Transcriptional analysis and promoter activity of the *Spodoptera littoralis* multicapsid nucleopolyhedrovirus ecdysteroid UDP-glucosyltransferase gene

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The ecdysteroid UDP-glucosyltransferase gene (*egt*) of *Spodoptera littoralis* multicapsid nucleopolyhedrovirus (SpliMNPV) is a homologue of the *Autographa californica* MNPV (AcMNPV) *egt* gene, which has been found to block insect moulting. Infection of larvae with an *egt*-deleted AcMNPV resulted in enhanced mortality as compared to infection with the wild-type virus. Consequently, deletion of an *egt* gene has been proposed as a tempting approach for enhancing the insecticidal properties of baculoviruses. In a previous report we described the mapping and sequencing of the SpliMNPV *egt* gene. Here we use time-course Northern blot and biochemical analyses to show the production of *egt* transcripts and protein. The SpliMNPV *egt* transcription start sites were mapped to 22 and 25 nucleotides downstream of the TATA box by primer extension. Transient expression assays of chimeric *egt* promoter–chloramphenicol acetyltransferase (*cat*) reporter gene constructs revealed low promoter activity that was trans-activated by AcMNPV immediate-early viral protein IE-1.

*Spodoptera* species are important lepidopteran pests which cause severe damage to major crops in many parts of the world. Overuse of chemical insecticides for the control of these pests has led to insect resistance, as well as being an increasing threat to the environment. In searching for alternative methods for pest control the use of naturally occurring insect viruses of the *Baculoviridae* has been suggested (Miller et al., 1983). Members of this family, including a *Spodoptera littoralis* multicapsid nucleopolyhedrovirus (SpliMNPV), have the potential to serve as pest-control agents. However, their efficiency is low relative to chemical insecticides. In an attempt to improve the insecticidal properties of baculoviruses, the cloning of foreign genes under the control of promoters of nonessential viral genes was suggested, as reviewed by Wood & Granados (1991). In most of the engineered baculoviruses, expression is based on the strong, very late promoters of the p10 or polyhedrin genes. The use of early transcribed viral genes as sites for cloning has the potential to further improve the virus insecticidal properties, due to the expression of an insecticidal gene(s) at earlier stages of infection.

The ecdysteroid UDP-glucosyltransferase gene (*egt*) of *Autographa californica* MNPV (AcMNPV) (O’Reilly & Miller, 1989) is a nonessential early-expressed gene (O’Reilly & Miller, 1990). Removal of the AcMNPV *egt* gene renders the virus more virulent (O’Reilly & Miller, 1991). Consequently, it has been suggested as a candidate for genetic manipulation of the virus. We previously reported the identification and nucleotide sequence of a SpliMNPV *egt* gene (Faktor et al., 1995). In this report we show the time-course expression of this *egt* gene, and properties of its promoter function in DNA-transfected cells.

The enzyme EGT catalyses the conjugation of sugars to ecdysteroid hormones such as ecdysone. To determine whether SpliMNPV encodes a functional EGT enzyme, a UDP-glucosyltransferase activity assay was performed (O’Reilly & Miller, 1990). Medium samples of virus-infected and mock infected Si-2 cells (Chejanovsky & Gershburg, 1995) were subjected to the assay. The assay was performed on a TLC plate with Phosphorimager. Samples were separated into three categories: free ecdysone, ecdysone-sugar conjugates and a sample from AcMNPV-infected SF9 cells (8 h p.i.). The data showed that SpliMNPV-infected cell medium contains both free ecdysone and ecdysone-sugar conjugates, indicating that the enzyme EGT is active in the virus-infected cells.

**Fig. 1.** EGT activity in SpliMNPV-infected cell medium. A Phosphorimager picture showing the separation of EGT reaction products on a TLC plate. E, free [3H]ecdysone; CE, [3H]ecdysone–sugar conjugates; P, a sample from AcMNPV-infected SF9 cells (8 h p.i.); M, mock-infected Si-2 cells; 3, 11, 22 and 72 denote time (h) p.i. of tested samples.
were collected at various times post-infection (p.i.) and 20 µl of each sample was incubated with a mixture of UDP-glucose, UDP-galactose and [3H]ecdysone for 3 h. The substrate for the reaction was not defined; therefore, two UDP-sugars were added to ensure that sufficient substrate was present to allow the reaction to continue. The reaction products were separated by thin-layer chromatography and visualized by a PhosphorImager (FUJIX Bas 1000). The final figure was further processed by Adobe Photoshop software (Fig. 1). UDP-glucosyltransferase activity, as determined by the presence of conjugated ecdysone, was first detected 3 h p.i., and increased with time (CE, Fig. 1). Mock infected cells showed no enzymatic activity (Fig. 1, lane M). These results strongly suggest that the EGT activity detected in medium of virus-infected cells resulted from a virus-encoded enzyme. The assay showed the production of a functional EGT enzyme, which is produced at the early stages of virus infection and is secreted by the infected cells, as has been shown for AcMNPV EGT (O’Reilly & Miller, 1990).

