ATG start codon. This *polh* fragment has *Sst*II and *Bam*HI cohesive ends and was subcloned upstream of the *GFP* coding sequence in pGFP-1 (Clonetech, Palo Alto, CA). This resulted in the addition of 7 aa to the N-terminus of the GFP protein. After digestion with *Mlu*I and *Eco*R47III and then endfilling the *Mlu*I site to create a blunt end, this *polh*::*GFP* cassette was subcloned into the *Eco*RV site of pBluescript generating a plasmid with several sites directly upstream and downstream of the *polh*::*GFP* cassette that could be used to inactivate genes. The cassette was isolated on a *Sma*I/*Eco*RI fragment and cloned into the *Sun*I and *Eco*RI sites within the *PEP* gene after endfilling the *Sun*I site to generate a blunt end. To create the vGFP:PEP- isolate, Ld652Y cells were transfected with 2.5 μg of A21-MPV viral DNA and 2.5 μg of pDB172 plasmid DNA using the Lipofectin reagent (Bethesda Research Laboratories, Gaithersburg, MD) as described previously (Bischoff and Slavicek, 1996). At 7 days post-infection (p.i.), the cells and medium (3 ml) were collected and diluted with 12 ml fresh medium in a T75 flask. The nonoccluded virus was collected at 7 days p.i. and plaque purified. Recombinant plaques were identified by fluorescence microscopy ($\lambda_{ex} = 395$, $\lambda_{em} = 512$ nm).

A second recombinant virus was also constructed in which the *PEP* gene was partially deleted. pDB179 was derived from pDB171 with a 0.6-kb *Apo*I/*Eco*RI N-terminal deletion of the *PEP* gene. This construct lacks the first 178 aa of PEP. To create the vPEP- strain, cells were transfected with vGFP:PEP- DNA and pDB179 as described above. Recombinant plaques were identified by lack of fluorescence.

### *Viral DNA Isolation and Southern Blot Analysis*

Nonoccluded virus was isolated from infected *Ld*652Y cells as described previously (Bischoff and Slavicek, 1994) and used as a source of genomic DNA for restriction analysis. The viral DNA was digested with restriction endonucleases and fractionated on 1.0% agarose-TBE gels. Southern blot analysis was performed on nitrocellulose using a probe labeled with a Nick-Translation kit (Bethesda Research Laboratories) and [$\alpha$-$^{32}$P]dCTP (NEN).

### *Analysis of Polyhedra Production*

T25 flasks were seeded with $1 \times 10^6$ cells/flask. The cells were allowed to attach for 1 h before addition of the virus at ten 50% tissue culture infectious dose ($TCID_{50}$) units per cell. The virus was removed after a 1-h adsorption period and the cells were covered with fresh medium. Cells were examined and polyhedra recovered at 7 days p.i. Polyhedra were isolated, purified, and quantitated as previously described (Slavicek

*et al.*, 1996). Briefly, polyhedra were pelleted by centrifugation at 550*g* for 5 min; the polyhedra pellet was resuspended in complete medium and then sonicated for 30 s. The number of polyhedra present was determined using a hemacytometer.

### *Scanning and Transmission Electron Microscopy*

Groups of fourth-instar *L. dispar* larvae were infected by injection of 5 µl of nonoccluded virus from isolates A21-MPV, vGFP:PEP-, or vPEP-. Dead larvae were collected daily and frozen. Twenty dead larvae from each group were pooled, and polyhedra were isolated as previously described (Slavicek *et al.*, 1992). Briefly, the larvae were homogenized and the homogenate was passed through a Buchner funnel lined with cheesecloth. The remaining debris was extensively washed, and the flow through was combined with the primary eluent. Polyhedra were then isolated and quantitated as described above.

For analysis by scanning electron microscopy (SEM), the polyhedra were placed on a specimen stub and coated with gold in a SPI sputter module (SPI 11430-AB). They were viewed with a LEO 435VP scanning electron microscope operating at 12.5 kV. The diameter of 100 polyhedra from each of the isolates was directly measured from photographs after SEM analysis. From these measurements, the average polyhedral volume was calculated assuming the polyhedra were spherical in shape.

