The Ecdysteroid UDP-Glucosyltransferase Gene Promoter from *Autographa californica* Multicapsid Nucleopolyhedrovirus

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The ecdysteroid UDP-glucosyltransferase (*egt*) gene promoter fragments of different lengths were generated from the genomic DNA of the *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV) by PCR. After being purified and enzymatic digestion, they were cloned into the pGEM-3Z vector for construction of reporter plasmids pAc*egt*542-*luc*, pAc*egt*309-*luc* and pAc*egt*159-*luc* with the luciferase gene driven by the AcMNPV *egt* promoter. The results of transient expression in the *Spodoptera frugiperda* cell line-21 (Sf21) showed that the transcriptional activity of the AcMNPV *egt* promoter required the transactivation of viral factor(s). The expression of luciferase gene driven by the AcMNPV *egt* promoter was first detected at 24 h post infection. The *egt* promoter from the *Bombyx mori* nucleopolyhedrovirus (BmNPV), closely related to AcMNPV, revealed similar properties to that of the AcMNPV *egt* promoter. When BmNPV homologous region 3 was subcloned downstream the luciferase gene, the luciferase activity of the reporter plasmid was enhanced by over 1000-fold, but the property of the promoter was not changed. As a substrate of ecdysteroid UDP-glucosyltransferase (EGT), foreign insect ecdysone showed negative effects on *egt* promoter transcriptional activity. Ecdysone of 1.0–2.0 μg/ml significantly down-regulated the promoter activity. Promoter activities of different lengths showed that an AcMNPV *egt* promoter fragment of 159 bp has the basal transcriptional activity but it was almost abolished only about 0.2% of that of 309 bp and 542 bp, respectively, and the nucleotide sequence from – 159 to – 309 nt upstream the translation initiation site includes the main *cis*-acting elements interacting with viral factors.

Key words: Baculovirus, Ecdysteroid UDP-Glucosyltransferase Gene, Promoter

Introduction

The ecdysteroid UDP-glucosyltransferase (*egt*) gene of the baculovirus encodes ecdysteroid UDP-glucosyltransferase (EGT) (O'Reilly and Miller, 1989; O'Reilly, 1995). EGT is synthesized by the baculovirus in the host insect cells, existing mainly as an oligomer of three to five subunits, and secreted into the host haemolymph (O'Reilly and Miller, 1990; Evans and O'Reilly, 1999). EGT catalyses the conjugation of ecdysone and UDP-sugar forming the inactive ecdysone-22-*O*-β-D-glucoside thus controls the molting of the host and lengthens the feeding time of the host, and therefore facilitates the propagation of the baculovirus (O'Reilly and Miller, 1989; Evans and O'Reilly, 1998). The baculoviral *egt* gene is non-essential for replication of the virus, so deletion of *egt* gene does not interfere with the virus replication but enables the virus to kill its host in less time than the wilde-type (wt) virus (O'Reilly and Miller, 1991; Eldridge *et al.*, 1992; Flipsen *et al.*, 1995; Slavicek *et al.*, 1999), indicating that the efficiency of baculoviruses as potential pesticides may be improved by deletion of *egt* gene.

The *Autographa california* nucleopolyhedrovirus (AcMNPV) *egt* gene is the first one identified from baculovirus and has been extensively studied (O'Reilly and Miller, 1989). It is an early-expressed gene since its transcription does not depend on the synthesis of viral protein and DNA replication (O'Reilly and Miller, 1990). The *egt* transcriptional products are two 5′-co-terminal mRNAs of 1.8 kb and 3 kb and the transcription level declines late in infection. EGT activity is observed at 3 h post infection (hpi) in the virus infected cells and extracellular fluid (O'Reilly and Miller, 1990). The *Bombyx mori* nucleopolyhedrovirus (BmNPV) is the closely related baculovirus of AcMNPV. The newly pupated silkworm pu-

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pae infected with wt AcMNPV lead to artificial diapaused pupae (Zhang *et al.*, 1993). This metamorphosis change is resulted from the expression of AcMNPV *egt* gene (Shikata *et al.*, 1998).

