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Deletion of the *Lymantria dispar* Multicapsid Nucleopolyhedrovirus Ecdysteroid UDP-Glucosyl Transferase Gene Enhances Viral Killing Speed in the Last Instar of the Gypsy Moth

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The *Lymantria dispar* multicapsid nucleopolyhedrovirus (Ld*M*NPV) is used on a limited basis as a gypsy moth (*L. dispar*) control agent. In an effort to improve the efficacy (i.e., killing speed) of the Ld*M*NPV, we generated a recombinant viral strain (vEGT-) that does not produce the enzyme ecdysteroid UDP-glucosyltransferase (EGT). We compared the potency and efficacy of vEGT- to wild-type virus (A21-MPV) and the impact of vEGT- and A21-MPV on larval weight gain. Bioassay of *L. dispar* 1st, 4th, and 5th instars showed no significant difference in the LC_{50} values in larvae infected with vEGT- or A21-MPV. The LT_{50} values of 1st and 4th instar larvae infected with either virus were similar, but the LT_{50} value of 5th instar larvae infected with vEGT- was significantly lower by ca. 33% compared with larvae infected with A21-MPV. Female 4th and 5th instar larvae infected with vEGT- gained significantly less weight than those infected with A21-MPV, whereas male 4th and 5th instar larvae infected with either virus showed no significant difference in weight gain. Larval weight gain was also used as an indicator of feeding activity through generation of FT_{50} values (time when 50% of larvae ceased feeding). Fifth instar larvae infected with vEGT- exhibited a decrease in the FT_{50} of ca. 32% compared with A21-MPV-infected larvae. Comparison of the total number of polyhedra produced in vEGT- or A21-MPV infected larvae showed that significantly more polyhedra were produced in 5th instars infected with A21-MPV. Deletion of the Ld*M*NPV *egt* gene generated an improved virus strain since the killing speed of vEGT- is significantly faster than wild-type virus in 5th instar *L. dispar* larvae, and larvae infected with vEGT- stopped gaining weight earlier than larvae infected with wild-type virus. Because the majority of foliage consumption by *L. dispar* larvae occurs in the 5th instar, the use of vEGT- may offer better foliage protection than wild-type virus in the field.

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INTRODUCTION

The gypsy moth (*Lymantria dispar* L.) is a major exotic forest pest in the United States. First introduced in 1869 in Medford, Massachusetts, the gypsy moth has since expanded its range to include all of the northeastern United States and is currently spreading into the mid-Atlantic and midwestern states. The affected region is increasing at a rate of 8 to 24 km/year. Additionally, another strain, the Asian gypsy moth, was inadvertently introduced into the Pacific Northwest and North Carolina in recent years. The gypsy moth is a serious tree-defoliating insect pest and can feed on over 480 tree species (Liebhold *et al.*, 1995). Near complete defoliation of forest stands occurs in years of high population densities, which can result in high levels of tree mortality. In 1981 and 1990 approximately 5 and 3 million ha of forest were defoliated, respectively, by the gypsy moth.

Baculoviruses are a class of insect viruses that have been used on a limited basis for control of insect pests in agriculture and forestry. One of these viruses, the *Lymantria dispar* multicapsid nucleopolyhedrovirus (Ld*M*NPV) has been registered by the U.S. Forest Service for use as a gypsy moth control agent. The Ld*M*NPV has the significant advantage of being specific for the gypsy moth in contrast to other control agents (Lewis and Podgwaite, 1981). Consequently, Ld*M*NPV is the agent of choice for use in environmentally sensitive areas. However, it is not used extensively for gypsy moth control due to high production costs and low potency (Podgwaite, 1981). Another disadvantage of the Ld*M*NPV compared with chemical controls is that the virus requires a long time period, approximately 8 to 14 days, to kill its host. In order to mitigate the disadvantages of Ld*M*NPV as a control

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agent, efforts are being made to develop improved viral strains (Slavicek *et al.*, 1996).

One method which has previously been used to enhance the efficacy (killing speed) of baculoviruses involves the *ecdysteroid UDP-glucosyltransferase* (*egt*) gene. Ecdysteroids are hormones that, in concert with juvenile hormone, control the larval-to-larval and larval-to-pupal molts of holometabolous insects (Smith, 1985; Koolman and Karlson, 1985). Upon increases in ecdysone titers, larvae cease to eat in preparation for molting. The *Autographa californica M* NPV (Ac*M* NPV) contains an *egt* gene which encodes a protein that inactivates ecdysteroids by sugar conjugation in infected larvae (O'Reilly and Miller, 1989, 1990). Because of this glycosylation, Ac*M* NPV-infected insects fail to molt and as a consequence continue eating and growing in the time that they normally would be in the wandering premolting condition. This is thought to provide a selective advantage for the virus, as the larger larvae produce more progeny virus. Insects infected with a strain of Ac*M* NPV lacking the *egt* gene exhibit reduced feeding and earlier mortality compared to wild-type Ac*M* NPV-infected larvae (O'Reilly and Miller, 1991; O'Reilly, 1995).

With an interest in generating an improved strain of Ld*M* NPV, we determined if viral efficacy could be enhanced through deletion of the *egt* gene. A homologue of the Ac*M* NPV *egt* gene in the Ld*M* NPV has been identified and characterized (Riegel *et al.*, 1994a). The predicted Ld*M* NPV-encoded EGT protein exhibits a 42% amino acid identity with the Ac*M* NPV EGT polypeptide. In this paper we describe the generation of a recombinant LdMNPV that does not produce an *egt* gene product and the impact of this gene's deletion on viral production, potency, and efficacy.

MATERIALS AND METHODS

Maintenance of Cells and Virus

All *in vitro* virus growth was carried out in *L. dispar* 652Y cells (Ld652Y). These cells were grown in Goodwin's IPL52B medium (JRH Biosciences, Lenexa, KS) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA) and 6.0 mM glutamine (Gibco BRL, Gaithersburg, MD) at 27°C. LdMNPV strain A21-MPV was used as the wild-type virus in this study (Slavicek *et al.*, 1996).

Generation of an EGT- Recombinant Virus

All cloning was carried out using standard techniques. The starting point for the generation of the recombinant virus was the plasmid pEGT4.9 (Riegel *et al.*, 1994a). The *Kpn*I site located in the polylinker portion of pEGT4.9 (a pUC18 derivative) was eliminated by cloning the *Bam*HI to *Eco*RI fragment of the polylinker from pBluescript KS+ (Stratagene, La Jolla, CA) into the *Bam*HI and *Eco*RI sites of pEGT4.9, creating pEGT4.9K- (Fig. 1). A linker oligonucleotide pair, 5′CTGGTACCAAAGATCTGC and ATAGGAC-CATGGTTTCTAGACGCCGG5′, which, when annealed, has *Bst*XI (compatible with the *Bst*XI site found within the Ld*M* NPV *egt* gene) and *Not*I overhangs and contains a *Kpn*I site and a *Bgl*II site, was synthesized on an ABI Model 380A DNA synthesizer. This linker was cloned into the *Bst*XI and *Not*I sites of pEGT4.9K- in place of 872 bases of the coding sequence of the *egt* gene. This new plasmid, pEGT4.9K-link, was digested with *Kpn*I and *Bgl*II. The digested plasmid was ligated with the gel-purified *Kpn*I to *Bam*HI fragment of pCH110 (Pharmacia Biotech, Piscataway, NJ) containing the *Escherichia coli lac*Z gene. This construction created a translational fusion between the first 22 amino acids of EGT and β-galactosidase. This plasmid, pEGT-LacZ, was then used in lipofectin (Gibco BRL)-mediated cotransfections of Ld652Y cells with DNA from isolate A21-MPV. Five micrograms of plasmid DNA was mixed with 5 μg A21-MPV viral DNA and mixed in 100 μL TE. This DNA mixture was mixed with 100 μL of lipofectin and added to 2.1×10^5 Ld652Y cells which were in 1.5 mL serum-free medium without gentimycin. The medium from transfections in P6 wells was used to carry out plaque assays. Individual plaques were propagated in 96-well microtiter plates. An aliquot (30 μls) of budded virus-containing medium was dot blotted and probed with a [α-^{32}P]dCTP (DuPont NEN, Boston, MA)-labeled fragment from pCH110 (*Kpn*I to *Bam*HI) that contains the *lac*Z gene DNA (nick translation kit, Gibco BRL). Three positive plaques were found. These plaques were amplified in tissue culture flasks, and re-plaque purified. These plaques were dot blotted as above and four positive plaques were obtained. These were propagated and one was chosen as the vEGT- strain of Ld*M* NPV.

