The ecdysteroid UDP-glucosyltransferase gene of *Autographa californica* nucleopolyhedrovirus alters the moulting and metamorphosis of a non-target insect, the silkworm, *Bombyx mori* (Lepidoptera, Bombycidae)

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The *Autographa californica* nucleopolyhedrovirus (AcMNPV) does not infect the silkworm and molecular studies on silkworm insusceptibility have not been performed. In cultured cells of the silkworm, the expression of viral genes has been reported. The expression of AcMNPV genes and their effect in vivo and in vitro was studied. In this study, the early gene, the ecdysteroid UDP-glucosyltransferase (egt) gene of AcMNPV, which inactivates the insect moulting hormone by sugar conjugation, was examined to determine whether it would alter the growth of the silkworm. Using wild-type (wt) AcMNPV, the egt gene deletion virus (vEGTDEL), and the virus carrying the egt promoter–lacZ cassette in vEGTDEL (vEGTZ), the egt promoter-driven expression in cultured cells and in non-productive infection of the silkworm was characterized. Infection of cultured cells with vEGTZ at three different doses occurred in a single cell manner. When budded wt AcMNPV was injected into the fourth and fifth instar larvae, an increase in the amount of virus occurred and caused abnormal larval growth, which resulted in the prolongation or skipping of the larval instar, premature pupation, or death during the pupal stage. For infection of the fourth instar larvae, precocious metamorphosis was observed. When the same amount of vEGTDEL was injected, the alteration of growth did not occur. These results suggest that the egt gene was expressed in the primary infected cells of the silkworm, and that the EGT was secreted into the haemocoel, which significantly altered larval growth.

**Introduction**

Baculoviruses replicate in arthropods, specifically in lepidopteran insects. The number of host insect species of the baculovirus is considered to be low. In the replication cycle, baculovirus exhibits two types of morphology, budded virus (BV) and occlusion body (OB) embedded virus particles in a proteinaceous crystal body. The BV causes cell to cell infection in vivo and in vitro and the OB causes host to host infection. The ecdysteroid UDP-glucosyltransferase (egt) gene is involved in virus propagation in the host insect (O’Reilly & Miller, 1989; O’Reilly et al., 1991). The egt gene of the baculovirus extends the feeding period by inactivating the ecdysteroids using a sugar conjugation reaction which results in excess production of virus in the host insects compared to egt deletion virus (O’Reilly et al., 1991, 1992). Therefore, the baculovirus has been considered to maintain the egt gene during the course of evolution for its targeting of lepidopteran insects (Riegel et al., 1994; Barrett et al., 1995; Faktor et al., 1995; Clarke et al., 1996; Hu et al., 1997; Smith & Goodale, 1998).

In the baculovirus replication cycle, viral genes are expressed as a cascade and in three temporal phases; early, late and very late (Friesen & Miller, 1986). Early genes share a common promoter function with host genes; therefore no viral gene products are necessary for the expression of early genes. Morris & Miller (1992) studied promoter influence on *Autographa californica* nucleopolyhedrovirus (AcMNPV)-mediated gene expression in permissive and non-permissive insect cell lines and demonstrated that late-promoter-driven expression was greater than early-promoter-driven expression.
in some non-permissive cell lines. They also demonstrated AcMNPV DNA replication in these non-permissive cell lines (Morris & Miller, 1993), which indicated that late-promoter-driven expression might have depended on viral DNA replication in the permissive cell lines. It would be useful to determine the amount of expression of the baculovirus early promoters and the mechanism of adoption of the gene expression machinery in non-permissive insects to determine the host species of baculoviruses and to design baculovirus transfer vectors. In this study, we characterized an early gene of the AcMNPV egt to examine its effect on virus infection in the non-permissive insect Bombyx mori. The results demonstrated that egt promoter-driven expression occurred in BmN4 cells after a single infection and that EGT, when expressed or secreted, altered the growth of the silkworm as demonstrated by the prolongation or skipping of the instar or death in the pre-pupa or pupal stage.

Methods

Viruses, cultured cells and insects. The standard genotype L1 of AcMNPV (wt AcMNPV), a virus with a deletion in the egt gene (vEGTDEL), and a virus in which the egt gene was replaced with Escherichia coli lacZ gene driven by the egt promoter (vEGTZ) (O’Reilly & Miller, 1990; O’Reilly et al., 1991) were generously provided by D. O’Reilly by permission of L. K. Miller (University of Georgia, GA, USA). The Bombyx mori NPV D1 strain (wt BmNPV) was used as the experimental control (Hashimoto et al., 1994). AcMNPV BV and OB were propagated by infection of IPLB-Sf9 cells (Sf9) (Vaughn et al., 1977) and BmNPV OB were propagated by infection of the silkworm. To remove the EGT in the BV suspension, which was secreted from Sf9 cells infected with wt AcMNPV, the BV was precipitated twice by ultracentrifugation and was re-suspended in fresh culture medium. The BV titre was determined by infection of the cells in a 96 multi-well plate and was represented by its TCID\(_{50}\). The amount of OB was counted in triplicate using a Thoma haemocytometer. The silkworm eggs of a bi-voltine strain (Kinshu × Showa or Shunrei × Shogetsu) were purchased from Kanebo and the silkworms were reared on an artificial diet (Silkmate 2S, Nihon-Nosan-Kogyou).