Previously it has been shown that promoter activities of *egt* genes from AcMNPV and *Spodoptera littoralis* nucleopolyhedrovirus (SpliMNPV) require the transactivation of viral immediate-early gene product IE-1. In SpliMNPV infected cells, EGT activity was detected at 3 hpi in cell medium but the *egt* transcripts were first detected at 8 hpi (Toister-Achituv and Faktor, 1997), while the transcripts of *Lymantria dispar* nucleopolyhedrovirus (LdMNPV) *egt* were observed first at 16 hpi and lasted to 48 hpi (Slavicek *et al.*, 1999). The transcription of LdMNPV *egt* occurs in the presence of viral DNA synthesis inhibitor aphidicolin but not in the presence of protein synthesis inhibitor cycloheximide (Riegel *et al.*, 1994). In the genome of *Lacanobia oleracea* granulovirus, transcription of *egt* gene is mainly from a late gene promoter motif GTAAG (Smith and Goodale, 1998).

Because the importance of baculovirus in the biological control of insects and the *egt* gene is up to now the only one known which affects the hormone metabolism of host insects, a number of baculovirus *egt* genes has been characterized and exhibits different transcriptional properties. We report the characterization of AcMNPV *egt* promoter by transient expression in an insect cell line. In addition, we have investigated the effects of BmNPV homologous region 3 (*hr3*) and ecdysone on the promoter activity of AcMNPV *egt* and determined the main *cis*-acting region of the promoter.

Materials and Methods

Virus, bacterial, vectors, silkworm and reagents

AcMNPV, BmNPV ZJ-8, Sf21 and *Bombyx mori* ovary-derived cell line-5 (Bm5), *E. coli* TG1, pGEM-3Z, pSK-*hr3* containing a *hr3* from BmNPV ZJ-8 (Zhang *et al.*, 1995) and pUL220-*luc* containing a luciferase gene (Lei *et al.*, 1993) were maintained in our laboratory. The normalization plasmid pSK-*hsp70-LacZ-hr3* containing a β-galactosidase gene driven by *hsp70* promoter and enhanced by BmNPV ZJ-8 *hr3* was previously constructed in our laboratory (Zhou *et al.*, 2003).

Enzymes, cell culture medium TC-100, fetal bovine serum (FBS) and lipofectin were from Invitrogen. The E4030 kit for the luciferase assay was from Promega. The ecdysone, 20-β-hydroxyl-ecdysterone, was prepared by the Sericultural Research Institute. Other reagents were from Sigma Chemical. Luciferase activities were measured by a Beckman LS-600TA liquid scintillation spectrometer.

Construction of reporter plasmids

Genomic DNA of AcMNPV and BmNPV were prepared as previously described (Summers and Smith, 1987). Primers for PCR amplification of AcMNPV *egt* promoter were designed based on AcMNPV genomic nucleotide sequence (GenBank accession number: NC001623): Ac*egt*542 primer (forward-1) 5′-TC*GAATTC*CTTGTACCGATGCACGCGAA-3′, Ac*egt*309 primer (forward-2) 5′-CC*GAATTC*CAACGGTTTGACGTGCA-3′, Ac*egt*159 primer (forward-3) 5′-AT*GAATTC*CCACATCATGTCGACG-3′, corresponding to the region between nucleotides – 542 and – 522 nt, – 309 and – 291 nt, – 159 and – 141 nt relative to the translation initiation site of *egt*, respectively, there is an *Eco*RI site at 5′-end of each. And the reverse primer 5′-AG*GGATCC*AATTTTGCTTCAAACCGAATAACTG-3′, complementary to the region between nucleotides – 22 upstream the translation initiation site and + 3 nt containing a *Bam*HI site at 5′-end, ATG was mutated to ATT. Each of the above three forward primers pairs with the reserve one.

PCR amplification of AcMNPV *egt* promoter fragments was performed with above primers using the denatured genomic DNA of AcMNPV as template under normal condition. Thus AcMNPV *egt* promoter fragments of 542 bp, 309 bp and 159 bp were generated, respectively. After *Eco*RI-*Bam*HI digestion, they were subcloned into the pGEM-3Z vector as previously described (Sambrook *et al.*, 1989) for construction of pAc*egt*542, pAc*egt*309 and pAc*egt*159. After identification and sequencing of cloned AcMNPV *egt* promoters, a complete luciferase gene separated from pUL220-*luc* by digestion of *Bam*HI was subcloned into pAc*egt*542/*Bam*HI, pAc*egt*309/*Bam*HI and pAc*egt*159/*Bam*HI downstream the *egt* promoter, respectively, for construction of transient expression plasmids pAc*egt*542-*luc*, pAc*egt*309-*luc* and pAc*egt*159-*luc*. For construction of *hr3* enhanced reporter plasmid pAc*egt*542-*luc*-*hr3*, BmNPV *hr3* separated from pSK-*hr3* by *Pst*I di-

gestion was subcloned into pAc*egt*542-*luc* downstream the *luc*.