For examination by transmission electron microscopy, polyhedra were fixed in sodium cacodylate buffer (SCB), pH 7.2 (0.05 M sodium cacodylate, 0.5 mM HCl), containing 1% glutaraldehyde for 1 h at 4°C. The samples were washed 3× in SCB over a 1-h time period. The samples were postfixed in SCB containing 2% osmium tetroxide for 2.5 h at ambient temperature, and then washed 3× in SCB over a 1-h 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

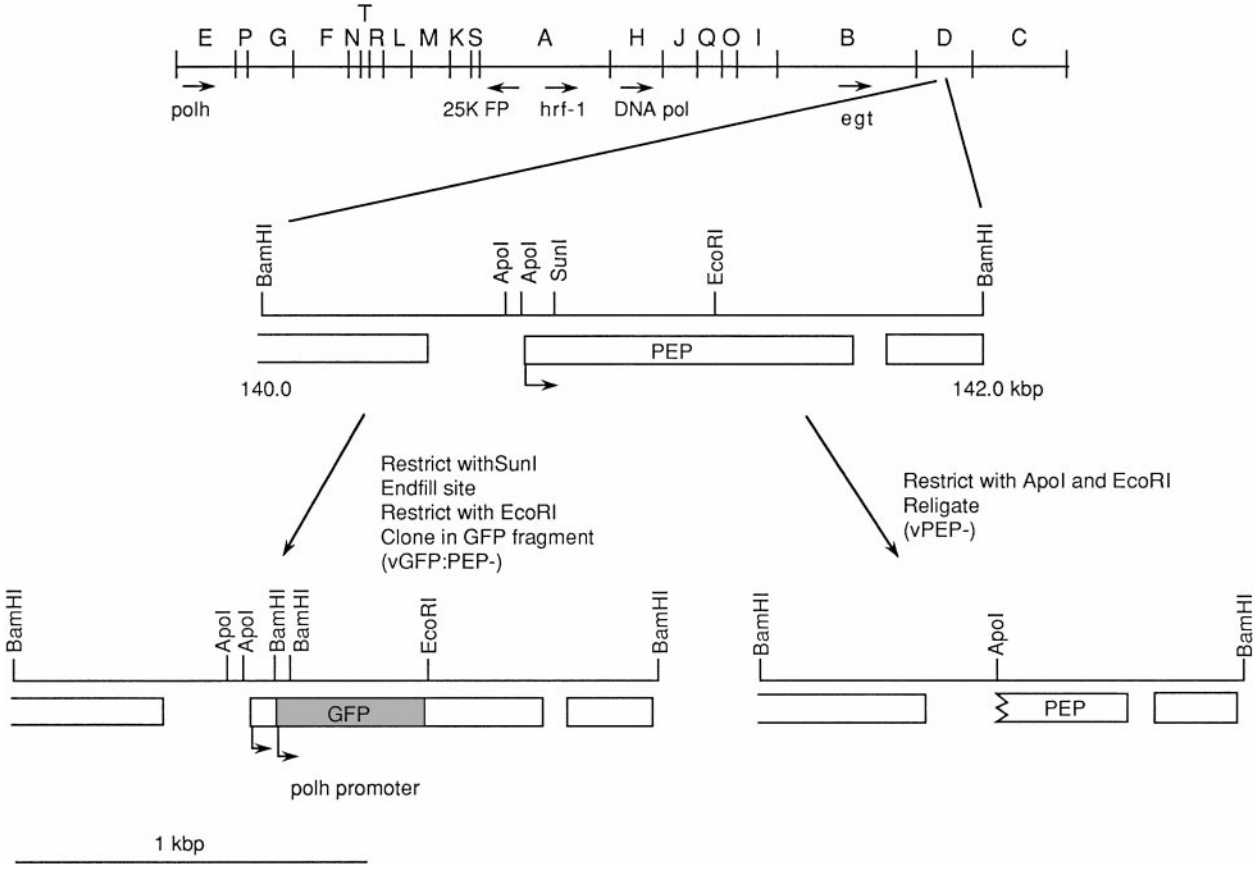


**FIG. 1.** Location of the Ld*M*NPV *PEP* gene and construction of vGFP:PEP- and vPEP-. The enlarged map below the A21-MPV *Bg*/II restriction map (Bischoff and Slavicek, 1996) shows the location of the *PEP* open reading frame at 140.0 to 142.2 kb on the viral genome (Bjornson and Rohrmann, 1992). Other known Ld*M*NPV genes are indicated as follows: polh, *polyhedrin* (Smith *et al.*, 1988); 25K FP, *25K few polyhedra* (Bischoff and Slavicek, 1996); EGT, *ecdysteriod UDP-glucosyltransferase* (Riegel *et al.*, 1994); DNA pol, *DNA polymerase* (Bjornson *et al.*, 1992); and hrf-1, *host range factor 1* (Thiem *et al.*, 1996). vGFP:PEP- was constructed by insertion of sequences containing the *GFP* gene between the *Sun*I (after end filling) and *Eco*RI site. vPEP- was created by deletion of the region between the first *Apo*I and the *Eco*RI sites.