Southern, Northern, and Sequence Analysis of the Recombinant Virus

Budded virus was harvested from Ld652Y cells infected with isolates A21-MPV or vEGT- as previously described (Bischoff and Slavicek, 1994) and used as a source of genomic DNA for restriction analysis. Viral DNA was digested with restriction endonucleases and fractionated on 0.8% agarose–Tris–borate–EDTA gels. Southern blot analysis was performed on nitrocellulose with probes labeled by nick translation with [α-^{32}P]dCTP.

Tissue culture flasks (25 cm^2) were seeded with 2×10^6 Ld652Y cells. The cells were infected with either LdMNPV isolate A21-MPV or vEGT- at 10 tissue culture infectious doses ($TCID_{50}$) units per cell for 1 h at 27°C. At that time the inoculum was replaced with 5 ml of fresh medium. Cells were harvested at 0, 4, 16, 24,

and 48 h post-infection (h p.i., counted from the end of the 1-h adsorption period). Total cytoplasmic RNA was isolated following the method of Friesen and Miller (1985). RNA was separated on a 20-cm 1.2% agarose gel containing formaldehyde by overnight electrophoresis at 30 V, and the gel was blotted. A 0.9-kb *Bst*XI to *Not*I fragment (which was the deleted region in vEGT-) and a 3.5-kb *Hin*dIII to *Bam*HI fragment containing the *lac*Z gene from pCH110 were labeled with [α-^{32}P]dCTP by nick translation. These probes were hybridized to duplicate blots and washed according to the phosphate buffer procedure of Mahmoudi and Lin (1989). The blot was exposed to Kodak XAR-5 film in cassettes containing X-Omatic intensifying screens (Kodak, Rochester, NY).

vEGT- DNA was purified from budded virus as previously described (Bischoff and Slavicek, 1994). DNA sequencing was carried out directly on the viral DNA using Taq sequencing (fmol kit, Promega, Madison, WI) and [γ-^{32}P]ATP end-labeled internal sequencing primers from the *egt* gene.

Assay of Larval Weight Gain

Polyhedra were isolated from Ld652Y cells infected with isolates vEGT- or A21-MPV as previously described (Slavicek *et al.*, 1995) and were used to infect 4th instar *L. dispar* larvae to generate *in vivo*-produced polyhedra. These polyhedra were isolated as previously described (Bischoff and Slavicek, 1997) and used for larval weight gain studies and bioassays. Larvae were chosen at random, placed into individual containers, and starved overnight. Fourth and 5th instar larvae were infected with isolates vEGT- or A21-MPV *per os* by allowing them to feed on a diet plug containing 8×10^6 or 1.6×10^7 polyhedral inclusion bodies (PIBs), respectively. Control larvae were mock infected by allowing them to feed on virus-free diet plugs. Fifty larvae were used for each treatment. Larvae that failed to consume all of their diet within 24 h were discarded. Larvae were weighed each day at the same time until they died or pupated. Fourth instar larvae were used 1 or 3 days after molting (L4D1 or L4D3) and 5th instars were used 3 days after molting (L5D3). Larvae were separated into male and female groups based on their weight at the time of infection. Control larvae were raised to adults and the gender was recorded.

Bioassay Analysis of vEGT-

Neonate bioassays were conducted using the diet incorporation method as previously described (Bischoff and Slavicek, 1997). Fourth and 5th instar larvae were bioassayed using the droplet feeding method (Hughes *et al.*, 1986; Popham *et al.*, 1997). Larvae were fed known concentrations of PIBs (10^4 to 10^9 PIBs/ml) suspended in 5% sucrose and 5% green food color (McCormick & Co., Inc., Hunt Valley, MD). Larvae were placed on a clean plastic surface near a droplet of the PIB suspension (10–15 µl). When larvae had ingested the droplet (usually within 5 min) they were transferred to individual cups with fresh diet. Five virus concentrations with 30 insects/dose were tested for both viruses. Larvae were monitored until all larvae had either died or pupated and dead larvae were counted and removed daily. The 50% lethal concentrations (LC_{50}s) were determined by Probit analysis (Finney, 1971) by using the POLO-PC program (LeOra Software, Berkeley, CA; Russell *et al.*, 1977), and 50% lethal times (LT_{50}s) and 50% feeding times (FT_{50}s) were determined using the ViStat program (version 2.1; Boyce Thompson Institute, Cornell University, Ithaca, NY [Hughes, 1990]). Larval weights and weight gains and LT_{50} values were analyzed by analysis of variance (ANOVA, Fisher's PLSD) using the StatView program from Abacus Concepts (Berkeley, CA).

RESULTS

Generation of vEGT-

Generation of the transplacement vector used to construct a Ld*M*NPV strain lacking an *egt* gene is outlined in Fig. 1. This plasmid, pEGT-LacZ, was then cotransfected in Ld652Y cells with DNA from isolate A21-MPV to generate an EGT- viral strain. Virus harvested from the transfection was used to infect Ld652Y cells, and an agarose overlay containing X-gal was applied. After a 2-week incubation period, no β-galactosidase activity was detected. Consequently, individual plaques were picked and used to inoculate Ld652Y cells, and budded virus from these infections was screened by hybridization with a *lac*Z gene probe to identify recombinant virus. Virus from plaques that hybridized to the probe were repurified by plaque assay, and one was designated vEGT-.

The construction of the vEGT- was confirmed by Southern analysis (Fig. 2). A single blot was generated containing two lanes each of vEGT- and of isolate A21-MPV DNA digested with *Hin*dIII and *Bam*HI. After the gel was blotted, the blot was cut in half and one half was probed with the *Bst*XI to *Not*I fragment deleted from vEGT-. This probe hybridized only to a 8.0-kb fragment in the lane containing A21-MPV, as expected, thus confirming the deletion of a portion of the *egt* gene in vEGT-. The other half of the blot was probed with a 3.5-kb fragment isolated from pCH110 containing the *lac*Z gene. This probe hybridized only to a 10.5-kb fragment (the size of fragment expected if the segment of the *EGT* gene was replaced with the *lac*Z gene) in the lane containing vEGT-, thus confirming that the *egt* fragment had been removed and replaced with the *lac*Z gene in vEGT-.

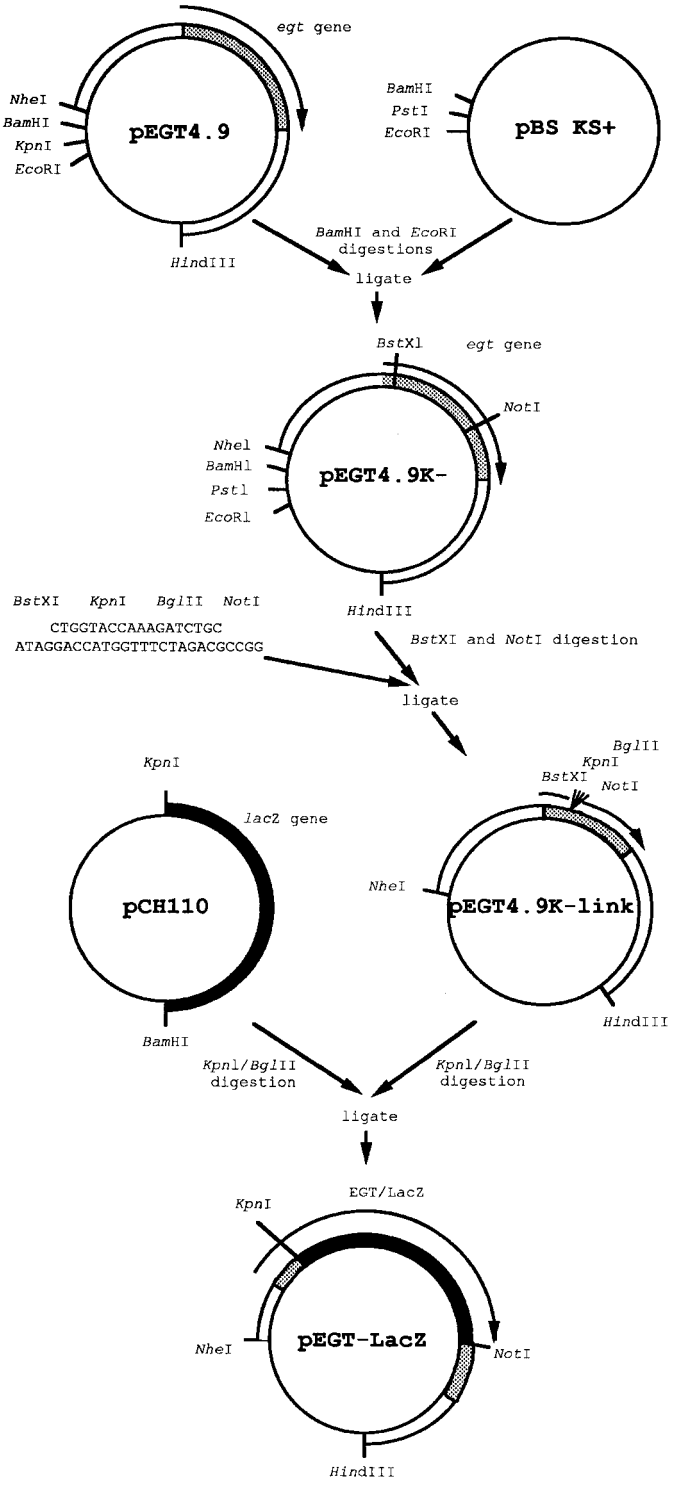

FIG. 1. Detailed schematic of the construction scheme used to generate vEGT-.