Infection of BmN4 cells with wt AcMNPV or vEGTZ. BV of wt AcMNPV or vEGTZ was used to infect BmN4 cells to determine egt promoter-driven expression and virus replication. To determine the expression of virus genes, BmN4 cells (1 × 10\(^4\)) were infected with wt AcMNPV or vEGTZ at 0·014, 0·14 or 1·4 TCID\(_{50}\) per cell and the egt promoter-driven expression of lacZ was examined by X-Gal staining at 36, 72 and 120 h post-infection (p.i.). The staining was performed by adding X-Gal solution to the cell suspension at a final concentration of 0·2 mg/ml and incubating the cells at 37 °C overnight. The wells were examined using an inverted microscope and the stained and un-stained cells were counted.

Bioassay of the fourth and fifth instar larvae by BV of wt AcMNPV and vEGTDEL. A series of tenfold dilutions of the BV suspension at 5 × 10\(^4\) to 5 × 10\(^8\) TCID\(_{50}\)/ml or at 5 × 10\(^5\) to 5 × 10\(^8\) TCID\(_{50}\)/ml was prepared and 10 μl or 100 μl of the suspension was subcutaneously injected into the fourth or fifth instar larvae, respectively. The larvae were provided with a virus-free diet in a Styrofoam cup until death or emergence. To determine the change in the cell titres in the haemolymph of the larvae, 10 μl or 100 μl of the BV suspension at 1·0 × 10\(^9\) TCID\(_{50}\)/ml was injected into the fourth or fifth instar larvae, respectively, and 50 μl of the haemolymph was collected at 0, 48, 120 and 148 h p.i. from the fourth instar larvae and at 0, 36, 72 and 108 h p.i. from the fifth instar larvae. The time-point 1 h after injection was used as 0 h p.i.

Bioassay of the fourth and fifth instar larvae by OB of wt AcMNPV or vEGTDEL. The fourth instar larvae were provided with 1·0 × 10\(^5\) or 1·0 × 10\(^7\) OB of the three types of AcMNPV in 5 μl of sterilized water mixed with 0·2 g of the artificial diet. These amounts of OB were equivalent to those of the wt BmNPV which caused 55% and 90% mortality, respectively. The fifth instar larvae were provided with 1·0 × 10\(^5\) or 1·0 × 10\(^7\) OB of the three types of AcMNPV in 5 μl of sterilized water mixed with 2 g of the artificial diet. These amounts of OB were equivalent to those of the wt BmNPV which caused 35% and 85% mortality, respectively. After 24 h of feeding, 20 larvae which had consumed the diet with OB were used for the bioassay. For mock-infection, the larvae were provided with an artificial diet mixed with 5 μl of sterilized water for 24 h. Thereafter, all larvae tested were provided with a virus-free artificial diet in a small Styrofoam cup until mock-infected larvae emerged.

Results and Discussion

egt promoter-driven expression of AcMNPV in BmN4 cells

To determine if the egt gene promoter was functional, the cells infected with vEGTZ were stained with X-Gal. Five hundred BmN4 cells infected with 0·014, 0·14 or 1·4 TCID\(_{50}\) per cell were examined at 36, 72 and 120 h p.i. and the blue cells were counted (Table 1). The cultures at 0·014 TCID\(_{50}\) per cell had no blue cells at any time post-infection, the cultures at 0·14 TCID\(_{50}\) per cell had 8·7, 7·8 and 7·3% of cells stained blue.

Expression of lacZ was confirmed by staining the infected BmN4 cells with X-Gal as shown in Methods. BmN4 cells (1 × 10\(^4\)) were infected with vEGTZ or wt AcMNPV at 0·014, 0·14 or 1·4 TCID\(_{50}\) per cell. The cells were collected at 36, 72 and 120 h p.i. and stained with X-Gal (0·2 mg/ml) at 37 °C overnight. Five hundred cells were examined under an inverted microscope and the percentages of blue-stained cells are shown. The result of wt AcMNPV infection was 0% and only the result at 1·4 TCID\(_{50}\) per cell is shown in this table.

