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Ecdysteroid Stimulates Virus Transmission in Larvae Infected with *Bombyx mori* Nucleopolyhedrovirus.

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Most baculoviruses have an ecdysteroid UDP-glucosyltransferase (*egt*) gene, whose product inactivates ecdysteroid within the infected host. *Bombyx mori* larvae infected with BmEGTZ, a mutant *B. mori* nucleopolyhedrovirus (BmNPV) in which the *egt* gene has been inactivated, die more rapidly compared to larvae infected with wild-type BmNPV. In this study, the profile of hemolymph proteins, and progression of virus infection in BmEGTZ- and BmNPV-infected *B. mori* larvae, was analyzed by SDS-PAGE and histochemically. These analyses showed that virus-encoded and virus-induced proteins were expressed quicker in BmEGTZ-infected larvae than in BmNPV-infected larvae. This suggests that the decrease in time to death, following BmEGTZ infection, results from the stimulation of virus-specific protein expression. In order to examine the effect of ecdysteroid on virus transmission, the profile of hemolymph proteins, and progression of virus infection, were analyzed following an ecdysteroid injection of BmEGTZ- or BmNPV-infected larvae. In the BmNPV-infected larvae, ecdysteroid treatment had no apparent effect on hemolymph protein expression. This suggests that the injected ecdysteroid was inactivated by the BmNPV-expressed ecdysteroid UDP-glucosyltransferase. An Ecdysteroid injection into BmEGTZ-infected larvae increased the speed of virus-specific protein expression and virus transmission. These results suggest that ecdysteroid stimulates protein expression, which in turn results in the stimulation of virus transmission.

Keywords: BmNPV, *Bombyx mori* nucleopolyhedrovirus, Ecdysteroid UDP-glucosyltransferase, *egt*

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Introduction

Baculoviruses are invertebrate-specific viruses characterized by circular, double-stranded DNA genomes and enveloped, rod-shaped virions. In general, following baculovirus infection of a susceptible insect larva, larval molting and pupation are blocked. The virus-encoded enzyme (ecdysteroid UDP-glucosyltransferase (EGT)), which catalyzes the conjugation of a sugar molecule to the insect molting hormone ecdysteroid, is thought to be involved in this block since sugar-conjugated ecdysteroid is functionally inactive. Following the initial identification of the *egt* gene in the baculovirus *Autographa californica* nucleopolyhedrovirus (AcMNPV) (O'Reilly & Miller, 1989), an *egt* gene homologue was identified in most of the other baculoviruses (Clarke *et al.*, 1996).

When larvae are infected with a mutant AcMNPV, which lacks a functional *egt* gene, these larvae stop feeding and prepare to molt or pupate (O'Reilly & Miller, 1989). O'Reilly *et al.* (O'Reilly & Miller, 1991; O'Reilly *et al.*, 1998) speculated that the *egt* gene product of the wild-type AcMNPV prolongs feeding, resulting in an enlarged larva which yields greater numbers of progeny virions. It was also been shown that larvae infected with an *egt* deletion mutant are killed faster than larvae infected with wild-type virus (O'Reilly & Miller, 1991; Park *et al.*, 1996). We previously reported that *Bombyx mori* larvae injected with BmEGTZ, a *B. mori* NPV (BmNPV) deletion mutant of *egt*, also die faster (by about 12 h) than larvae infected with wild-type BmNPV (Kang *et al.*, 1998). Flipsen *et al.* (1995) showed histochemically that an AcMNPV *egt* deletion mutant induces the degeneration of Malpighian tubules in *Spodoptera exigua* larva. Furthermore, since this phenomenon is not observed in wild-type AcMNPV infected larvae, they suggested that the Malpighian tubule degeneration is involved in the rapid killing. However, the mechanistic basis for this reduced time to death is still unclear. The aim of this study is: (1) Elucidate the physiological basis for the reduced time to death of

BmEGTZ-infected larvae, compared to BmNPV-infected larvae. (2) Examine the role, if any, of ecdysteroid in this process. In order to accomplish this, the profile of hemolymph proteins, and progression of virus infection, were examined by SDS-PAGE and histochemical analyses of newly molted fifth instar *B. mori* larvae; which were virus-infected, or virus-infected and subsequently ecdysteroid treated.