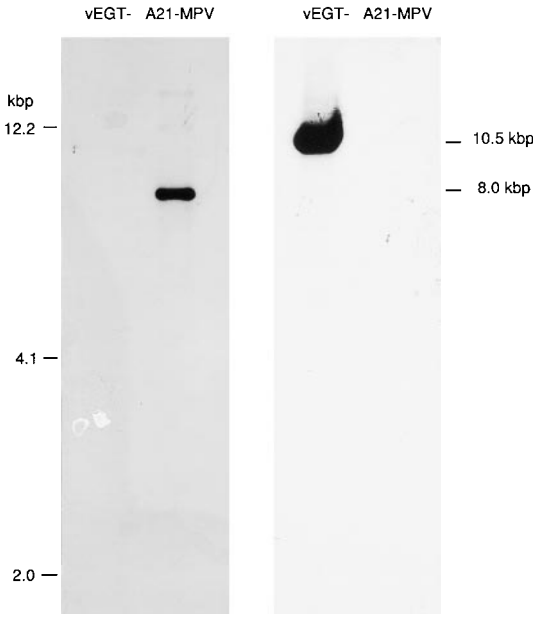

FIG. 2. Southern analysis of vEGT- and A21-MPV genomic DNA. Budded-virus DNA isolated from cells infected with either A21-MPV or vEGT- was restricted with *Bam*HI and *Hin*dIII. After DNA transfer, the blots were probed with the 0.9-kb *Bst*XI to *Not*I fragment deleted from vEGT- (left half) or the 3.5-kb *Hin*dIII to *Bam*HI fragment from pCH110 containing the *lac*Z gene (right half). The lanes contain the viral DNA as marked. DNA size markers are indicated on the left in kb, and the size of viral fragments that hybridized with the probes are shown on the right.

Analysis of lacZ and egt Gene Transcription

The recombinant virus was tested by Northern analysis for *lac*Z and *egt* gene transcription. Duplicate Northern blots were generated using total cellular RNA isolated at 0, 4, 16, 24, and 48 h p.i. from both A21-MPV and vEGT- infected *L. dispar* 652Y cells. These blots were probed using the same *egt* and *lac*Z gene probes used in the Southern analysis above. Probing with the *egt* gene fragment revealed a transcript only in the cells infected with A21-MPV, starting at 16 h p.i. and continuing through 48 h p.i. (data not shown). The Northern blot probed with the *lac*Z gene probe revealed no *lac*Z gene transcripts from cells infected with isolates A21-MPV or vEGT- (data not shown). The reason for the lack of a transcript from the *EGT/lac*Z fusion gene is unknown; however, this finding provides a basis for the lack of blue viral plaques. Sequencing of the *EGT/lac*Z gene junctions of vEGT- revealed no errors (data not shown). One possible explanation is that the *EGT/lac*Z gene fusion RNA generated by vEGT- is very unstable. Regardless of the basis for the apparent lack of an *EGT/lac*Z fusion transcript, the goal of generating a Ld*M* NPV that lacks a functional *egt* gene was achieved.

Larval Weight Gain

Newly molted 4th instar larvae (L4D1) were infected *per os* with diet contaminated with either A21-MPV or vEGT- virus. Larvae were weighed daily and the amount of weight gained was expressed as the cumulative weight gain. The standard deviations of the mean values for cumulative weight gains were large. The starting weights of male and female mock-infected larvae were analyzed to determine if the large standard deviations of the means were due, in part, to significant differences in the amount of weight gained by male compared to female larvae. The average weight of male larvae at the beginning of the study was found to be approximately half that of female larvae, and this difference was highly significant (Table 1). To reduce the magnitude of the standard deviations of the means due to differences between male and female larval weights, larvae were divided into male and female groups. At the beginning of the weight gain study, 95% of male larvae weighed less than 160 mg and 95% of female larvae weighed more than 160 mg in the mock-infected group. Based on this finding, virally infected larvae were classified as male or female based on their starting weight. Larvae that weighed more than 160 mg were classified as females, and larvae under 160 mg were classified as males.

Female mock-infected larvae gained significantly ($F = 442.3$; $df = 1, 48$; $P < 0.0001$) more weight than male mock-infected larvae (Figs. 3A and 4A). Female larvae had gained approximately 2750 mg compared to a gain of approximately 725 mg by the males at the time of pupation. Virally infected female larvae gained significantly ($F = 840.6$; $df = 1, 55$; $P < 0.0001$ and $F = 609.5$; $df = 1, 55$; $P < 0.0001$ for vEGT- and A21-MPV, respectively) less weight than mock-infected female larvae (Fig. 3A). Female larvae infected with vEGT- gained approximately 27% less weight than female larvae infected with A21-MPV (Fig. 3B). This difference was significant ($F = 4.9$; $df = 1, 38$; $P < 0.03$). Female larvae infected with vEGT- exhibited a slightly shorter time of virus-induced mortality compared to larvae infected with A21-MPV; however, when the 4th instar LT_{50} values were statistically analyzed, no difference was found (Fig. 3B). Male mock-infected larvae gained significantly ($F = 256.9$; $df = 1, 38$; $P < 0.0001$ and $F = 167.7$; $df = 1, 39$; $P < 0.0001$ for vEGT- and A21-MPV, respectively) more weight than virally infected larvae (Fig. 4A). There was no significant difference in the amount of weight gained or in the time of virus-induced mortality in male larvae infected with vEGT- compared to larvae infected with A21-MPV (Fig. 4B).

To determine if the day of viral infection impacts the efficacy of vEGT-, weight gain was also investigated in mid-4th instar larvae (L4D3). Larvae weighing less than 185 mg were classified as male and larvae weighing greater than 185 mg as female (Table 1). Female larvae infected with vEGT- gained significantly less weight ($F = 4.8$; $df = 1, 38$; $P < 0.035$) than larvae infected with A21-MPV (Figs. 5A and 5B). The average total weight gain of female larvae infected with vEGT- was approximately 38% less than that of larvae infected with A21-MPV. There was no significant difference in the weight gain of male larvae infected with vEGT- or A21-MPV (Figs. 6A and 6B). Larvae infected with vEGT- or A21-MPV molted to the 5th instar at approximately the same time (data not shown). There was no significant difference in the time of virus-induced mortality in larvae infected with vEGT- compared to A21-MPV-infected larvae.

Larval weight gain was investigated in 5th instar larvae to determine if the larval to pupal transition has an impact on the efficacy of vEGT- (the laboratory strain of *L. dispar* normally goes through five instars prior to pupation). Larvae weighing less than 650 mg were classified as male and larvae weighing greater than 650 mg as female (Table 1). Female larvae infected with vEGT- gained significantly less weight ($F = 41.1$; $df = 1, 68$; $P < 0.0001$) than larvae infected with A21-MPV (Fig. 7A). The average total weight gain of female larvae infected with vEGT- was approximately 25% less than that of larvae infected with A21-MPV. There was

TABLE 1

Weights of Mock-Infected Female and Male *Lymantria dispar* Larvae Used in the Weight Gain Studies

	Male			Female					
Larval stage[a]	Average weight ± SD[b]	Range	*N*	Average weight ± SD	Range	*N*	*F*	*df*	*P*[c]
L4D1	121.6 ± 35.4	68–191	17	210.5 ± 36.2	153–278	33	55.5	1,48	<0.0001
L4D3	161.5 ± 41.1	121–204	13	219.4 ± 38.3	163–320	33	20.4	1,44	<0.0001
L5D3	531.5 ± 69.3	339–682	20	993.1 ± 211.7	681–1336	30	88.2	1,48	<0.0001

[a] Insects used in this analysis were larval instars 4 or 5 at the 1st or 3rd day after molting.
[b] The average weight is in mg ± the standard deviation of the mean.
[c] ANOVA, Fisher's PLSD.