To further study the temporal expression of the SpliMNPV egt gene, Sl-2 insect cells were infected with SpliMNPV. Total RNA was extracted at various times p.i. by the guanidinium–phenol extraction method using Tri-reagent (Molecular Research CE). For Northern blot hybridization, 20 µg RNA samples were separated on agarose gels containing 6% formaldehyde and transferred to Hybond-N membranes (Amersham). Hybridization was performed in a 50% formamide solution at 42 °C, utilizing a [32P]-labelled egt-specific single-strand DNA probe (Sambrook et al., 1989). The template for the probe labelling reaction was a plasmid containing a BamHI–XhoI DNA fragment from nucleotide position 898 to 1578 of the egt gene (Faktor et al., 1995). Following washing under stringent conditions, the blot was exposed to a PhosphorImager screen and the results were visualized and processed as already described. The egt transcripts were observed 8 h p.i. (Fig. 2a). Our previous results on the detection of the SpliMNPV polyhedrin gene transcripts later than 48 h p.i. (Faktor et al., 1996), in tissue cultured cells, indicated relatively slow kinetics of infection and led us to regard the SpliMNPV egt as an early expressed gene. This characteristic has been demonstrated for egt genes of other baculoviruses (O’Reilly & Miller, 1990; Riegel et al., 1994; Barrett et al., 1995). The size of the RNA transcript was estimated to be 1.8 kb, corresponding to the size predicted by sequence analysis (Faktor et al., 1995). The inability to detect egt mRNA before 8 h p.i., while EGT activity was detected earlier, could result from the different sensitivities of the two detection methods.

A primer extension assay was used to determine the egt mRNA start site(s). Owing to the apparently low level of egt expression (Fig. 2a, lanes 3 and 8), polyadenylated RNA was isolated on an oligo(dT)12–18 cellulose column (Invitrogen),
Transcriptional analysis of SpliMNPV egt gene

(a)

<table>
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<tr>
<th>Virus</th>
<th>Promoter Sequence</th>
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<tr>
<td>SpliMNPV</td>
<td>TGGCACACGTGACACATTTTTTATAAAATGGGAGCAGTATCCCCGTCGCCCAAGCATCGTGGTGATCAGTCAATCAAGT</td>
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<td>AcMNPV</td>
<td>CGATGGTGGAAATTTTAGGGCTATATAAAAGGTACCCCTAAAAATTTTGTACATAAACTAAATTCATGACGTTTTGAGAAAAAC</td>
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<tr>
<td>CfMNPV</td>
<td>GGGCCCATTGAGCAAAATATACCCAGAGCTGCGAACAGACTACGATCGGCAAGCTCAGG</td>
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<td>CfDEFMNPV</td>
<td>ACCGGCAGTCTAGGGCGCTATATAAAACGGGTACGGCTACTAAGCAACAGTTTTTTAAGGACAGGCTTAAGCTCAAGAAGCTG</td>
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<tr>
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<td>MbMNPV</td>
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<td>OpMNPV</td>
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(b)

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<th>CAT Activity</th>
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<tbody>
<tr>
<td>IE-1</td>
<td>-</td>
<td>+</td>
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Fig. 3. Sequence comparison and activity of egt promoters. (a) Comparison of egt promoter sequences from SpliMNPV (Faktor et al., 1995), AcMNPV (O’Reilly & Miller, 1990), CfMNPV and the defective virus CfDEFMNPV (Barrett et al., 1995), LdMNPV (Riegel et al., 1994), MbMNPV (EMBL/GenBank accession number U41999) and Orgyia pseudotsugata MNPV [OpMNPV (EMBL/GenBank accession number D17353)]. Translation-initiation sites (ATG) are shown in dark boxes, TATA boxes are underlined and CAGT or imperfect motifs are shown in boxes. Transcription-initiation sites are indicated with arrows. (b) CAT activity assay of Sf9 cells transfected with the AcMNPV early 39K promoter–CAT (p39CAT), AcMNPV egt promoter–CAT (pAcgtCAT), SpliMNPV egt promoter–CAT (pSlegtCAT). Each of the constructs was assayed by transfection alone (-) or by cotransfection with AcMNPV IE-1 gene (pIE-1, +).