Materials and Methods

Cells, viruses, and silkworms The *B. mori*-derived BmN cell line was maintained in Grace's medium (Gibco BRL, New York, USA) supplemented with 0.26% tryptose broth and 10% fetal bovine serum. The wild-type BmNPV T3 isolate (Maeda *et al.*, 1985), as well as the BmNPV deletion mutants BmCysPD (Ohkawa *et al.*, 1994) (lacking the endogenous cysteine protease gene and BmEGTZ in which the endogenous *egt* gene was disrupted by insertion of a lacZ gene cassette (Maeda *et al.*, unpublished)), were propagated and titrated on BmN cells, as described previously (Maeda, 1989; Koh *et al.*, 1998). Silkworm, *B. mori*, larvae were reared on an artificial diet at 27°C, as described previously (Choudary *et al.*, 1995).

Virus infection of larvae Female larvae on the third day, after the third or fourth larval ecdysis, were hemocoelically injected with 1 x 10^5 plaque forming units (PFU) of each virus (BmEGTZ, BmNPV or BmCysPD) in 20 ul of tissue culture medium. Control insects were injected with 20 ul of tissue culture medium.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) At 12, 24, 36, 48, 60, 72, 84, 96 108 or 120 h postinfection (p.i.), a proleg of treated larva was pierced with a sterilized pin and the hemolymph was collected into a chilled microfuge tube containing a few crystals of phenylthiourea in order to prevent melanization, as described previously (Choudary *et al.*, 1995). Hemolymph proteins were analyzed by vertical gel electrophoresis (12% acrylamide gels) in the presence of SDS, as described by Laemmli (1970). All of the samples were boiled for 5 min in the presence of 2% SDS, 6.25 mM Tris-HCl pH 6.7, 15% glycerol and 100 mM dithiothreitol before loading them onto the gel. Following electrophoretic separation, the proteins were stained with Coomassie brilliant blue R-250 (Bio-Rad, Richmond, USA).

Preparation and examination of larvae for lacZ expression Larvae were prepared for X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) staining by securing them individually onto styrofoam blocks with insect pins through the head capsule and terminal abdominal segment. Using scissors, a longitudinal incision was made through the dorsal cuticle. The cuticle was spread out to expose the internal tissues using four additional insect pins and the dissected larvae were covered with dissecting buffer (PBS: 0.5 M phosphate, 0.5 M NaCl, pH 6.0) for 3 min. The dissecting buffer was then removed and the tissues immediately infused with fixative (4% paraformaldehyde in PBS) for 20 min. Following fixation, the larvae were washed twice with PBS and infused with staining solution [5 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$, 2 mM $MgCl_2$ in distilled H_2O] containing 2 mg/ml X-Gal for 6-12 hr in total darkness, as described previously (Engelhard *et al.*, 1994). The stained larvae were observed under a dissecting microscope at 10-40x.

Ecdysteroid treatment Female larvae (newly emerged fifth instar) were first injected with 1×10^5 PFU of each virus (BmEGTZ, BmNPV T3 or BmCysPD), as described above. At 24 h post virus injection, the larvae were reinjected with 10 ul of a solution containing either 50 ng or 100 ng [200- or 100-fold dilution of 1 mg/ml ecdysteroid (Sigma, St. Louis, USA) in 5% methanol] of ecdysteroid. At 72, 96 or 120 h post ecdysteroid treatment, the larval tissues were subjected to X-Gal, staining as described above, or the hemolymph proteins were collected and subjected to SDS-PAGE, as described above.