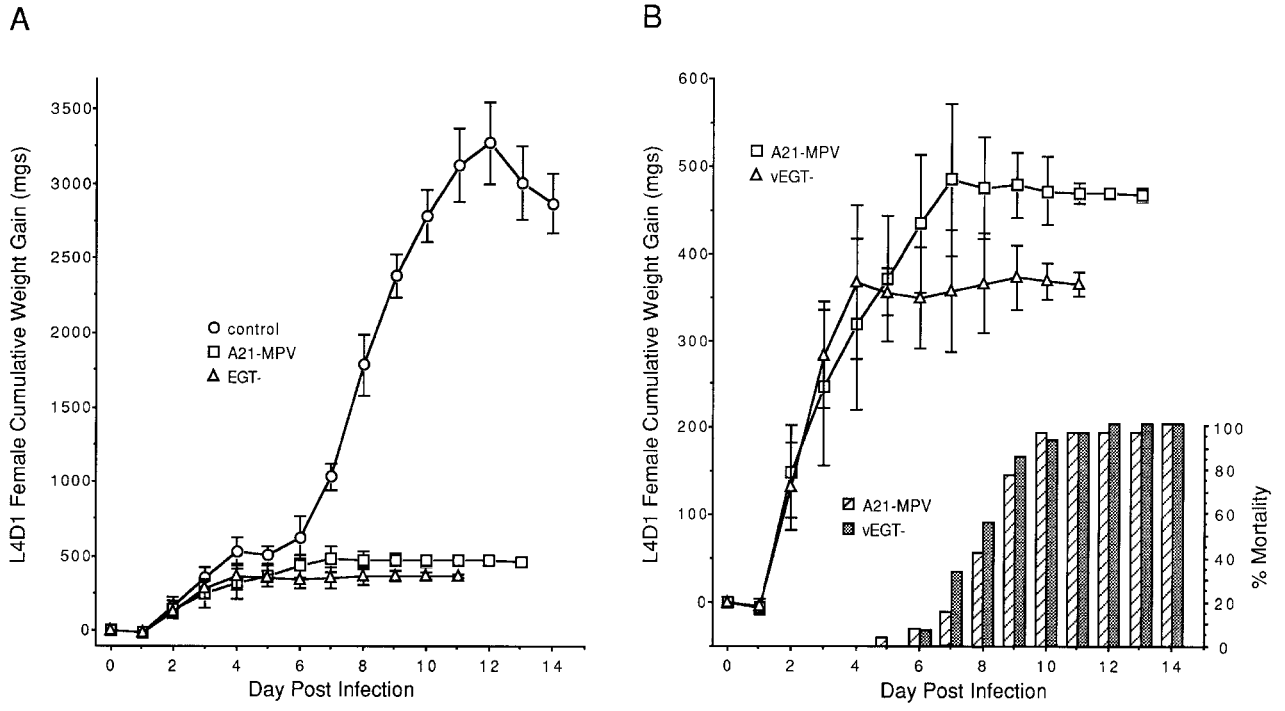

FIG. 3. Weight gain and mortality of female day 1 4th instar larvae. Twenty-seven, 26, and 33 larvae were infected with A21-MPV or vEGT- (*per os*) or were mock infected, respectively, 24 h after ecdysis to 4th instar larvae. The daily cumulative weight gains ± the standard deviation (A and B) and the daily cumulative mortality (B) observed in virally infected larvae are shown.

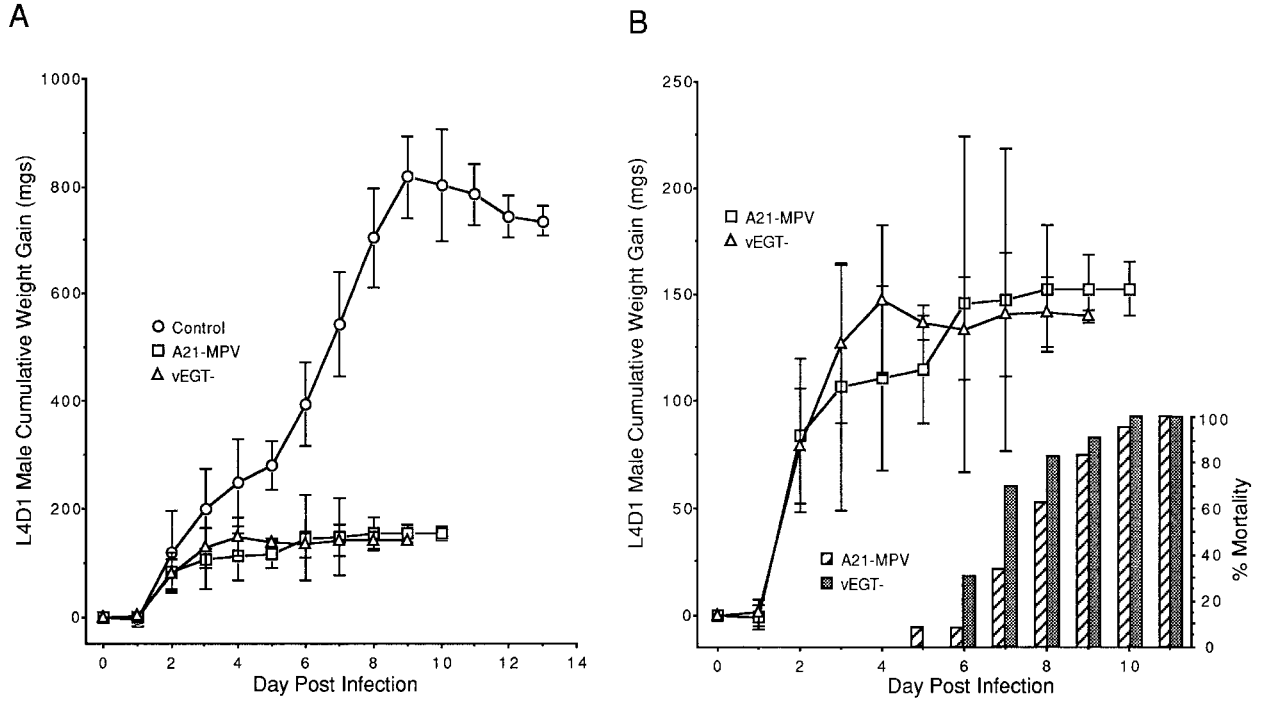

FIG. 4. Weight gain and mortality of male day 1 4th instar larvae. Twenty-three, 24, and 17 larvae were infected with A21-MPV or vEGT- (*per os*) or were mock infected, respectively, 24 h after ecdysis to 4th instar larvae. The daily cumulative weight gains ± the standard deviation (A and B) and the daily cumulative mortality (B) observed in virally infected larvae are shown.