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 h. The samples were then washed 3× (10 min each wash) in propylene oxide. The samples were infiltrated over a 24-h period with propylene oxide:Poly/Bed 812 (Polysciences, Warrington, PA) (25.55 g poly bed, 13.5 g dodecenylsuccinic anhydride, 10.9 g nadic methylanhydride) at 2:1, 1:1, and 1:2 ratios. 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 h, 45°C for 8 h, and finally at 60°C overnight. The samples were sectioned with a diamond knife on a Reichert-Jung Ultracut E43 microtome; the sections were stained for 20 m 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 JEOL JEM-1010 transmission electron microscope.

### *Insect Bioassays*

Fourth-instar *L. dispar* larvae were infected *per os* by placing them on a diet containing surface applied *in vitro* synthesized A21-MPV, vGFP:PEP-, or vPEP- polyhedra ($5 \times 10^6$ polyhedra total). The larvae were placed on fresh diet after 48 h on the virus-contaminated diet. Dead larvae were collected and used as a source of *in vivo* polyhedra for the bioassays. vGFP:PEP- polyhedra were initially tested for biological activity with the diet incorporation method (Ignoffo, 1965). Both vGFP:PEP- and vPEP- polyhedra were then bioassayed using the droplet feeding method (Hughes and Woods, 1981; Hughes *et al.*, 1986) as described previously (Bischoff and Slavicek, 1997). Dead larvae were removed and counted daily. The 50% lethal concentration ($LC_{50}$) and 50% lethal time ($LT_{50}$) values were determined for each virus by Probit analysis (Finney, 1971) using the POLO-PC program (LeOra Software, Berkeley, CA; Russell *et al.*, 1977). The 50% lethal dose ($LD_{50}$) was determined using the ViStat program (version 2.1; Boyce Thompson Institute, Cornell University, Ithaca, NY [Hughes, 1990]).

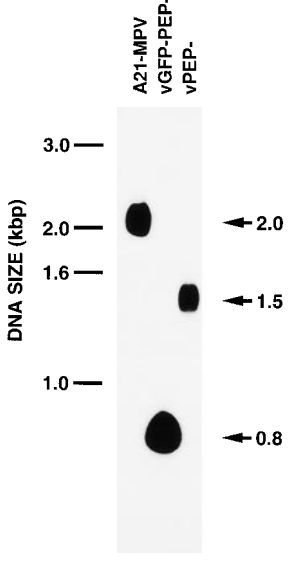


**FIG. 2.** Southern blot analysis of A21-MPV and the *PEP* mutant viruses. Viral DNA was restricted with *Bam*HI and probed with the 0.8-kb *Bam*HI/*Sun*I fragment to confirm that the mutations were transferred to the viral genome. Size markers (in kb) are shown to the left and the size of the hybridizing fragments to the right.

A

Number polyhedra produced *in vitro*

| Virus | Mean number of polyhedra ($1 \times 10^8$) | Std. Dev. ($1 \times 10^8$) | N |
|---|---|---|---|
| A21-MPV | 1.02 | 0.15 | 4 |
| vGFP:PEP- | 1.47 | 0.76 | 4 |
| vPEP- | 1.30 | 0.15 | 4 |

B

Diameter of polyhedra (μm)

| Virus | Mean Diameter (μm) | Std. Dev. | N | *P*-Value |
|---|---|---|---|---|
| A21-MPV | 2.14 | 0.38 | 100 | |
| vGFP:PEP- | 1.47 | 0.24 | 100 | <0.0001 |
| vPEP- | 2.00 | 0.38 | 100 | 0.0035 |

C

Volume of polyhedra ($\mu m^3$)

| Virus | Volume ($\mu m^3$) | volume % of wild type |
|---|---|---|
| A21-MPV | 5.13 | 100 |
| vGFP:PEP- | 1.66 | 32.4 |
| vPEP- | 4.19 | 81.7 |

**FIG. 3.** Polyhedra production characteristics of the A21-MPV, vGFP:PEP- or vPEP- isolates. (A) Number polyhedra produced *in vitro*. (B) Diameter of polyhedra measured from photographs after scanning electron microscopic analysis. (C) Calculation of polyhedral volume.