Results and Discussion

Comparison of the hemolymph protein profiles between BmEGTZ- and BmNPV T3- infected larvae In order to establish a biochemical basis for the accelerated death of BmEGTZ-infected *B. mori*, the hemolymph protein profiles (between BmEGTZ- and BmNPV-infected larvae) were compared by SDS-PAGE. At 48 h p.i. and earlier, the differences in the hemolymph protein profiles of larvae (injected with either BmEGTZ or BmNPV on the second day after the third larval ecdysis) were not observed (data not shown). Thereafter, temporal differences were found in the profiles; virus-expressed or virus-induced proteins were found at least 12 h earlier in BmEGTZ-infected larvae (Fig. 1A). For example, virus-specific proteins of 16, 31, 40, and 50 kDa were detected at 60 h p.i. in BmEGTZ-infected larvae; whereas, these proteins were undetected in BmNPV-infected larvae until 72 h p.i. or later. The expression of polyhedrin (a major, very late, virus structural protein of 31 kDa) in BmEGTZ-infected larvae at 84 h p.i., however, was not as high compared to that in BmNPV-infected larvae at 96 h p.i. A similar pattern of the accelerated appearance (by at least a 12 h) of virus-expressed, or virus-induced proteins, was found in the hemolymph of larvae injected with BmEGTZ on the third day, after the fourth larval ecdysis when compared to BmNPV-injected larvae (Fig. 1B).

Histochemical analysis of virus transmission following BmEGTZ or BmCysPD infection Virus transmission in BmEGTZ- or BmCysPD-infected larvae was compared at 2, 3, 4 or 5 days p.i. by histochemical detection (with X-Gal) of beta-galactosidase expression. In these experiments, a control virus (BmCysPD) was used, which carries a lacZ gene cassette identical to that of BmEGTZ at the cysteine protease gene locus. BmCysPD has indistinguishable growth characteristics compared to BmNPV (Ohkawa *et al.*, 1994). Following the injection of *B. mori* larvae (5th instar, day 3) with either BmEGTZ or BmCysPD, beta-galactosidase expression was clearly detected in the fat body and tracheal tissues of BmEGTZ-infected larvae at 2 days p.i. (Fig. 2B),

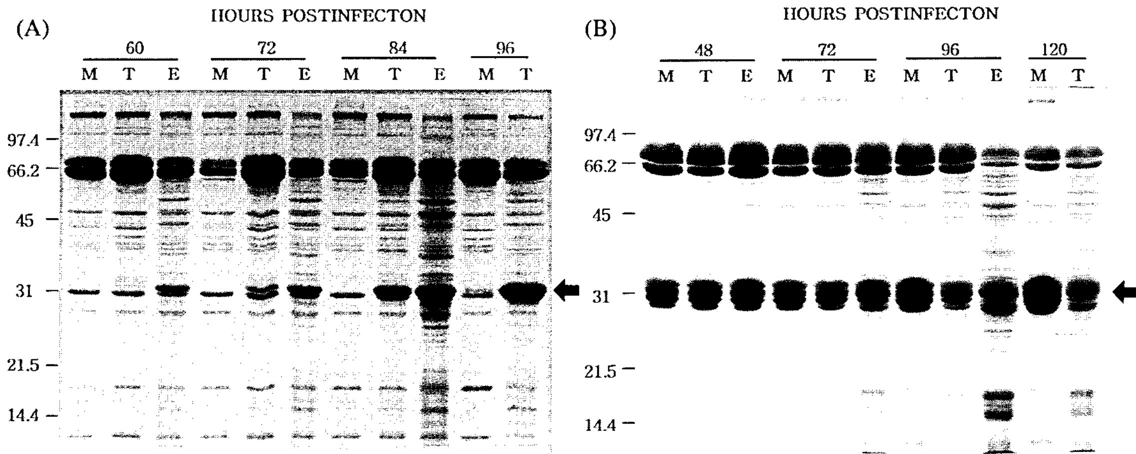

Fig. 1. SDS-PAGE analysis of hemolymph proteins from *B. mori* larvae that were mock- (M), wild-type BmNPV- (T) or BmEGTZ-infected (E) on the second day of the third ecdysis (A), or the third day of the fourth larval ecdysis (B). The hemolymph was collected at 48, 60, 72, 84, 96, or 120 h p.i. The arrowhead indicates the migration of polyhedrin. Protein molecular masses in kilo Daltons are shown at the left.

