Int. J. Indust. Entomol. Vol. 8, No. 2, 2004, pp. 169~174

International Journal of
Industrial Entomology

Characterization of Ecdysteroid UDP-Glucosyltransferase Gene Promoter from *Bombyx mori* Nucleopolyhedrovirus

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***Bombyx mori* nucleopolyhedrovirus (BmNPV) ecdysteroid UDP-glucosyltransferase gene (*egt*) promoter fragments of different lengths were amplified from BmNPV ZJ-8 genomic DNA by PCR. Reporter plasmids pBm*egt*542-*luc*, pBm*egt*309-*luc* and pBm*egt*159-*luc* with *luciferase* (*luc*) driven by *egt* promoters were constructed. Both *in vitro* and *in vivo* expressions showed that BmNPV *egt* promoter activity requires the transactivation of viral factor(s), and expression of *luc* was detected earliest at 24 hrs post infection (pi). BmNPV ZJ-8 homologous region 3 (*hr3*) increased the expression of *luc* by over 1,600-fold. Molting hormone of 1.0 ~ 2.0 μg/ml can dramatically down regulate expression of *luc*. Juvenile hormone analogue of 0.5 ~ 2.0 μg/ml increased expression of *luc* by 145.8% to 75.7%. Deletion assay revealed that the promoter fragment of 159 bp contains the basal promoter structure; Promoter fragments of 309 bp and 542 bp showed similar but much higher transcriptional activities than that of 159 bp, suggesting that nucleotide from –159 to –309 nt upstream the translation initiation site harbors the main *cis*-acting elements.**

Key words: *Bombyx mori*, Baculovirus, Ecdysteroid UDP-glucosyltransferase gene, Promoter, Transient expression, Insect hormones

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Introduction

Insect baculovirus *egt* genes encode ecdysteroid UDP-glucosyltransferase (EGT) (O'Reilly *et al.*, 1989). EGT existing mainly as an oligomer of three to five subunits is synthesized in host insect cells by the virus and secreted into the host hemolymph (O'Reilly *et al.*, 1990; Evans *et al.*, 1998, 1999). EGTs conjugate UDP-galactoses to host ecdysteroids forming inactive ecdysteroid-galactosides, by which the viruses control the molting and metamorphism of the host insects to prolong the feeding time of host and therefore, increase the yield of progeny virus (O'Reilly *et al.*, 1989, 1990, 1991). *Autographa californica* nucleopolyhedrovrius (AcMNPV) *egt* is an immediate-early gene, its transcription does not depend on synthesis of viral proteins and DNA replication but declines late in infection (O'Reilly *et al.*, 1991; Evans *et al.*, 1998). AcMNPV *egt* is non-essential for viral replication and the 50% survival time for insects infected by *egt*-minus AcMNPV is 20 – 30% shorter than for insects infected by wild-type (wt) virus (O'Reilly *et al.*, 1991; Eldridge *et al.*, 1992). The promoter activity of *Spodoptera littoralis* nucleopolyhedrovrius (SpliMNPV) *egt* is weak and requires the transactivation of immediate-early protein IE-1, and its transcription occurs earliest at 8 hrs pi, but the EGT activity is detected at 3 hrs pi (Toister-Achituv *et al.*, 1997). However, the transcription of *Lymantria dispar* nucleopolyhedrovrius (LdMNPV) *egt* gene occurs from 12 to 48 hrs pi with the peak amount of mRNA at 16 hrs pi, and transcription continues in the presence of aphidicolin, but not in the presence of cycloheximide. LdMNPV *egt* is therefore classified as a delayed-early gene (Riegel *et al.*, 1994).

Bombyx mori nucleopolyhedrovirus (BmNPV) *egt* locates at the region from 6407 to 7925 nt of genomic DNA (Gomi *et al.*, 1999), and nucleotide sequence of

BmNPV ZJ-8 *egt* gene shows 95% homology with that of AcMNPV (Ayres *et al.*, 1994; Ji *et al.*, 2000). Here we report the characteristics of BmNPV *egt* promoter and effects of BmNPV *hr3*, MH and JHA on the promoter activity.