## RESULTS AND DISCUSSION

### *Construction of LdMNPV Isolates Lacking the Polyhedral Envelope Protein*

To ascertain the effect of the polyhedral envelope protein on Ld*M*NPV potency, two viruses were constructed in which the *PEP* gene had been disrupted (Fig. 1). The *PEP* gene is located at approximately 140.0 to 142.0 kb on the viral genome. A 2.0-kb *Bam*HI fragment encompassing this region was subcloned and the *PEP* gene was inactivated by replacement of the 0.46-kb *Sun*I/*Eco*RI fragment with a cassette containing the *GFP* gene under control of the *polh* promoter. In this case, *GFP* was used as a reporter gene to identify recombinant viruses by fluorescence microscopy (data not shown). The vPEP- isolate was constructed using a

plasmid in which the 0.6-kb *Apo*I/*Eco*RI fragment of *PEP* was removed. This allele, when transferred to the vGFP:PEP- viral genome, replaced the *GFP* gene, allowing recombinant viruses to be identified by the lack of fluorescence.

The *PEP*-inactivated isolates were plaque purified and verified by Southern blot analysis using the 0.8-kb *Bam*HI/*Sun*I fragment as a probe (Fig. 2). Viral DNA from A21-MPV, vGFP:PEP-, and vPEP- was isolated and restricted with *Bam*HI. In Lane 1, (A21-MPV), the 2.0-kb *Bam*HI fragment hybridized as expected. The vGFP:PEP- construct (Lane 2) has additional *Bam*HI sites at the start of the *GFP* gene; therefore, a 0.8-kb fragment hybridized. In vPEP- (Lane 3), a 1.5-kb fragment hybridized corresponding to the 2.0-kb *Bam*HI fragment with a 0.5-kb deletion within the *PEP* gene. These Southern blot results confirmed that the mutations were transferred to the viral genome and that the *PEP* gene was disrupted in both these isolates.

### *Analysis of Polyhedra Generated by the vGFP:PEP- and vPEP- Isolates*

Plaque-purified isolates of A21-MPV, vGFP:PEP-, and vPEP- were characterized by polyhedra production. There was no significant difference (ANOVA with Fisher's PLSD, $P < 0.05$) in the amounts of polyhedra produced by the *PEP* mutant isolates and isolate A21-MPV either *in vitro* (ranging from $8 \times 10^7$ to $2 \times 10^8$ polyhedra/flask; Fig. 3A) or *in vivo* (approximately $1 \times 10^9$ polyhedra per larvae). Visual examination by light microscopy revealed that cells infected with isolates vGFP:PEP- and vPEP- did not undergo cell lysis by 7 days p.i. and therefore do not release polyhedra into the medium (Fig. 4). Even after 2 weeks p.i., the cells did not lyse when infected with either the vGFP:PEP- or vPEP- viruses (data not shown). The lack of lysis appears to be due to inactivation of the *PEP* gene and not the expression of *GFP*, since both the vGFP:PEP- and vPEP- viruses exhibit this phenotype. Since there is no impact on the quantity and only a small impact on the size (see below) of the polyhedra produced in the vPEP- virus, the lack of cell lysis is probably not due to less pressure on the cell plasma membranes as a consequence of decreased polyhedral mass. Earlier investigations on *PEP* minus mutants of Ac*M*NPV (Zuidema *et al.*, 1989) and *Orgyia pseudotsugata M*NPV (Gross *et al.*, 1994) did not note this effect on cell lysis. *Ld*652Y cells infected with vGFP:PEP- did exhibit the characteristic GFP fluorescence when illuminated with blue light (data not shown).