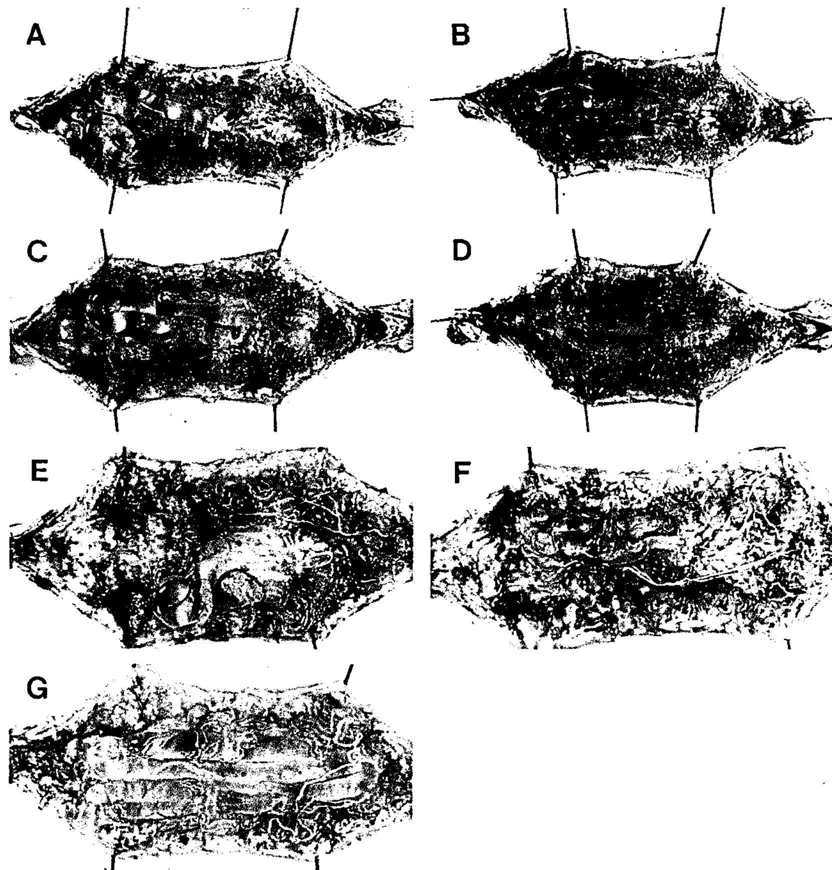

Fig. 2. Histochemical analysis of the progression of virus infection as determined by LacZ expression (blue staining) in *B. mori* larvae infected with control BmCysPD (A, C, E and G) or BmEGTZ (B, D and F) on the third day of the fourth larval ecdysis. Larvae were stained at 2 (A and B), 3 (C and D), 4 (E and F) or 5 (G) days p.i.

but only barely detectable in BmCysPD-infected larvae (Fig. 2A). At 3 days p.i., the staining of fat body and tracheal tissues in BmEGTZ-infected larvae intensified; staining was also detected in other tissues including midgut, silkgland, Malphigian tubules, nervous system, and gonads (Fig. 2D). The staining intensity of BmCysPD-infected larvae (Fig. 2C) was similar to that found in BmEGTZ-infected larvae at 2 days p.i. At 4 days p.i., similar differences were found in the