Materials and Methods

Bacterial, vectors, silkworms and reagents

BmNPV ZJ-8, *Bombyx mori*-derived Bm5 cells and silkworm variety JY-1 are maintained in our laboratory. Silkworm larvae were reared at 26 ± 1°C. *E. coli* TG1, Bluescript SK(M13-), pSK-*hr3* containing a BmNPV *hr3*, and pUL220-*luc* were provided by Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Science (Lei *et al.*, 1993, 1994; Zhang *et al.*, 1995). Vector pGEM-3Z was from Promega. And plasmid pSK-*hsp70-LacZ-hr3* containing β-galactosidase driven by *hsp70* promoter and enhanced by BmNPV ZJ-8 *hr3* was previously constructed in our laboratory (Zhou 2002; Tang *et al.*, 2003; Zhou *et al.*, 2003A).

Enzymes, insect cell culture medium TC-100, fetal bovine serum (FBS) and lipofectin were from Invitrogen. E4030 kit for luciferase assay was from Promega. The insect molting hormone (MH) 20-β-hydroxylecdysterone was prepared by the Sericultural Research Institute. Juvenile hormone analogue (JHA) ZR512 was kindly donated by Professor Cui Wei-Zheng from Shandong Agriculture University. Other reagents were from Sigma Chemical. Luciferase activities were measured by a Beckman LS-600TA liquid scintillation spectrometer.

Construction of reporter plasmids

BmNPV ZJ-8 genomic DNA was prepared as described previously (Zhang, *et al.*, 1995). The PCR primers for amplification of 542 bp BmNPV *egt* promoter were designed in accordance with nucleotides of BmNPV T3 (GenBank Accession Number: L33180) and *egt* of BmNPV ZJ-8, respectively (Gomi *et al.*, 1999; Ji *et al.*, 2000). Forward primer 1 (FP1, 5'-TCGAATTCCTTGTACCGATGCACGCGAA-3') corresponds to the region between nucleotides −544 and −524 relative to the translation initiation site of BmNPV T3 *egt* and contains an *Eco*RI site at 5'-end. Reverse primer 1 (RP1, 5'-AAGGATCCAATTTTGCTTCAACCCGAATAACTG-3') complementary to the region between nucleotides −22 and +2 relative to the *egt* translation initiation site of BmNPV ZJ-8, ATG was mutated to ATT, and contains a *Bam*HI site at 5'-end. A 542 bp *egt* promoter segment was generated and cloned into the polycloning site of pGEM-3Z vector after *Eco*RI-*Bam*HI digestion as described previously for construction of pBm*egt*542. Then *luc* segment separated from pUL220-*luc* by *Bam*HI digestion was cloned into pBm*egt*542/*Bam*HI downstream the *egt* promoter for construction of reporter plasmid pBm*egt*542-*luc*. BmNPV ZJ-8 *hr3* segment (Genbank Accession Number: U51238) separated from pSK-*hr3* by *Pst*I digestion was cloned into pBm*egt*542-*luc* downstream the *luc* for construction of reporter plasmid pBm*egt*542-*luc-hr3*.

For the deletion assay of BmNPV *egt* promoter, two other forward primers corresponding to the region between nucleotides −309 and −291, −159 and −141, relative to the translation initiation site of *egt* were designed, respectively. Forward primer 2 (FP2) for amplification of 309 bp *egt* promoter, 5'-CCGAATTCCAACGGTTTGACGTGCA-3' and forward primer 3 (FP3) for amplification of 159 bp *egt* promoter, 5'-ATGAATTCCCACATCATGTCGACG-3', matching the above RP1, respectively. The *egt* promoter segments of 309 bp and 159 bp were generated by PCR. And constructions of reporter plasmid pBm*egt*309-*luc* and pBm*egt*159-*luc* with *luc* driven by 309 bp and 159 bp length of *egt* promoter segments were generated as described above.

In vitro expression and insect hormone treatments

Bm5 cells were seeded into 12 cm^2 flasks with an approximately density of 5×10^5 cell/ml and cultured at 27°C over night. One μg reporter plasmid DNA and 0.5 μg normalization plasmid pSK-*hsp70-LacZ-hr3* DNA were mixed with 7.5 μl lipofectin in a 50 μl volume as transfection solution. After remove of primary medium and being washed twice with serum-free TC-100 medium, cells were transfected for 4 – 5 hrs and followed by infection of wt BmNPV (MOI = 1.0) for 1 hr. Then medium was replaced by 3 ml TC-100 medium containing 10% FBS and this moment was taken as zero time of infection. Cells were harvested at 48 hrs pi except for the temporal expression experiments. Cells transfected with pUL220 were taken as blank. Three replicates were done for each experiment. In hormone treatments, designed dosages of hormone were administrated after the replacement of medium. The non-hormone treatments were taken as control.