Scanning electron microscopic analysis of the polyhedra produced by vGFP:PEP- and vPEP- revealed that they are smaller than wild-type polyhedra (Fig. 5A). A21-MPV polyhedra have an average diameter of 2.14 μm; whereas vGFP:PEP- polyhedra are much smaller at 1.47 μm and the vPEP- polyhedra slightly smaller at 2.00 μm in diameter (Fig. 3B). If the polyhedra are considered to be spherical in shape, the volume of vGFP:PEP- polyhedra are 32% that of the wild-type; whereas those of vPEP- have 81% of the volume of wild-type polyhedra (Fig. 3C). Polyhedra produced by vGFP:PEP- and vPEP- isolates have a pitted surface in contrast to the smooth appearance of completed A21-MPV polyhedra (Fig. 5A). Incomplete A21-MPV polyhe-

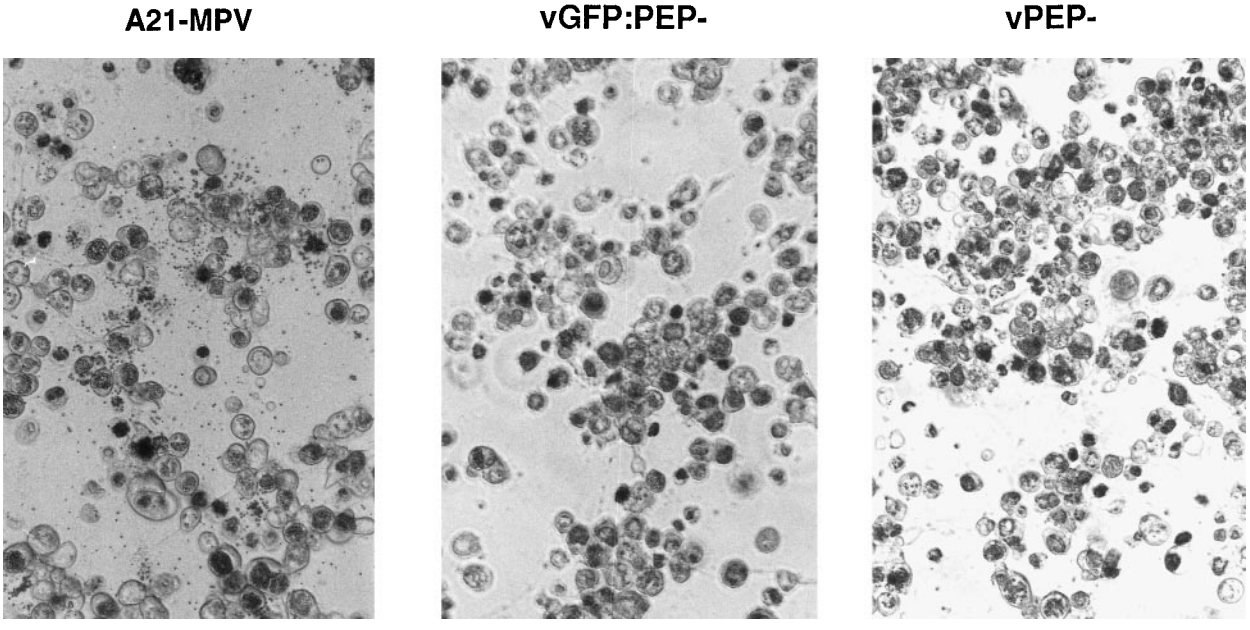


**FIG. 4.** Analysis of cells infected with A21-MPV, vGFP:PEP-, or vPEP-. Ld625Y cells were infected for 1 h at a multiplicity of infection of 10. At 7 days p.i., the cells were examined by light microscopy and photographed at 100× magnification.