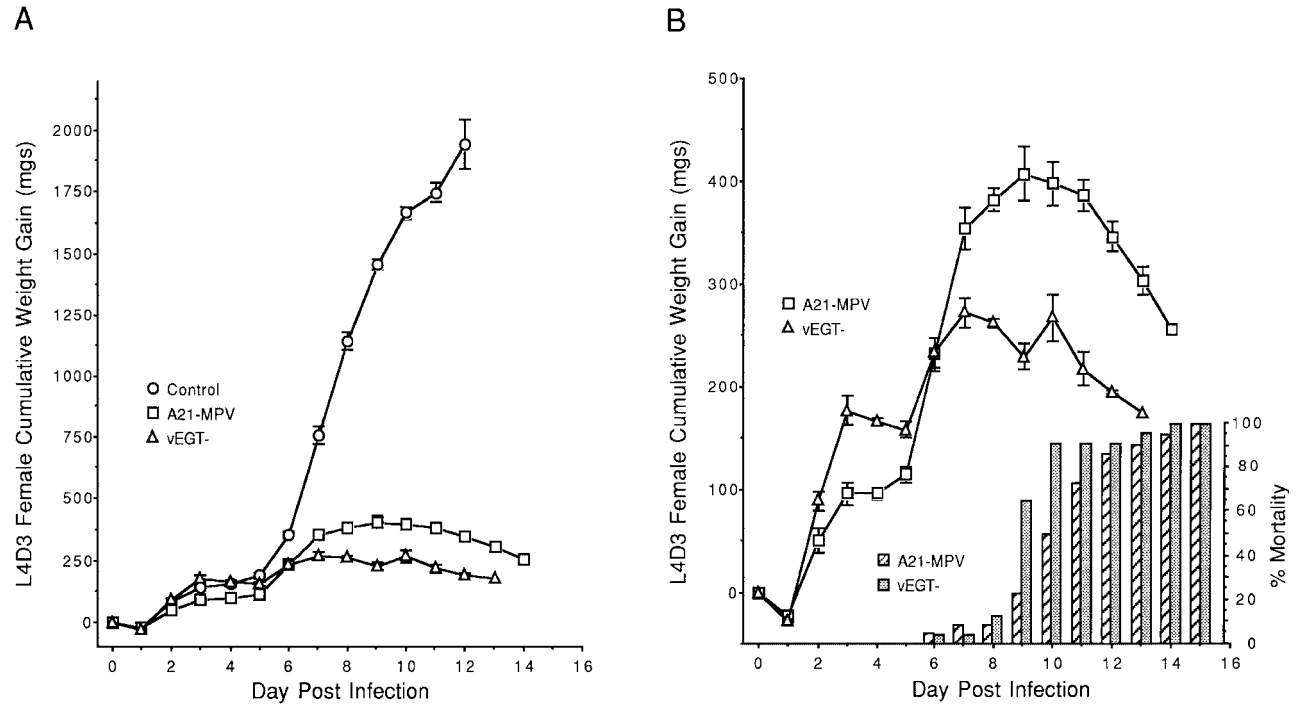

FIG. 5. Weight gain and mortality of female day 3 4th instar larvae. Three groups of 50 larvae were infected with A21-MPV or vEGT- (*per os*) or were mock infected, respectively, 3 days after ecdysis to 4th instar larvae. The daily cumulative weight gains ± the standard deviation (A and B) and the daily cumulative mortality (B) observed in virally infected larvae are shown.

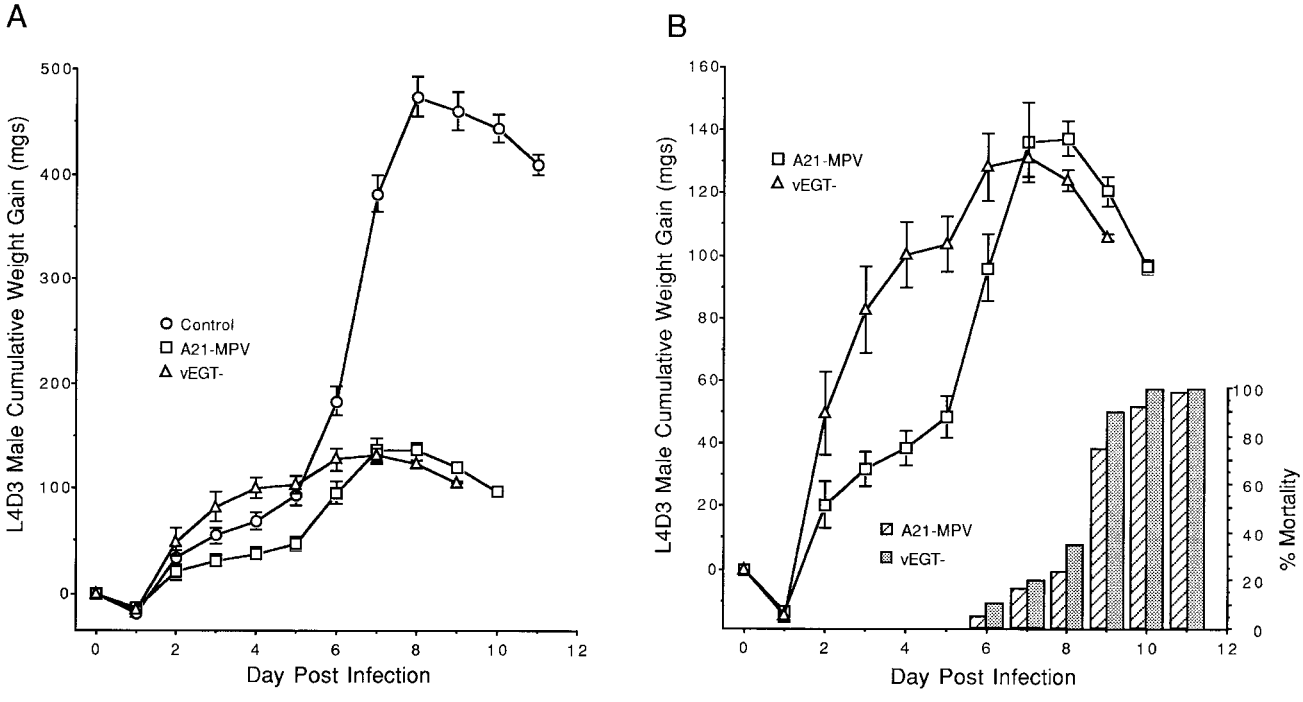

FIG. 6. Weight gain and mortality of male day 3 4th instar larvae. Three groups of 50 larvae were infected with A21-MPV or vEGT- (*per os*) or were mock infected, respectively, 3 days after ecdysis to 4th instar larvae. The daily cumulative weight gains ± the standard deviation (A and B) and the daily cumulative mortality (B) observed in virally infected larvae are shown.

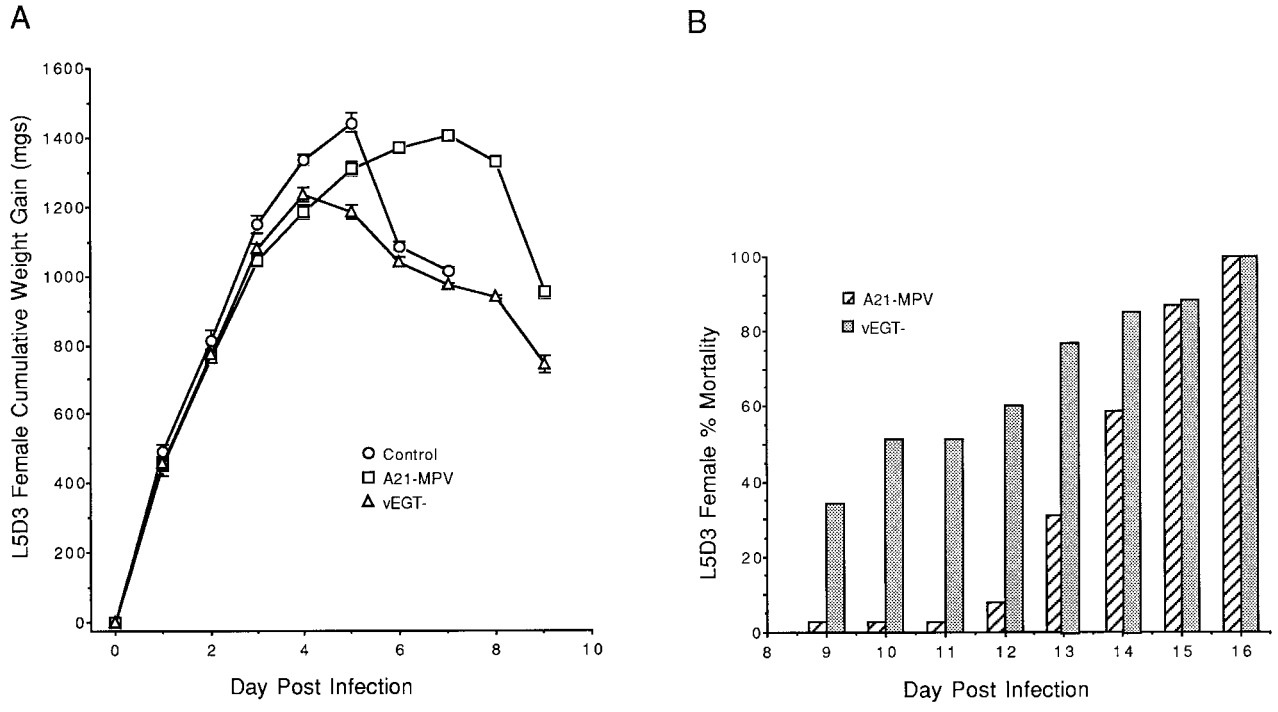

FIG. 7. Weight gain and mortality of female 5th instar larvae. Three groups of 50 larvae were infected with A21-MPV or vEGT- (*per os*) or were mock infected, respectively, 3 days after ecdysis to 5th instar larvae. The daily cumulative weight gains ± the standard deviation (A and B) and the daily cumulative mortality (B) observed in virally infected larvae are shown.

no significant difference in the weight gain of male larvae infected with vEGT- or A21-MPV (Fig. 8A). Both female and male larvae infected with vEGT- died significantly faster than A21-MPV-infected larvae ($F = 141.4$; $df = 1, 8$; $P < 0.0001$).