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at 36, 72 and 120 h p.i., respectively, and the cultures at 1:4 TCID<sub>50</sub> per cell had 98±8, 98±7 and 98±2% at 36, 72 and 120 h p.i., respectively. The cells infected with the same amounts of wt AcMNPV and examined by X-Gal staining did not stain blue. These results indicate that lacZ expression did not occur during incubation, but it depended on the amount of virus. The proportion of blue cells in each culture was equal to the amount of virus used to infect the culture, which indicated that there was a single infection of BmN4 cells with wt AcMNPV BV as demonstrated previously (Volkman & Goldsmith, 1982). The possible secondary infection of the BmN4 cells is consistent with the in vitro host range expansion of AcMNPV to BmN cells by the alteration of a single amino acid residue of the putative DNA helicase gene, p143 (Kamiya & Maeda, 1997). The intensity of the blue colour of the cells after overnight incubation varied, which suggested heterogeneous egt promoter-driven expression of lacZ in the cells (data not shown). The heterogeneity is consistent with the results of Morris & Miller (1993), i.e. the differences in AcMNPV gene expression in BmN4 cells are probably a function of random variation in the infection of the cells instead of a function of genetic heterogeneity in the cell population.

To dissect the non-productive replication of AcMNPV in non-permissive cells, Morris & Miller (1992) examined promoter influence on gene expression in BmN4 cells by using promoters that are active in the early, late or very late stages of virus replication. They demonstrated that late-promoter-driven expression, which is dependent on DNA replication, produced an insignificant amount of BV, no polyhedra, and a substantial amount of viral DNA (Morris & Miller, 1993). To determine the distribution of the expression of viral genes during the three replication phases, they used fluorescence-activated sorting technology and demonstrated semi-productive expression caused by late and very late gene promoters, but not by the early gene promoter. In this study, we demonstrated the efficient expression of lacZ caused by an early gene promoter, the egt gene, in BmN4 cells, which suggests that other early-gene-promoter expression involved in viral DNA replication and late gene expression might have occurred.

**AcMNPV infection of the silkworm larvae**

To determine the effect of injection of BV of AcMNPV on the silkworm larvae, various amounts of wt AcMNPV or vEGTDEL were injected into the fourth instar larvae (Figs 1, 2 and 4). When wt AcMNPV at 5·0×10<sup>2</sup>, 5·0×10<sup>3</sup> or 5·0×10<sup>6</sup> TCID<sub>50</sub> per larva was injected into the fourth instar larvae, they skipped the fifth instar stage and started the spinning stage. The average duration of the fourth instar group was extended 7 days, which was 2 days longer than that of the mock-infected larvae. After spinning, the larvae that were injected with wt AcMNPV at 5·0×10<sup>6</sup> TCID<sub>50</sub> per larva stopped growth in the pre-pupa stage and died, the larvae at 5·0×10<sup>5</sup> TCID<sub>50</sub> per larva pupated normally but died afterwards, and the larvae at 5·0×10<sup>4</sup> TCID<sub>50</sub> per larva pupated and emerged normally. When wt AcMNPV at 5·0×10<sup>3</sup>, 5·0×10<sup>4</sup> or 5·0×10<sup>5</sup> TCID<sub>50</sub> per larva was injected into 20 larvae and their growth patterns were measured every 24 h until death or emergence. The thickness of the bar corresponds to the number of larvae alive and the growth stages are indicated by the closed section of the line. The shape of the solid boundary lines for the closed sections indicates the number of larvae and the transition of the growth stage. The dotted boundary lines indicate the number of larvae and the start of spinning. This format also applies to Figs 2 and 4.

**Fig. 1.** Effect of BV injection of wt AcMNPV on the fourth instar silkworm growth pattern. Wt AcMNPV BV at 5·0×10<sup>4</sup>, 5·0×10<sup>5</sup>, 5·0×10<sup>6</sup>, 5·0×10<sup>7</sup> or 5·0×10<sup>8</sup> TCID<sub>50</sub> per larva was injected into 20 larvae and their growth patterns were measured every 24 h until death or emergence. The thickness of the bar corresponds to the number of larvae alive and the growth stages are indicated by the closed section of the line. The shape of the solid boundary lines for the closed sections indicates the number of larvae and the transition of the growth stage. The dotted boundary lines indicate the number of larvae and the start of spinning. This format also applies to Figs 2 and 4.

**Fig. 2.** Effect of BV injection of wt AcMNPV on the fifth instar silkworm growth pattern. Wt AcMNPV BV at 5·0×10<sup>2</sup>, 5·0×10<sup>4</sup> or 5·0×10<sup>5</sup> TCID<sub>50</sub> per larva was injected into 20 larvae and their growth patterns were measured every 24 h until death or emergence. See legend to Fig. 1 for description of format.
correlation between the amount of growth alteration, such as irregular moulting and metamorphosis, and the amount of virus used for injection. This suggested that the virus can replicate only in the primary infected cells, that secrete EGT, which alters their growth. The alteration seemed to occur only when the haemolymph EGT concentration increased over the threshold level. The probable single AcMNPV infection of the silkworm cells was consistent with the result in the in vitro experiment (Table 1). The increased amount of virus that is necessary to alter the growth of the fifth instar larvae also supports this correlation. We suggest that the egt gene of AcMNPV was expressed in the cells of the larvae that were susceptible to virus infection and that EGT, secreted into the haemolymph, impaired the hormonal control of the larva by inactivating the ecdysteroids by sugar conjugation.