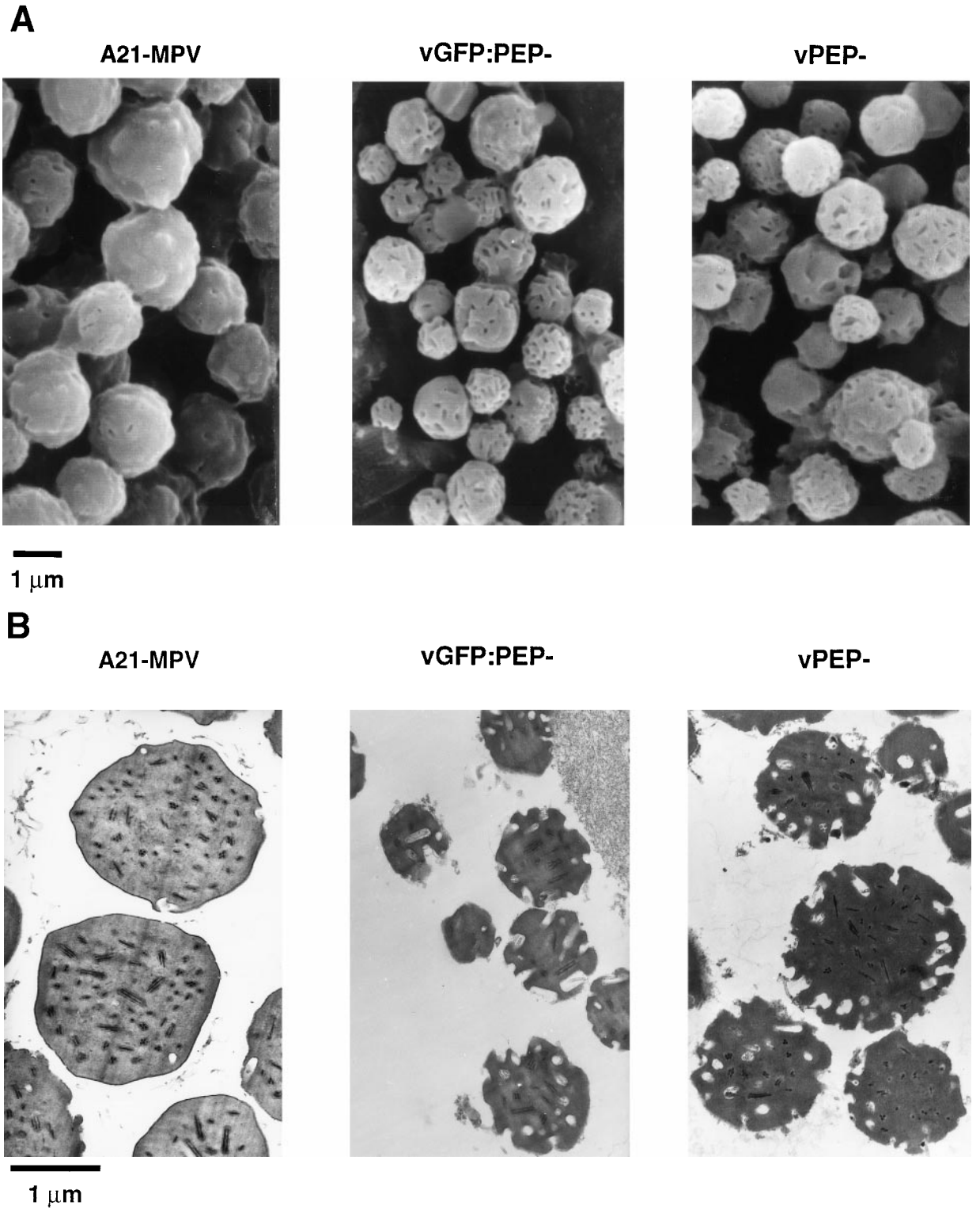


**FIG. 5.** Scanning and transmission electron microscopic analysis of polyhedra. The polyhedra were isolated from larvae infected with A21-MPV, vGFP:PEP-, or vPEP-. (A) Polyhedra were subjected to sputter coating with gold, visualized by scanning electron microscopy, and photographed at 7,000× magnification. (B) Polyhedra were sectioned, analyzed by transmission electron microscopy, and photographed at 12,000× magnification.

**TABLE 1**

Dose–Mortality Response of *Lymantria dispar* Larvae Infected *per os* with LdMNPV Isolates A21-MPV, vGFP:PEP−, or vPEP−

| Bioassay[a] | Virus | $LC_{50}$[b] | 95% FL[b] | Potency ratio[c] |
|---|---|---|---|---|
| 1 | A21-MPV | 5.4 | 3.8–7.6 | |
| 1 | vGFP:PEP− | 32.0 | 22–46 | 5.9 |
| 2 | A21-MPV | 6.9 | 2.6–12.4 | |
| 2 | vGFP:PEP− | 6.8 | 1.8–13.5 | 0.99 |
| 2 | vPEP− | 7.2 | 3.0–12.5 | 1.04 |
| 3 | A21-MPV | 135.0 | 64–1052 | |
| 3 | vGFP:PEP− | 235.0 | 125–839 | 1.74 |
| 3 | vPEP− | 63.0 | 37–130 | 0.47 |

[a] Assay 1, diet incorporation; assays 2 to 3, droplet feeding.
[b] Values are numbers of polyhedra per microliter of diet (assay 1) or solution (assays 2 and 3). FL, fiducial limits.
[c] vGFP:PEP− or vPEP− virus $LC_{50}$/A21-MPV $LC_{50}$.

dra that had not been completely enveloped also displayed pitted surfaces (Figs. 5A and 5B). However, complete A21-MPV polyhedra exhibited continuous envelopes and smooth surfaces.