Similarly, according to the nucleotide sequence of BmNPV T3 (GenBank Accession number: L33180) and *egt* of BmNPV ZJ-8 (Ji *et al.*, 2000), two primers were designed as follows: Bm*egt*542 forward 5′-TCGAATTCCTTGTACCGATGCA-CGCGAA-3′, corresponds to the region between nucleotides – 544 and – 524 relative to the translation initiation site of BmNPV *egt* and contains an *Eco*RI site at 5′-end. Bm*egt*542 reverse 5′-AAGGATCCAATTTTGCTTCAACCCGAATA-ACTG-3′ is 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. And a BmNPV *egt* promoter fragment of 542 bp was generated with the primers using the denatured genomic DNA of BmNPV as template. Finally, the reporter plasmid pBm*egt*542-*luc* with a *luc* driven by BmNPV *egt* promoter was constructed.

Cell culture, transfection and transient expression

The methods for routine Sf21 and Bm5 cell culture and transfection were as previously described (Summers and Smith, 1987; Zhou *et al.*, 2002). Cells were seeded into 12 cm^2 flasks at a density of 5×10^5 cells/ml (3 ml per flask) and cultured over night. Before transfection, the medium was removed and the cells were washed twice with serum-free TC-100 medium. Then cells were transfected with 50 μl transfection solution containing 1 μg reporter plasmid DNA and 7.5 μl lipofectin in 1 ml serum-free medium for 4–5 h followed by infection of wt NPV (MOI = 1.0) for 1 h. Then serum-free medium was replaced by 3 ml TC-100 medium containing 10% FBS and this moment was set as zero time of infection.

In all experiments except for the hormone treatments, 0.5 μg normalization plasmid pSK-*hsp70-LacZ-hr3* DNA was accompanied with reporter plasmid for transfection (Zhou *et al.*, 2003). Cells transfected with pUL220-*luc* served as the blank. Three replicates were done for each experiment. In the hormone treatments, designed dosages of ecdysone were administrated into the medium immediately after medium replacement.

Preparation of cell extracts and assay of luciferase activity

Cells were harvested at 48 hpi except for the time-course expression by centrifugation at $9{,}000 \times g$ for 5 min at 4 °C. Cell extracts were prepared with a kit (E4030, Promega) and the lysates were processed with a freeze-thaw cycle at – 20 °C and room temperature followed by centrifugation at 4 °C to remove cell debris. The supernatants were used for luciferase assay. β-Galactosidase specific activity of the normalization plasmid and protein of the lysates were estimated as previously described (Idahl *et al.*, 1986; Zhou *et al.*, 2002).

Results

Promoter activities of AcMNPV and BmNPV egt transactivated by viral factor(s)

Sf21 cells were transfected for 4–5 h with the mixture of lepofectin and reporter plasmid pAc*egt*542-*luc* combined with the normalization plasmid pSK-*hsp70-LacZ-hr3*. pUL220-*luc* transfected cells served as the blank. 48 h after medium replacement, cells were harvested for assay of luciferase activity. The luciferase activity from cells transfected with reporter plasmid and from the blank were 24.0 ± 14.4 and 18.7 ± 6.1 cpm, respectively, indicating that luciferase gene did not express in uninfected cells. Similar result was also obtained from Bm5 cells transfected with pBm*egt*542-*luc* (data not shown).

In contrast with the uninfected control, cells infected with wt NPV after transfection with pAc*egt*542-*luc* or pBm*egt*542-*luc*, at 48 hpi luciferase activities were 19,772 ± 1,637.7 and 11,706 ± 1,498.5 cpm per μg extract, respectively, after deduction of the blank and being modified by β-galactosidase specific activity of normalization plasmid and protein content of lysates. These results strongly suggested that the transcriptional activities of *egt* promoters both from AcMNPV and BmNPV require the transactivation of baculovirus factor(s).