according to the manufacturer’s instructions, from total RNA prepared at 8 h and 24 h p.i. For reverse transcriptase reactions (AMV-RT Promega), a γ-32P-end-labelled 20 base oligonucleotide (5’ CCGAGTTTCTCAGACGTGC 3’), complementary to the region between nucleotides 31 and 50 downstream of the initiation codon, was annealed to 3 μg of polyadenylated RNA. The elongated DNA products were separated on a 6% denaturing polyacrylamide gel and visualized by a PhosphorImager. The extension reaction with cell-culture-derived mRNA did not reveal an extended product (not shown). Assuming that a higher level of egt expression could be obtained in infected larvae, the reaction was repeated with polyadenylated RNA prepared from infected larvae. Stage-3 larvae were infected with 10^6 polyhedral inclusion bodies. At 96 and 120 h p.i., larvae were pulverized under liquid N₂ and RNA was extracted from the powder by the method described earlier for cultured cells. Selection of larval polyadenylated RNA was performed with some modifications due to the formation of insoluble precipitants in the RNA preparation. Total RNA was mixed with oligo(dT) cellulose in an Eppendorf tube. Washes and elutions were performed by alternately spinning down and re-suspending the RNA–
oligo(dT) mixture and buffers. Following reverse transcription of 10 μg polyadenylated mRNA and gel electrophoresis, two initiation sites were detected (Fig. 2b). Utilizing the egt sequence ladder, transcription start sites were localized to nucleotides A (position −25) and C (position −28) relative to the translation initiation codon. These are situated 25 and 22 bases, respectively, downstream of the putative TATA box. This finding confirmed our previous suggestion that an initiation codon present immediately downstream of the TATA box is not part of the SpliMNPV egt ORF (Faktor et al., 1995). These sites are similar to the localized transcription start site of the AcMNPV egt (O’Reilly & Miller, 1990), but differ from the site found in the Lymnantria dispar (Ld) MNPV egt gene transcript (Riegel et al., 1994). Comparison of the sequences of available egt promoters revealed that all promoters contained a TATA box (Fig. 3a). We could not find common sequences or common potential cis-regulatory elements in sequences upstream of the TATA box, although in several egt promoters potential cis-elements could be found, such as a CAGTCG motif (G-box) in SpliMNPV (Faktor et al., 1995), an ATTGTGTAA in AcMNPV (O’Reilly & Miller, 1990) and an AAGTGATT in Choristoneura fumiferana (Cf) MNPV (Barrett et al., 1995). Downstream of the TATA box several CAGT or CAGT-imperfect motifs (Blissard et al., 1992) were detected. Because none of the specific egt transcription-initiation sites were mapped to any of these motifs, their role in transcription-initiation from egt promoters is unclear. The distance between the putative TATA boxes and the site of translation initiation ranged between 67 and 44 bp in the different promoters.

To further study the transcriptional regulation of egt promoters, chimeric promoter–cat reporter gene constructs were generated. In pSlegCAT, a BamHI–BglII DNA fragment containing the SpliMNPV egt promoter sequences from nucleotides −11 to −1500 relative to the translation start site was cloned upstream of the cat gene in pSVOCAT (Gorman et al., 1982). This promoter fragment was generated by PCR using a 25 base oligonucleotide (5′ CCTAGATCTATGACGAGCAGATGC 3′), complementary to the region between nucleotides −11 and −26 relative to the initiation codon and containing a BglII restriction site. The second oligonucleotide was the T7 promoter primer (Stratagene). In pAegtCAT, a PstI–BglII DNA fragment of the AcMNPV egt promoter from nucleotides −10 to −1420 was cloned upstream of the cat gene in pSVOCAT. This DNA fragment was generated by PCR using a 26 base oligonucleotide (5′ CAGATCTAAACCGAATAACTGTACTGG 3′) complementary to the region between nucleotides −10 and −28 relative to the initiation codon, and containing a BglII restriction site. The second oligonucleotide was the pUC universal sequencing primer (New England Biolabs). p39CAT (Guarino & Summers, 1986), containing the 39K early promoter of AcMNPV, was used as a positive control. SF9 cells were transfected with the above constructs by the calcium phosphate coprecipitation method (Graham & van der Eb, 1973) and cell extracts were assayed 48 h later for CAT activity, as described previously (Gorman et al., 1982). In the transient transfection assay both egt promoters were not active (Fig. 3b), or gave very low activity (data not shown). These results indicated that egt promoters are weak and that a further increase of promoter activity may require a viral product.

Expression of several early genes in baculoviruses, including the AcMNPV 39K promoter, has been shown to require the immediate-early viral protein IE-1 (Guarino & Summers, 1986; Nissen & Friesen, 1989; Blissard & Rohrmann, 1991). To establish the possible role of IE-1 in egt promoter activity, each of the above described constructs was cotransfected with a plasmid containing the AcMNPV IE-1 gene (pIE-1; Guarino & Summers, 1986) into SF9 cells. IE-1 was found to activate transcription from the egt promoters of both AcMNPV and SpliMNPV (Fig. 3b).

In summary, we established that SpliMNPV egt is a functional, early-transcribed gene. The enzyme is secreted into the medium of virus-infected cells. The low activity of the egt promoter in transient transfection assays could be further transactivated by the heterologous AcMNPV IE-1 protein. Alignment of several egt promoter sequences showed high sequence divergence, except for the location of the TATA box and transcription start site(s). As AcMNPV IE-1 is thought to mediate promoter activation via the TATA box and downstream sequences, this finding strengthens the possible role of an SpliMNPV IE-1-like gene in the activation of the SpliMNPV egt promoter.

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References


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