The length of the 5th instar of mock-infected male and female larvae was analyzed to determine if the lengths were different. Female larvae spent an average of 10.8 ± 0.7 days in the 5th instar compared with an average of 9.1 ± 0.6 days for the males. This difference was significant ($F = 78.8$; $df = 1, 48$; $P < 0.0001$).

Biological Activity of the Recombinant Virus

The biological activity of vEGT- was determined through bioassay of *L. dispar* larvae. The LC_{50} values for vEGT- were slightly greater than the values obtained for A21-MPV-infected neonate larvae (Table 2). However, this difference was not significant. The LC_{50} values of vEGT- and A21-MPV were essentially the same in 4th and 5th instar *L. dispar* larvae (Table 2).

No significant difference was found in the LT_{50} values in neonate or 4th instar larvae infected with viral isolates A21-MPV or vEGT- (Table 3). In one of the L1D1 bioassays, vEGT- infected insects died earlier than larvae infected with A21-MPV; yet at another dose in the same bioassay, there essentially was no difference in the LT_{50} values. In a second bioassay, the vEGT- virus exhibited a LT_{50} value approximately 21% less than the A21-MPV virus. However, at a higher dose the A21-MPV virus exhibited a LT_{50} value that was about 8% lower than vEGT-. Essentially, the same results were obtained with 4th instar larvae (Table 3). In two bioassays vEGT- exhibited slightly lower LT_{50}s. In the third bioassay vEGT- exhibited a lower LT_{50} at one dose (1×10^6 PIBs/ml) and a higher LT_{50} value than A21-MPV virus at a higher dose (1×10^7 PIBs/ml). In contrast, consistent results were observed in 5th instar bioassays (Table 3). In three separate bioassays, using two infection methods, vEGT- exhibited lower LT_{50} values compared to A21-MPV. vEGT- exhibited a statistically significant ($F = 141.4$; $df = 1, 8$; $P < 0.001$) LT_{50} reduction of ca. 33% compared to A21-MPV. Both female and male larvae infected with vEGT- exhibited similar LT_{50} reductions (data not shown).

Cessation of Larval Weight Gain after Infection with vEGT- or A21-MPV

The time at which larvae infected with virus stopped gaining weight was used as an indication of larval feeding cessation. Fourth instar larvae infected with virus continued feeding until a day or two prior to death at which time the larvae ceased feeding and become immobile. Fifth instar larvae stopped feeding and became immobile several days prior to death.

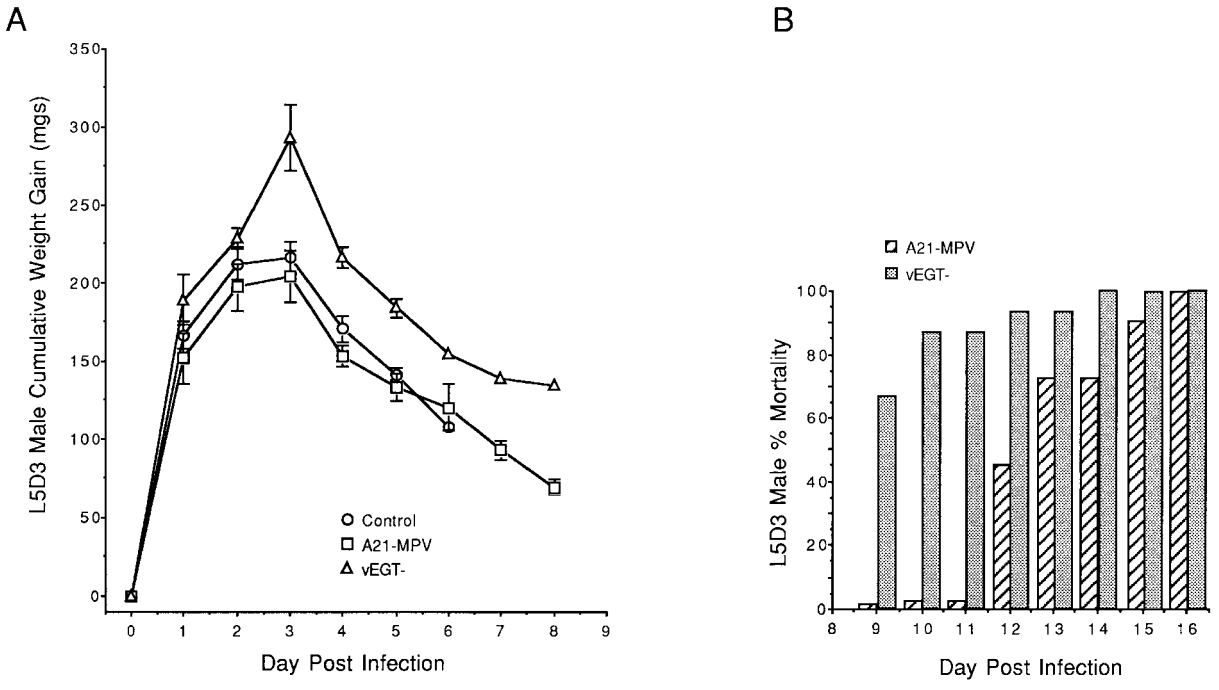

FIG. 8. Weight gain and mortality of male 5th instar larvae. Three groups of 50 larvae were infected with A21-MPV or vEGT- (*per os*) or were mock infected, respectively, 3 days after ecdysis to 5th instar larvae. The daily cumulative weight gains ± the standard deviation (A and B) and the daily cumulative mortality (B) observed in virally infected larvae are shown.

As a consequence of this behavior, the first point at which virally infected larvae began to lose weight is a better indicator of feeding cessation than LT_{50} values. The day after a larva's peak weight was used to calculate FT_{50} values (mean time at which 50% of larvae ceased feeding). In bioassays of 4th instar larvae, those infected with vEGT- exhibited lower FT_{50} values compared with larvae infected with A21-MPV (Table 4). Fifth instar larvae infected with vEGT- exhibited a 2-day decrease in the FT_{50} value compared to larvae infected with A21-MPV (Table 4).

Polyhedra Synthesis in Larvae Infected with vEGT- or A21-MPV

Polyhedra were isolated from the 4th and 5th instar larvae used in the larval weight gain study and quantified. In both male and female larvae, larvae infected with A21-MPV yielded more polyhedra than larvae infected with vEGT- (Fig. 9). The differences observed in 4th and 5th instar female larvae and in 5th instar male larvae were significant ($F = 9.0$; $df = 1, 42$; $P < 0.005$; $F = 89.2$; $df = 1, 72$; $P < 0.0001$; and

TABLE 2

Dose-Mortality Response of *Lymantria dispar* Larvae Infected *per os* with LdMNPV Isolates vEGT- or A21-MPV

Larval instar[a]	Bioassay	Virus	LC_{50} (PIBs/ml)	95% Fiducial limits	Slope
L1D1	1	A21-MPV	3.2×10^2	2.3×10^2–4.4×10^2	1.3 ± 0.1
		vEGT-	1.0×10^3	2.7×10^2–2.7×10^3	0.6 ± 0.1
L1D1	2	A21-MPV	3.2×10^2	1.9×10^2–5.1×10^2	1.2 ± 0.1
		vEGT-	1.6×10^3	8.0×10^2–3.0×10^3	0.8 ± 0.1
L4D2	5	A21-MPV	1.7×10^6	8.8×10^5–3.4×10^6	0.8 ± 0.1
		vEGT-	1.6×10^6	8.5×10^5–2.9×10^6	0.9 ± 0.1
L5D2	6	A21-MPV	9.7×10^6	4.0×10^6–2.0×10^7	1.0 ± 0.2
		vEGT-	1.1×10^7	2.6×10^6–3.6×10^7	1.1 ± 0.2
L5D2	7	A21-MPV	8.0×10^6	8.3×10^5–7.1×10^7	0.6 ± 0.1
		vEGT-	2.6×10^7	1.0×10^7–7.4×10^7	0.5 ± 0.1

[a] Insects used in the bioassays were larval instar 1, 4, or 5 at the 1st or 2nd day after molting.