In vivo expression

Silkworm larvae of second day in the 5th instar were transfected with 20 μl transfection solution containing 1 μg reporter plasmid pBm*egt*542-*luc* or pBm*egt*542-*luc-hr3* and 5 μl lipofectin. Two hrs later, 1×10^6 pfu of wt BmNPV was injected into the hemocoel. Larvae transfected only with reporter plasmid were set as control, and larvae transfected only with pUL220 as blank. Haemolymph of five larvae was harvested and pooled.

Three replicates were used for each treatment.

Preparation of cell extracts and assay of luciferase activity

Cells were harvested by centrifuging at 9 000 g for 5 min at 4°C. Cell extracts were prepared with a kit (E4030, Promega), and lysates were processed with a freeze-thaw cycle at −20°C and room temperature followed by centrifugation at 4°C to remove cell debris and supernatants were used for luciferase assay. The β-galactosidase activity of the normalization plasmid and amount of total protein of the lysates were measured as previously described (Idahl *et al.*, 1986; Zhou 2002a; Tang *et al.*, 2003; Zhou *et al.*, 2003b). Same procedures were accomplished with hemolymph of silkworm larvae.

Results

BmNPV *egt* promoter activity requires transactivation of virus factor(s)

Bm5 cells were transfected with reporter plasmid pBm*egt*542-*luc* for 4 to 5 hrs. Thereafter, cells were infected with wt BmNPV. One hr later cell medium was replaced by fresh TC-100 containing 10% of FBS. At 48 hrs pi, the normalized luciferase activity per μg protein of cell extract was 11,706 ± 1,498.5 cpm. However, in uninfected cells luciferase activity was not detected. This revealed that activity of BmNPV *egt* promoter activity requires the transactivation of virus factor(s).

Temporal expression of *luc* driven by BmNPV *egt* promoter

Bm5 cells were transfected with pBm*egt*542-*luc* and infected with wt BmNPV as described above. The transfected but uninfected cells were taken as control. Cells were harvested at 2, 6, 12, 18, 24, 48 and 60 hrs pi, respectively. The luciferase assay results showed that luciferase activity was detected earliest at 24 hrs pi, and increased rapidly. At 48 hrs pi luciferase activity reached 19,708.5 ± 1,941.4 cpm (Fig. 1). But in uninfected cells, luciferase activity was not detected. This indicated that the expression *luc* driven by BmNPV *egt* promoter is late and demonstrated again that BmNPV *egt* promoter activity requires the transactivation of virus factor(s).

BmNPV *egt* promoter activity enhanced by BmNPV *hr3*

Bm5 cells were transfected with reporter plasmid pBm*egt*542-*luc-hr3* or pBm*egt*542-*luc* and infected with wt BmNPV setting uninfected cells as control. At 48 hrs pi, luciferase activity in cells transfected by pBm*egt*542-*luc-hr3* was $3.20 \times 10^7 \pm 1.92 \times 10^6$ cpm per ìg protein of cell extract, which is 1,600-fold of that of pBm*egt*542-*luc* transfected cells ($1.97 \times 10^4 \pm 5.4 \times 10^3$ cpm). It indicated that BmNPV *hr3* has significant enhancement effect on the activity of BmNPV *egt* promoter. But in all uninfected cells luciferase activity were not detected. This further proved that the activity of BmNPV *egt* promoter requires the transactivation of viral transfactor(s).

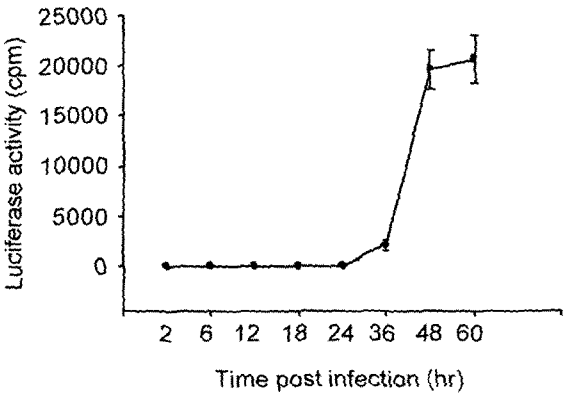

Fig. 1. Temporal expression of *luciferase* driven by BmNPV *egt* promoter. The harvesting hrs post infection (pi) are indicated on the X axis. The luciferase activities of pBm*egt*542-*luc* in Bm5 cells are indicated on the Y axis. The data are the average of three replicates normalized by β-galactosidase activity of normalization plasmid and per μg protein of extract. Luciferase activity was earliest detected at 24 hrs pi.