Analysis of polyhedral cross-sections by transmission electron microscopy revealed that vGFP:PEP- and vPEP- polyhedra lacked the polyhedral envelope seen as a dark ring around the cross-sections of wild-type polyhedra (Fig. 5B). These polyhedral cross-sections also have rod-shaped holes in which nucleocapsids have been lost during sample preparation, possibly indicating a lack of nucleocapsid stability within polyhedra due to loss of the polyhedron envelope. Therefore, inactivation of the *PEP* gene resulted in the production of smaller polyhedra that lacked a polyhedron envelope, and prevented virus-induced lysis of infected Ld*M*NPV cells. Since the polyhedra produced by vGFP:PEP- are even smaller than those produced by vPEP-, expression of *GFP* also has an effect on the size of the polyhedra produced by that virus.

### *Biological Activity of the A21-MPV, vGFP:PEP-, and vPEP- Isolates*

Diet incorporation and droplet feeding bioassays were conducted with *L. dispar* larvae infected with either A21-MPV or one of the *PEP* mutant isolates and the potency of the isolates was determined by Probit analysis (Table I). The vPEP- isolate had similar $LC_{50}$ (Table I) and $LD_{50}$ values (data not shown) to A21-MPV with overlapping fiducial limits within each bioassay. The vGFP:PEP- isolate exhibited a decrease of approximately sixfold in potency in the diet incorporation (Bioassay 1) and a decrease of about twofold in one of the droplet feeding bioassays (Bioassay 3). This decrease in potency was not found to be significant by analysis of variance (ANOVA; $P < 0.05$) and may be due to the smaller polyhedral volume of the vGFP:PEP- polyhedra in comparison to the A21-MPV polyhedra. Analysis of the time–mortality response shows similar killing speeds for all three viruses (data not shown). Therefore, even with the altered phenotype of the polyhedra produced in the Ld*M*NPV *PEP* mutant vi-