To determine the amount of virus replication, the fourth and fifth instar larvae were injected with wt AcMNPV at 5 × 10^6 or 5 × 10^7 TCID_{50} per larva and the change in the BV titres of the haemolymph was monitored for 168 h p.i. At 0 h p.i., the virus titres of the haemolymph was collected from the larvae, and the BV titres in the haemolymph were determined at 0, 48, 120 and 168 h p.i. for the fourth instar larvae and at 0, 36, 72 and 108 h p.i. for the fifth instar larvae, respectively. Fig. 3 shows the growth of the larvae infected with wt AcMNPV, which confirmed the alteration of moulting and metamorphosis (Figs 1 and 2). The change in growth of the vEGTDEL-injected larvae was not demonstrated when compared to the mock-infected larvae (Fig. 4). Because vEGTDEL infection of the BmN4 cells manifested a virus growth curve which was similar to that of wt AcMNPV and vEGTZ (data not shown), vEGTDEL might not have replicated in the larvae and probably changed the virus titres in a similar manner to that of wt AcMNPV.

To determine if growth alteration can be induced by per os infection of wt AcMNPV or vEGTDEL OB, the fourth or fifth instar larvae were provided with an artificial diet contaminated with the OB at 1 × 10^7 or 1 × 10^6 per larva, respectively. The larvae provided with the viral OB did not manifest any alteration of growth (data not shown), which suggests that AcMNPV cannot infect the silkworm larvae by a conventional pathway.

To determine if the growth alteration of the silkworm by injection of wt AcMNPV was caused by the egt gene, wt AcMNPV or vEGTDEL at 1 × 10^6 or 1 × 10^7 TCID_{50} per larva was injected into the fourth and fifth instar larvae, respectively. Fig. 4 shows the growth of the larvae infected with wt AcMNPV, which confirmed the alteration of moulting and metamorphosis (Figs 1 and 2). The change in growth of the vEGTDEL-injected larvae was not demonstrated when compared to the mock-infected larvae (Fig. 4). Because vEGTDEL infection of the BmN4 cells manifested a virus growth curve which was similar to that of wt AcMNPV and vEGTZ (data not shown), vEGTDEL might not have replicated in the larvae and probably changed the virus titres in a similar manner to that of wt AcMNPV.

EGT of baculovirus is necessary as a growth regulator in the infection of the target insects. This association between a baculovirus gene product and the hormonal control system in susceptible insects has been studied previously. In the fourth instar larvae of a gypsy moth infected with Lymantria dispar NPV (LdMNPV), in a similar manner to the egt-minus LdMNPV infection, ecdysteroid titres in the haemolymph increased more than those of mock-infected larvae, which suggested an insensitivity of the insect with the normal hormonal system caused by virus infection (Park et al., 1993). However, during larval–pupal moulting the ecdysteroid titres in the haemolymph of the infected insects did not rise above basal level. In contrast, in the fifth instar larvae of the fall armyworm infected with AcMNPV, ecdysteroid levels remained low throughout infection (O’Reilly et al., 1992). These results indicate that ecdysteroid titres in the haemolymph of insects infected with a baculovirus could increase or decrease, depending on a combination of the virus and the susceptible insect or the growth stage of the insect used for infection. In
this study, we used AcMNPV and the silkworm as a non-susceptible insect and demonstrated that the egt gene of AcMNPV can alter the growth of the silkworm when BV is injected. Interestingly, the growth alteration of the fourth instar larvae injected with wt AcMNPV is similar to the metamorphosis of the instar larvae which had their corpora allata removed (Fukuda, 1944), and juvenile hormone (JH) is not present but a relatively large amount of ecdysteroids is present. Because the productive infection of baculovirus in a homologous combination of virus and insect does not induce metamorphosis but blocks larval to larval molting and no affect of baculovirus infection on JH titre has been reported, it is possible in our results that the haemolymph JH titre of the infected larvae is affected by non-productive infection by AcMNPV. The determination of the haemolymph JH titre and ecdysteroid titre in silkworms infected with AcMNPV may reveal how altered hormones can contribute to the induction of irregular growth. The results would be a useful example of hormonal regulation by baculovirus in the absence of productive infection compared to the homologous combination of baculovirus and host insect.

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References


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