Effects of foreign insect hormones on BmNPV *egt* promoter activity

Bm5 cells were transfected with pBm*egt*542-*luc* and then 0.5, 1.0 and 2.0 μg per ml medium of MH or JHA were administrated during medium replacement. The results showed that luciferase activity indexes of cells treated with MH 0.5, 1.0 and 2.0 μg/ml were 123.9 ± 31.1%, 72.2 ± 5.8% and 28.3 ± 4.8% relative to non-hormone treatments set as 100%, respectively (Table 1). While in cells treated with JHA of 0.5 ~ 2.0 μg/ml, luciferase activity increased by 145.8% to 75.7%. But with the increase of JHA concentration, the luciferase activity augment declined (Table 2).

Deletion assay of BmNPV *egt* promoter

Bm5 cells were transfected with one of these three plasmids, pBm*egt*542-*luc* (542 bp), pBm*egt*309-*luc* (309 bp) and pBm*egt*159-*luc* (159 bp), for transient expression as described above. For each plasmid, uninfected cells were set as control and cells transfected with pUL220 were made as blank. Cells were harvested at 48 hrs pi and ready for assay of luciferase activity. The data showed that in uninfected cells, all three plasmids did not express. On the

Table 1. Effects of molting hormone on the activity of BmNPV *egt* promoter

MH concentration (μg/ml)	0.0	0.5	1.0	2.0
Luciferase activity (cpm)	8,426.0 ± 709.9	10,442.9 ± 2,621.8	6,082.5 ± 487.2	2,383.5 ± 403.8

Note: Cells transfected with reporter plasmid pBm*egt*542-*luc* for 4 ~ 5 hrs and then infected with wt BmNPV for 1 hr, follow by fresh medium (TC-100 contains 10% FBS) replacement and administration of molting hormone (MH). At 48 hrs pi cells were harvested for luciferase assay. The data represents the average of three replicates per ìg protein of cell extract. The pUL220 transfected cells were taken as blank.

Table 2. Effects of juvenile hormone analogue on the activity of BmNPV *egt* promoter

JHA concentration (μg/ml)	0.0	0.5	1.0	2.0
Luciferase activity (cpm)	11,449.9 ± 3,673.7	28,146.6 ± 7,057.1	24,753.1 ± 1,906.6	20,120.7 ± 2,070.1

Note: Cells transfected with reporter plasmid pBm*egt*542-*luc* for 4 ~ 5 hrs and then infected with wt BmNPV for 1 hr, follow by fresh medium (TC-100 contains 10% FBS) replacement and administration of juvenile hormone analogue (JHA). At 48 hrs pi cells were harvested for luciferase assay. The data represents the average of three replicates per ìg protein of cell extract. The pUL220 transfected cells were taken as blank.

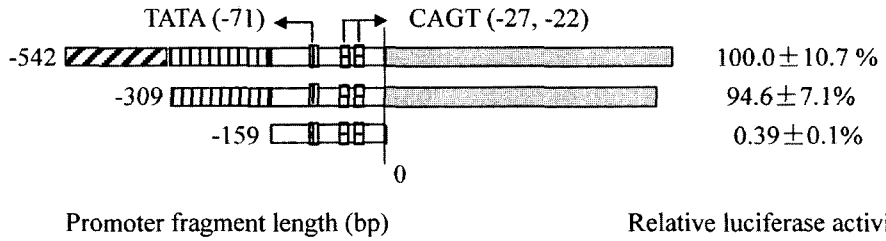

Fig. 2. Expression of *lucifrase* driven by BmNPV *egt* promoters of different lengths. The deletions of BmNPV *egt* promoter are shown on the left. TATA box and CAGT motifs are pointed out. And the relative activities of luciferase activities are shown on the right. The luciferase activity of pBm*egt*542-*luc* is set as 100%, that of pBm*egt*309-*luc* and pBm*egt*159-*luc* are 94.6 ± 7.1% and 0.39 ± 0.1%, respectively.

contrary, in the infected cells luciferase activity was detected but obviously different from plasmids. Luciferase activities of pBm*egt*542-*luc*, pBm*egt*309-*luc* and pBm*egt*159-*luc* were 11,000.3 ± 1173.4, 10,401.3 ± 780.3 and 43.2 ± 9.4 cpm, respectively (Fig. 2). The results indicated that nucleotide region from −1 to −159 nt upstream the translation initiation site ATG of BmNPV *egt* has the basal transcriptional activity, i.e. this region harbors basal promoter elements.