TABLE 3

Time-Mortality Response of *Lymantria dispar* Infected *per os* with LdMNPV Isolates A21-MPV or vEGT-

Bioassay[a]	Virus	Larval stage[b] at Infection	% Mortality	LT_{50} ± SE[c] d.p.i.	Slope	LT_{50}[d] % +/−
1	A21-MPV	L1D1	62.5	7.9 ± 0.4	4.7 ± 0.5	
1	vEGT-	L1D1	35.2	7.1 ± 0.3	6.7 ± 0.9	−10.1
1	A21-MPV	L1D1	99.0	8.1 ± 0.1	11.4 ± 1.1	
1	vEGT-	L1D1	56.9	8.0 ± 0.5	5.3 ± 0.9	−1.2
2	A21-MPV	L1D1	32.3	9.1 ± 0.3	7.7 ± 0.9	
2	vEGT-	L1D1	28.3	7.2 ± 0.4	4.8 ± 0.7	−20.9
2	A21-MPV	L1D1	62.5	7.7 ± 0.2	9.8 ± 0.9	
2	vEGT-	L1D1	35.2	8.3 ± 0.3	6.1 ± 0.7	+7.8
3	A21-MPV	L4D1	100	7.8 ± 0.2	11.4 ± 0.9	
3	vEGT-	L4D1	100	7.2 ± 0.2	12.4 ± 1.1	−7.7
4	A21-MPV	L4D3	92.0	8.8 ± 0.3	7.2 ± 0.9	
4	vEGT-	L4D3	86.0	8.4 ± 0.2	12.3 ± 1.6	−4.5
5	A21-MPV	L4D2	48.5	12.8 ± 0.5	11.7 ± 2.5	
5	vEGT-	L4D2	43.8	10.5 ± 0.3	20.0 ± 4.8	−18.0
5	A21-MPV	L4D2	74.2	11.2 ± 0.4	11.6 ± 2.1	
5	vEGT-	L4D2	73.7	12.0 ± 0.5	7.9 ± 1.3	+7.1
4	A21-MPV	L5D3	100	13.3 ± 0.2	13.7 ± 1.7	
4	vEGT-	L5D3	100	10.0 ± 0.3	8.0 ± 1.0	−24.8
6	A21-MPV	L5D2	45.1	12.2 ± 0.7	8.4 ± 2.0	
6	vEGT-	L5D2	38.3	8.1 ± 0.3	11.0 ± 2.2	−33.6
6	A21-MPV	L5D2	90.0	12.6 ± 0.5	8.2 ± 1.4	
6	vEGT-	L5D2	83.3	8.1 ± 0.2	15.6 ± 2.8	−35.7
7	A21-MPV	L5D2	35.5	14.2 ± 0.9	8.5 ± 2.2	
7	vEGT-	L5D2	42.9	10.1 ± 0.6	7.2 ± 1.6	−28.9
7	A21-MPV	L5D2	80.7	15.1 ± 0.5	10.9 ± 1.9	
7	vEGT-	L5D2	53.1	8.9 ± 0.4	10.6 ± 2.3	−41.1

[a] Bioassays 1 and 2 were performed using the diet incorporation method, 3 and 4 were performed using fixed-volume virus-contaminated diet plugs to infect larvae, and bioassays 5–7 were performed using the droplet feeding method.
[b] Insects used in the bioassays were larval instars 1, 4, or 5 at the 1st, 2nd, or 3rd day after molting.
[c] The LT_{50} values are expressed as days post infection (d.p.i.) ± the standard error of the mean.
[d] The percentage of increased (+) or decreased (−) LT_{50} value of vEGT-infected larvae relative to insects infected with A21-MPV.

$F = 25.2$; $df = 1, 23$; $P < 0.0001$ for 4th female, 5th female, and 5th male, respectively). There was no significant difference between 4th instar male larvae infected with vEGT- or A21-MPV. The largest difference was found in 5th instar female larvae. Larvae infected with vEGT- yielded approximately ninefold fewer polyhedra compared with larvae infected with A21-MPV. The difference was much less pronounced in 4th instar larvae compared to 5th instar insects. In both males and females, 4th instar larvae infected with A21-MPV yielded about twice as many polyhedra as larvae infected with vEGT-.

TABLE 4

Weight Gain Cessation of *Lymantria dispar* Infected *per os* with LdMNPV Isolates A21-MPV or vEGT-

Bioassay[a]	Virus	Larval[b] stage	FT_{50} ± SE[c]	Slope	FT_{50}[d] % +/−
3	A21-MPV	L4D1	5.8 ± 0.4	3.8 ± 0.5	
3	vEGT-	L4D1	4.6 ± 0.2	6.2 ± 0.8	−20.7
4	A21-MPV	L4D3	7.3 ± 0.4	5.2 ± 0.7	
4	vEGT-	L4D3	6.7 ± 0.2	8.5 ± 1.1	−8.2
4	A21-MPV	L5D3	6.5 ± 0.3	6.1 ± 0.8	
4	vEGT-	L5D3	4.4 ± 0.5	8.9 ± 1.2	−32.3

[a] Bioassays were performed using fixed-volume virus-contaminated diet plugs to infect larvae.
[b] Larvae used in the bioassays were larval instars 4 or 5 at the 1st or 3rd day after molting.
[c] The FT_{50} values are expressed as days post-infection ± the standard error of the mean.
[d] The percentage of decreased (−) or increased (+) FT_{50} value of vEGT-relative to A21-MPV.

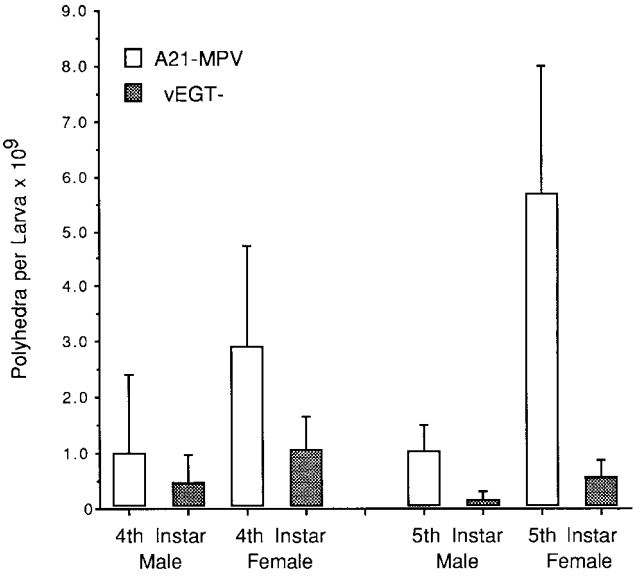

FIG. 9. Polyhedra production in 4th and 5th instar larvae. Polyhedra were isolated from the L4D3 and L5D3 larvae used in the weight studies described in Figs. 5–8 and quantified. The values are the averages of from 11 to 22 determinations from male larvae and from 21 to 38 determinations from female larvae. One standard deviation is shown.

DISCUSSION

The ecdysteroid UDP-glucosyl transferase gene was first identified in the Ac*M*NPV and is believed to alter host ecdysteroid levels (O'Reilly and Miller, 1989). Deletion of the *egt* gene from Ac*M*NPV enabled the virus to kill its host in less time than wild-type virus (O'Reilly and Miller, 1991). This finding indicates that the efficacy of a baculovirus may be improved through deletion of the *egt* gene. An *egt* gene has been identified in Ld*M*NPV and characterized (Riegel *et al.*, 1994a). In an effort to generate a Ld*M*NPV strain with improved efficacy, we have deleted the *egt* gene and analyzed its biological properties.

The weight gain in larvae infected with vEGT- was compared with that of larvae infected with Ld*M*NPV isolate A21-MPV to assess feeding behavior. The majority of diet consumed by the laboratory strain of *L. dispar* larvae occurs in the penultimate (4th) and ultimate (5th) instars, 21 and 70%, respectively (J. Slavicek, unpublished data; the majority of feral *L. dispar* female and male larvae have six and five instars, respectively). Consequently, an improved Ld*M*NPV strain would need to exhibit enhanced efficacy in the ultimate instar in order to have an advantage over wild-type virus as a biological insecticide for the gypsy moth. Therefore, larval weight gain was assessed in 4th and 5th instar larvae. In agreement with findings with 4th and 5th instar *Spodoptera frugiperda* (Smith) larvae infected with an *egt* gene deletion Ac*M*NPV strain (O'Reilly and Miller, 1991), 4th and 5th instar Ld*M*NPV larvae infected with vEGT- gained less weight than larvae infected with A21-MPV. However, in *L. dispar* larvae, only female larvae infected with vEGT- exhibited a decrease in weight gain. Fourth instar female larvae infected with vEGT- gained approximately 32% less weight compared to larvae infected with A21-MPV, and 5th instar female larvae gained approximately 25% less weight when infected with vEGT-.