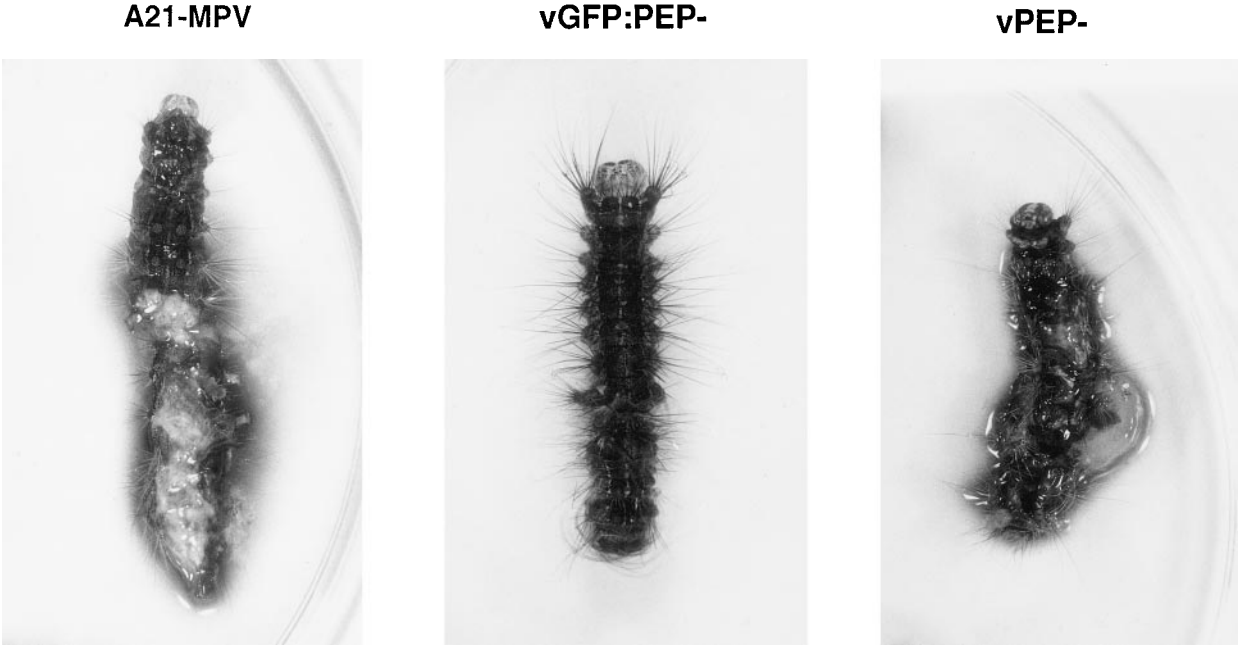


**FIG. 6.** Appearance of *Lymantria dispar* larvae infected with A21-MPV, vGFP:PEP-, or vPEP-. Fourth-instar *Lymantria dispar* larvae were infected by injection with 5 µl of nonoccluded virus from A21-MPV, vGFP:PEP-, or vPEP-, and photographed after death.

ruses, there is no significant difference in the potency or killing speed of these viral isolates compared to wild-type virus.

During the bioassays it was apparent that larvae infected with vGFP:PEP- did not readily release polyhedra upon death as is typically seen in a wild-type infection. In addition, larvae infected with the vGFP:PEP- did not fluoresce when illuminated with blue or UV light (data not shown) as has been seen with larvae of the diamondback moth, *Plutella xylostella* L. (Chao *et al.*, 1996) and *Trichoplusia ni* (Hübner) (Barrett *et al.*, 1998), after infection with recombinant GFP-expressing Ac*M*NPV. Normally the release of polyhedra to the environment is due to a process referred to as "liquefaction" in which viral-encoded enzymes (cathepsin and chitinase) degrade and liquefy the larvae after death (Hawtin *et al.*, 1997). To further document the lack of liquefaction by vGFP:PEP-, fourth-instar *L. dispar* larvae were injected with nonoccluded virus and photographed after death. Larvae infected with A21-MPV or vPEP- liqueified after death (Fig. 6), whereas those infected with vGFP:PEP- did not liquefy but shriveled upon death. The reason for the lack of liquefaction in larvae infected by this isolate is unknown. Since infection with vPEP- does result in liquefaction of larvae, it is not due to the inactivation of the *PEP* gene alone. One possibility is that the large amount of GFP produced under the *polh* promoter is interfering directly or indirectly with the function of the cathepsin or chitinase in Ld*M*NPV-infected larvae. Another possibility is that both the inactivation of the *PEP* gene and the high levels of *GFP* expression together result in the lack of larval liquefaction. It is interesting to note that inactivation of the *PEP* gene was sufficient to prevent lysis of infected cells in culture with or without expression of *GFP*, yet larval liquefaction was only affected when *GFP* was expressed.

The results of this study demonstrate that the same genetic manipulation in one baculovirus will not necessarily have the same effect in another baculovirus or host under investigation. Previously it was reported that inactivation of the *PEP* gene in the Ac*M*NPV resulted in a virus that had a sixfold increase in potency compared to wild-type virus. The results of our study indicate that inactivation of the Ld*M*NPV *PEP* gene did have an effect on polyhedron size and structure but did not impact viral potency. The increase in potency of the Ac*M*NPV *PEP* mutant virus was thought to be due to a quicker release of the viral particles due to the absence of the polyhedral envelope. Possibly the presence or absence of a polyhedral envelope in the Ld*M*NPV has no effect on the speed of dissolution of the polyhedra or release of nucleocapsids. Another alternative is that the nucleocapsids are released more quickly from *PEP*$^-$ Ld*M*NPV polyhedra than from wild-type polyhedra, but that the speed of release does not impact the rate of entry into gypsy moth midgut cells. In addition, the results of the vGFP:PEP- suggest that caution be exercised in the use of the GFP as a marker in studies of viral pathogenesis. In this investigation, expression of the *GFP* gene decreased polyhedral size and prevented larval liquefaction, which were independent of inactivation of the *PEP* gene. Again, this may be species dependent as this gene has been successfully used in other baculovirus/host systems.

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