Time-course expression of luciferase controlled by egt promoters from AcMNPV and BmNPV

Sf21 cells were transfected with pAc*egt*542-*luc* and infected with wt AcMNPV as described above. At 2, 6, 12, 18, 24, 36 and 48 hpi they were harvested and ready for extracts, respectively. Luciferase activity was first detected at 24 hpi

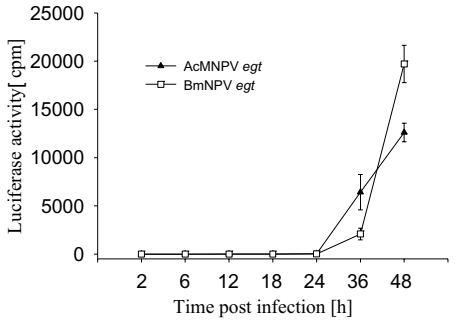

Fig. 1. Time-course expression of luciferase gene driven by *egt* promoter of AcMNPV and BmNPV. The harvesting hours post infection (hpi) are indicated on the X-axis. The luciferase activities in Sf21 cells transfected with pAc*egt*542-*luc* and in Bm5 cells transfected with pBm*egt*542-*luc* are indicated on the Y-axis (counts per minute, cpm). The figure shows the average luciferase activities of three separate treatments after deduction of the pUL220-*luc* transfected blank and being normalized by β-galactosidase activity of the normalization plasmid and protein of extract (mean ± S. D.). Luciferase activity was first detected at 24 hpi. ▲ represents pAc*egt*542-*luc*, □ represents pBm*egt*542-*luc*.

(32.9 ± 5.9 cpm per μg of extract) and followed by a rapidly increase within 48 h and reached 12,601.8 ± 967.7 cpm per μg of extract at 48 hpi.

In another parallel experiment, similar result was obtained in Bm5 cells transfected with pBm*egt*542-*luc* and infected with wt BmNPV. The modified luciferase activity was 19,708.5 ± 1,941.4 cpm (Fig. 1).

The effect of BmNPV hr3 on property of AcMNPV egt promoter

The above experiments showed that transcriptional activity of AcMNPV *egt* promoter was first detected at 24 hpi and required the transactivation of viral product(s). But in the Sf21 cells infected with AcMNPV, EGT activity was observed at 3 hpi (O'Reilly and Miller, 1990). There are some *hr*s in the genomic DNA of AcMNPV (Cocharn and Faulkner, 1983), that not only function as the origins of replication, but also enhance the viral gene transcription (Pearson *et al.*, 1992; Rodems and Friesen, 1993; Zhang *et al.*, 1995; Lu *et al.*, 1997). To analyze the effects of *hr*s on the activity of AcMNPV *egt* promoter, reporter plasmid pAc*egt*542-*luc-hr3* with BmNPV *hr3* downstream the *luc* was constructed for transient expression in Sf21 cells. The pAc*egt*542-*luc* transfected cells severed as the control and pUL220-*luc* transfected cells as the blank. The cells transfected but uninfected served as the control for each plasmid. Cells were harvested at 2, 6, 12, 18, 24, 36 and 48 hpi. In the uninfected treatments, luciferase activity of cells transfected with pAc*egt*542-*luc-hr3*, pAc*egt*542-*luc* and pUL220-*luc* were 16.0 ± 6.9, 17.0 ± 2.3 and 20.0 ± 4.0 cpm, respectively, without significant difference. On the contrary, in the virus infected cells, a very weak luciferase activity (24.0 ± 8.5 cpm after modification) was first detected at 18 hpi in cells transfected with pAc*egt*542-*luc-hr3* and increased within 48 h. By 24 hpi it reached 150.4 ± 10.2 cpm and by 48 hpi increased to $1.35 \times 10^7 \pm 1.31 \times 10^6$ cpm after modification, which is 1,071-fold of the control $1.26 \times 10^4 \pm 9.68 \times 10^2$ cpm. These results revealed that BmNPV *hr3* enhanced the promoter activity significantly but it did not change the property of AcMNPV *egt* promoter.