Why there is no reduction in weight gain in male larvae, in contrast to female larvae, infected with vEGT- larvae is unknown. As described below, vEGT- did not exhibit a lower LT_{50} value in 4th instar female larvae than A21-MPV and yet it did exhibit reduced weight gain. In addition, vEGT- exhibited a lower LT_{50} value in 5th instar male larvae, but there was no difference in larval weight gain between vEGT- and A21-MPV infected 5th instar male larvae. Therefore, a reduction in larval weight gain does not necessarily correlate with enhanced viral efficacy (i.e., killing speed). Several differences between *L. dispar* male and female larvae exist that could provide a basis for the weight gain results. Female *L. dispar* larvae are considerably larger than male larvae in the 4th and 5th instars (Table 1). It is interesting to note that in the larval weight gain studies virally infected 4th instar females exhibited an average peak weight gain of approximately 400–500 mg. In contrast, the virally infected 4th instar males exhibited a much smaller weight gain of approximately 120–150 mg (Figs. 5 and 7). In addition, virally infected 5th instar males exhibited an average peak weight gain of approximately 200 mg, far less than 4th instar females. Another difference is that females spent 1 day longer in the 5th instar than males. Consequently, the increase in ecdysteroid levels would likely occur sooner in males than females. If so, EGT levels may not have built up sufficiently in the males to allow for a detectable EGT-induced impact. Alternatively, the difference in the hormonal milieu of male and female *L. dispar* larvae may be the basis for the difference in weight gain between male and female larvae infected with vEGT-.

Earlier investigations have shown that an *egt* gene minus Ac*M*NPV strain exhibited reduced LT_{50} values in *S. frugiperda*, *Trichoplusia ni* (Hubner) (O'Reilly and Miller, 1991), and *Heliothis virescens* (F.) (Treacy *et al.*, 1997) compared to wild-type virus. A reduction in LT_{50} values was observed in 1st, 4th, and 5th *S. frugiperda* instar larvae and in 1st and 3rd instar *H. virescens* larvae. The increase in viral efficacy exhibited in *H. virescens* was characterized as slight (LT_{50} values were not calculated), and in 1st instar *S. frugiperda* an enhancement of approximately 21% was observed. Similar levels of efficacy enhancement were observed in 4th and 5th instar *T. ni* larvae (O'Reilly and Miller, 1991; LT_{50} values were not presented). In contrast, in the

present study, an enhancement in viral efficacy was consistently observed only in 5th instar *L. dispar*. These findings are in contradiction to our preliminary findings which suggested that vEGT- exhibited lower LT_{50} values than A21-MPV in 1st and 4th instar larvae (Riegel *et al.*, 1994b). The results of this investigation, in which the bioassays were repeated several times, clearly indicate that vEGT- does not consistently exhibit lower LT_{50} values than A21-MPV in the 1st and 4th larval instars.

It is possible that *L. dispar* 1st and 4th instar larvae infected with vEGT- exhibit a small decrease in LT_{50}, and since we sampled for mortality every 24 h the difference was missed. However, virally infected larvae died over a period of 6–14 days p.i.; consequently a small time period of larval mortality was not observed. If the enhancement in LT_{50} found in insects infected with Ld*M* NPV vEGT- was comparable to that observed in larvae infected with an EGT-Ac*M* NPV strain, the difference would be approximately 48 h. A difference of this magnitude would have been detected in the bioassays performed. Similar to the results with 1st and 4th instar larvae of our study, Popham *et al.* (1997) found that deletion of the *egt* gene from the *Helicoverpa zea* SNPV did not improve the killing speed of the virus in neonate larvae.

The basis for the different observations in 1st and 4th vs 5th instar larvae is not known. Our results suggest that a 5th instar-specific event may be occurring that is necessary for the manifestation of vEGT-'s increased viral efficacy. In *S. frugiperda*, larvae infected with an Ac*M* NPV *egt* gene deletion strain molted whereas insects infected with wild-type virus failed to molt (O'Reilly and Miller, 1991). In our study, all virally infected 4th instar larvae molted to 5th instar. This result suggests that viral *egt* gene expression is not impacting larval ecdysteroid levels sufficiently in the 4th instar to block molting to the 5th instar. The *egt* gene transcripts are difficult to detect in Ld652Y-infected cells, which suggests either a low-level transcription and/or a high mRNA turnover rate. Furthermore, transcription of the Ld*M* NPV *egt* gene occurs from approximately 12 to 48 h p.i. and maximally at about 16 h p.i. (Riegel *et al.*, 1994a) whereas the Ac*M* NPV *egt* gene is expressed predominately from 3 to 12 h p.i. and maximally at about 3 h p.i. (O'Reilly and Miller, 1990). The Ld*M* NPV exhibits a LT_{50} of approximately 12–15 days in 5th instar *L. dispar* (Table 3). In contrast, Ac*M* NPV exhibits a LT_{50} of approximately 5–6 days in the *S. frugiperda* host (O'Reilly and Miller, 1991). The onset of Ld*M* NPV DNA replication (18–20 h p.i.) and polyhedron formation (48 h p.i.) in cell culture (Riegel and Slavicek, 1997) is significantly delayed compared to the onset of DNA replication (6 h p.i.; Tjia *et al.*, 1979) and polyhedron formation (12 h p.i.) in Ac*M* NPV. The shorter time period for viral-induced mortality and the earlier onset and completion of Ac*M* NPV replication compared to Ld*M* NPV suggest that the Ld*M* NPV requires a longer period of time to complete systemic spread within its host. Burand and co-workers noted a difference in the time of the ecdysteroid peak in 4th and 5th instar larvae (Burand *et al.*, 1996). Ecdysteroid levels peaked in 4th instar larvae at 3–4 days after molting, whereas in 5th instar larvae the peak occurred 9 days after molting. A low level of *egt* gene expression in conjunction with a longer replication period exhibited by Ld*M* NPV and an earlier ecdysteroid peak may be the basis for the absence of vEGT- accelerated viral-induced mortality in pre-5th instar larvae.

O'Reilly and Miller (1991) have postulated that the function of the viral *egt* gene is to increase viral progeny production as measured by polyhedra production. Our findings on polyhedra synthesis by vEGT- and A21-MPV are consistent with this hypothesis. In both 4th and 5th instar larvae, those infected with A21-MPV yielded more polyhedra than larvae infected with vEGT- (Fig. 9). Fifth instar female larvae yielded approximately ninefold more polyhedra when infected with A21-MPV compared with larvae infected with vEGT-. With vEGT-, decreased polyhedra synthesis occurred in the presence (i.e., 5th instar males and females) and absence (i.e., 4th instar females) of increased viral efficacy and in the absence of decreased larval weight gain (i.e., 5th instar males).

Larval weight gain was used as an indicator of larval feeding activity for generation of FT_{50} values. Fifth instar larvae exhibited a 3-day earlier LT_{50} value and a 2-day earlier FT_{50} value when infected with vEGT- compared with A21-MPV. These results suggest that larvae infected with vEGT- stopped gaining weight and by inference stopped feeding approximately 5 days prior to death. Consequently, due to the accelerated cessation of larval weight gain when infected with vEGT- compared to infection by A21-MPV, vEGT- would be a better biological insecticide than wild-type virus.

In summary, through deletion of the Ld*M* NPV *egt* gene an improved virus strain was generated since the killing speed of vEGT- is significantly faster than wild-type virus in 5th instar *L. dispar* larvae, and larvae infected with vEGT- stopped gaining weight earlier than larvae infected with wild-type virus. Since the majority of foliage consumption by *L. dispar* larvae occurs in the 5th instar, the use of vEGT- may offer better foliage protection than wild-type virus in the field. Consequently, vEGT- would be an improved biological insecticide for the control of 5th instar *L. dispar* larvae. The results of this study indicate that larval life span and weight gains may not be appropriate measures of the mechanism by which viral EGT enhances viral progeny production in all instars. In addition, our results indicate that acceleration of viral-induced mor-

tality through deletion of the *egt* gene can be specific to a certain larval instar dependent upon the virus and host under investigation.

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