***In vivo* expression of *luc* driven by BmNPV *egt* promoter**

Reporter plasmids pBm*egt*542-*luc* and pBm*egt*542-*luc-hr3* were used to transfect silkworm lavae of 5th instar, respectively. Hemolymph was harvested at 8, 12, 18 and 24 hrs pi. In larvae transfected with pBm*egt*542-*luc-hr3* and infected with wt BmNPV, luciferase activity was earliest detected at 18 hrs pi, which was 1,356.9 ± 99.9 cpm per μg protein of extract. However, in larvae transfected with pBm*egt*542-*luc* and infected with wt BmNPV only a weak luciferase activity (40 ± 5.7 cpm) until 48 hrs pi.

Discussion

Previously it had been shown that AcMNPV *egt* is an immediate-early gene as reviewed (O'Reilly, 1997) and its transcription does not depend on synthesis of viral proteins and DNA replication (O'Reilly *et al.*, 1991). The transcription of SpliMNPV *egt* requires the transactivation of IE-1 (Toister-Achituv *et al.*, 1997). A recent study showed the transcripts of *Epiphyas postvitana* multicapsid nucleopolyhedrovirus *egt* and EGT activities are earliest detected at 9 hrs pi (Caradoc-Davise *et al.*, 2001). LdMNPV *egt* is a delayed-early gene and its transcription occurs from 12 to 48 hrs pi (Riegel *et al.*, 1994). In this experiment, *Bombyx mori*-derived Bm5 cells and larvae were used for transient expression assay. The results showed that BmNPV *egt* promoter activity required the transactivation of viral factor(s) and expression of *luc* driven by BmNPV *egt* promoter was very late, occurring at 24 hrs pi. On the other hand, there is one putative TATA box and two early CAGT motifs in BmNPV *egt* promoter region similar to that of AcMNPV *egt* (O'Reilly *et al.*, 1990; Blissard *et al.*, 1992; Chen *et al.*, 1997; Toister-

Achituv *et al.*, 1997). Thus we deduced that BmNPV *egt* is not an immediate-early but delayed-early gene at least. BmNPV homologous regions function as the origins of viral replication and enhancers of gene transcription (Zhang *et al.*, 1995; Lu *et al.*, 1997; Xiao *et al.*, 2001; Zhou *et al.*, 2003). In our experiments BmNPV *hr3* (pB*megt*542-*luc-hr3*) increased luciferase activity by 1,600-fold.

The balance of MH and JH regulates the development and metamorphosis of insects (Hiruma *et al.*, 1999). Our experiments showed that MH of 0.5 μg/ml has no significant effect, but MH of 1.0 – 2.0 μg/ml significantly decreased the activity of BmNPV *egt* promoter. This may because MH of high concentration caused a negative feedback to inhibit the activity of the *egt* promoter since MH is one of the substrate of EGT. JHA of 0.5 – 2.0 μg/ml increased expression of *luc* by 145.8% to 75.7%, but with the increase of JHA dose the augmentation declined. The main function of JH is to maintain the structure and function of cells and organs in silkworm larvae and therefore inhibits the metamorphosis of larvae and disintegration of cells and organs, extending the period of protein synthesis (Lü, 1991). So when relative low concentration of JHA was provided, a higher luciferase activity was observed in Bm5 cells. Meanwhile JHA increases the replication of nucleopolyhedrovirus (Zhou *et al.*, 2002). Thus when JHA concentration increased to a certain level, the replication of BmNPV was increased and BmNPV accelerated the disintegration of cells leading to a decline in augmentation of luciferase activity.

Deletion assays of BmNPV *egt* promoter revealed that nucleotide region from −1 to about −159 nt upstream the *egt* translation initiation site contains the basal structure of BmNPV *egt* promoter such as TATA box and two CTAG motifs as described above, but its transcriptional activity is almost abolished. Promoter segments of 309 bp and 542 bp showed similar transcriptional activities, which are 200-fold of that of 159 bp, revealing that nucleotide region from −159 to −309 bp upstream the translation initiation site is likely to contain the main *cis*-acting elements of transcription, for example the early gene conserved sequence ATTGTGTTA upstream the TATA box as that of AcMNPV *egt* (O'Reilly *et al.*, 1990; Lü 1998).

Acknowledgments

This work was supported by the grant from the National Natural Science Foundation of China (No.30271007).

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