Functional region of AcMNPV egt promoter

Reporter plasmids with AcMNPV *egt* promoter segments of different length, pAc*egt*542-*luc*, pAc*egt*309-*luc* and pAc*egt*159-*luc*, were used to transfect Sf21 cells. For each plasmid, uninfected cells served as the control, while pUL220-*luc* transfected cells served as the blank. In cells infected with AcMNPV, luciferase activities were detected for all three plasmids at 48 hpi. They were 19,064.4 ± 1,465.3, 16,305.9 ± 1,400.1 and 38.1 ± 4.7 cpm after modification, respectively. That of pAc*egt*542-*luc* was the highest, that of pAc*egt*309-*luc* was approaching to the former. And that of pAc*egt*159-*luc* was very weak (about 0.2% of former two) (Fig. 2). On the contrary, in uninfected cells no luciferase activity from any plasmid was detected. The results revealed that promoter fragment of 159 bp contains the basal elements of the promoter but its transcriptional activity was almost abolished. The nucleotide sequence from – 159 to – 309 bp upstream the translation initiation site likely includes the main *cis*-acting elements interacting with viral factors.

Effects of ecdysone, substrate of EGT, on AcMNPV egt promoter activity

Ecdysone promotes the development and metamorphosis of insects and it is one of the substrates of EGT. To investigate the effect of ecdysone on AcMNPV *egt* promoter activity, 20-β-hydroxyl-ecdysterone was administrated in medium to the fi-

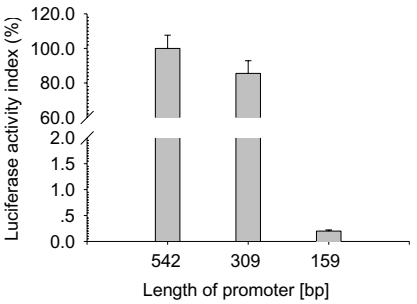

Fig. 2. Transcriptional activities of AcMNPV *egt* promoter of different lengths. The lengths of promoter regions are indicated on the X-axis. Promoter activities are indicated on the Y-axis. The data show the average luciferase activities of three separate treatments, relative to pAc*egt*542-*luc* serving as 100%, after deduction of the pUL220-*luc* transfected blank and being normalized by β-gal activity of the normalization plasmid and protein of extract (mean ± S. D.). The luciferase activities of 309 bp and 159 bp promoters are 85.5 ± 7.3% and 0.20 ± 0.02%, respectively.

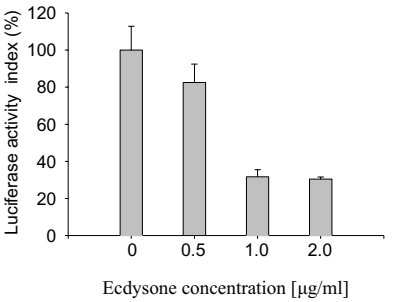

Fig. 3. Effects of foreign insect ecdysone on the activity of AcMNPV *egt* promoter. The ecdysone concentrations (μg/ml) are showed on the X-axis. The luciferase activity indexes of pAc*egt*542-*luc* in Sf21 cells are indicated on the Y-axis (%). The data show the average luciferase activities of three separate treatments, relative to non-hormone treatment serving as 100%, after deduction of the pUL220-*luc* transfected blank and being normalized by β-gal activity of the normalization plasmid and protein of extract (mean ± S. D.). The luciferase activities of cells treated with ecdysone of 0.5, 1.0 and 2.0 μg/ml are 82.58 ± 9.85%, 36.73 ± 9.02% and 35.27 ± 8.32%, respectively.

nal concentration of 0.5, 1.0 and 2.0 μg/ml after transfection of pAc*egt*542-*luc* and infection of wt AcMNPV. Cells without hormone treatment served as the control and cells transfected with pUL220-*luc* as the blank. The results showed that at 48 hpi luciferase activity indexes of cells treated with ecdysone at 0.5, 1.0 and 2.0 μg/ml were 82.58 ± 9.85%, 36.73 ± 9.02% and 35.27 ± 8.32% relative to the control, respectively (Fig. 3). It suggested that ecdysone of lower dose (0.5 μg/ml) has no significant effect on AcMNPV *egt* promoter activity. But ecdysone of higher dose (1.0 and 2.0 μg/ml) significantly decreased the activity of the promoter. This may be because high concentration of ecdysone caused a negative feedback to inhibit the promoter activity.

Acknowledgements

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