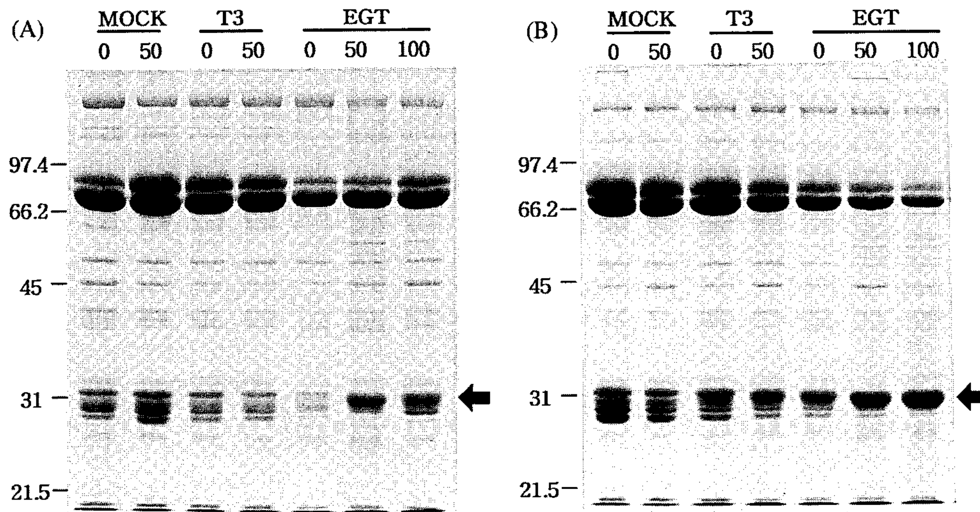

Fig. 3. SDS-PAGE analysis of hemolymph proteins at 96 h p.i. (A) or 120 h p.i. (B) from *B. mori* larvae that were mock- (MOCK), wild type BmNPV- (T3) or BmEGTZ-infected (EGT) on the day of the fourth larval ecdysis. At 24 h post virus or mock infection, individual larva was injected with zero, 50 or 100 ng of ecdysteroid as indicated. The arrowhead indicates the migration of polyhedrin. Protein molecular masses in kilo Daltons are shown at the left.

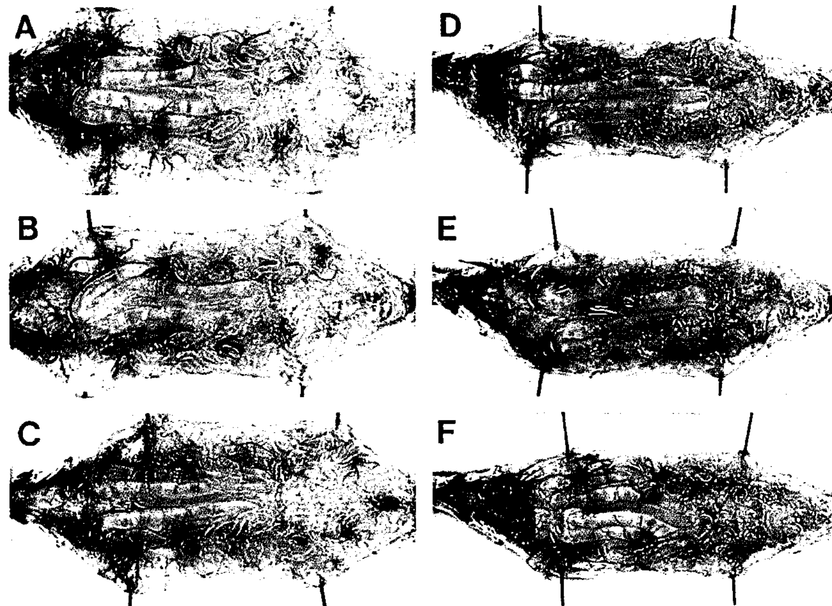

Fig. 4. Histochemical analysis of the progression of virus infection as determined by LacZ expression (blue staining) in *B. mori* larvae infected with BmEGTZ on the day of the fourth larval ecdysis and treated with zero (A and D), 50 (B and E) or 100 ng (C and F) of ecdysteroid per larva at 24 h p.i. The larvae were dissected at 3 (A, B, and C) or 4 (D, E, and F) days p.i.

staining intensities between BmEGTZ- and BmCysPD-infected larvae (Fig. 2F and E). At 5 days p.i., all of the BmEGTZ-infected larvae had died and were not subject to histochemical analysis. In contrast, all of the BmCysPD-infected larvae were still alive and showed a staining intensity that was similar to BmEGTZ-infected larvae at 4 days p.i. (Fig. 2G).

Effect of ecdysteroid treatment on hemolymph proteins

In order to examine how ecdysteroid affected the profile of proteins in the hemolymph of virus-infected *B. mori*, BmEGTZ- or BmNPV-infected 5th instar larvae were individually injected at 24 h post virus injection with a solution containing 0, 50, or 100 ng of ecdysteroid. Fifth instar larvae were used because at this larval stage endogenous ecdysteroid exists only in trace amounts (Kiguchi, 1983). The hemolymph proteins were collected at 96 h post virus infection and analyzed by SDS-PAGE (Fig. 3A). In mock-infected larvae, ecdysteroid treatment had no apparent effect in the profile of hemolymph proteins. This suggests that ecdysteroid itself does not affect the expression of hemolymph proteins. Ecdysteroid treatment also had no apparent effect on

hemolymph protein expression in BmNPV-infected larvae. This suggests that the injected ecdysteroid was inactivated by the BmNPV-expressed ecdysteroid UDP-glucosyltransferase. In contrast, ecdysteroid treatment appeared to accelerate virus-specific protein synthesis in BmEGTZ-infected larvae. For example, polyhedrin was detected in ecdysteroid treated larvae, but not in the non-ecdysteroid treated BmEGTZ-infected larvae at 96 h p.i. This level of polyhedrin expression was not detected in the non-ecdysteroid treated BmEGTZ-infected larvae until 120 h p.i. (Fig. 3B). Furthermore, this effect may be concentration dependent since the expression of polyhedrin appeared to be greater following ecdysteroid treatment at 100 ng/larva, when compared to treatment 50 ng/larva.

Histochemical analysis of the effect of ecdysteroid on virus transmission Ecdysteroid was injected into larvae at 24 h post BmEGTZ infection on the day of the fourth larval ecdysis. At 3 days p.i., tracheal and fat body tissues from ecdysteroid treated larvae (Fig. 4B and C), showed slightly stronger X-Gal-staining compared to non-ecdysteroid-treated larvae (Fig. 4A). Staining was also slightly more intense in larvae injected with a high dose (100 ng/larva) of ecdysteroid, in comparison to those injected with a low dose (50 ng/larva). At 4 days p.i., the staining intensity increased for all treatments, but was slightly more intense in the larvae treated with 100 ng of ecdysteroid (Fig. 4F), compared to the other treatments (Fig. 4D and E). These histochemical observations coincided with the SDS-PAGE analysis of the hemolymph proteins (Fig. 3). Furthermore, these findings indicated that virus transmission is affected by not only ecdysteroid, but by its concentration.

Larvae infected with a baculovirus carrying an inactivated *egt* gene succumb to viral infection quicker than larvae infected with a wild-type virus carrying an active *egt* gene (O'Reilly & Miller, 1991; Park *et al.*, 1996; Kang *et al.*, 1998). In this study, we found that in *B. mori* larvae the expression of virus-encoded, or virus-induced proteins, is accelerated following infection with a mutant BmNPV, in which the endogenous *egt* gene is inactivated. Furthermore, this accelerated protein expression apparently causes the accelerated transmission of the virus. This results in the early death of BmEGTZ-infected larvae, in comparison to BmNPV-infected larvae. Keeley and Vinson (1975) reported that the injection of 20-hydroxyecdysone into baculovirus-infected *Heliothis virescens* results in a delay in the onset of virus-induced pathology, as well as a decrease in the mortality of the infected insects. Similarly, by overexpressing prothoracicotropic hormone (PTTH) in baculovirus-infected larvae, O'Reilly *et al.* (1995) suggested that ecdysteroid has a direct inhibitory effect on viral replication. Our data, however, showed that following ecdysteroid treatment there is an acceleration in virus-specific protein synthesis, as well as virus transmission. This suggests that ecdysteroid plays a direct role in enhancing virus replication.

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