Analysis of promoter activity of selected *Cotesia plutellae* bracovirus genes

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In a previous study, we cloned 27 discrete genome segments of *Cotesia plutellae* bracovirus (CpBV) and provided the complete nucleotide sequences and annotation. Seven putative coding regions were predicted from one of the largest segments, CpBV-S30. The activity of promoters associated with six predicted ORFs from this segment were investigated using both transient and baculovirus expression assays with enhanced green fluorescent protein as a reporter gene. CpBV promoters showed activity earlier than the *polyhedrin* promoter and the activity of some of these promoters was superior to that of the *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) ie-1 promoter in the baculovirus expression assays. The promoter of ORF3004 showed the highest level of activity in insect cells, exhibiting 24 % of the activity obtained with the *polyhedrin* promoter in Sf9 cells. In *Spodoptera exigua* larvae, the ORF3006 promoter showed the highest activity, with about 35 % of the activity measured with the *polyhedrin* promoter. In addition, analysis of the ORF3006 promoter revealed that the region between −382 and −422 from the translation start point was critical for activity of this promoter. These results suggest that the CpBV-S30 promoters characterized here could be useful tools in a variety of biotechnological applications, such as gene expression analyses and insecticide development.

INTRODUCTION

Polydnaviruses (PDVs) are essential for the survival of some endoparasitoids in their lepidopteran host haemolymph and are implicated in attenuating host immune responses and altering host development (Beckage & Gelman, 2004; Lavine & Beckage, 1995; Lawrence & Lanzrein, 1993; Stettler et al., 1998; Stoltz & Vinson, 1979; Vinson, 1990; Webb, 1998). Because PDV genes play important regulatory roles in compromising host immunity and causing host developmental arrest, it has been suggested that these genes offer opportunities for enhancing the virulence of insect pathogens (Beckage & Gelman, 2004).

Evidence has been presented suggesting that some PDV genome segments could be used to transform insect cells and, possibly, larvae. Some PDV DNAs integrate into the genomic DNA of lepidopteran cells in culture (Doucet et al., 2007; Gundersen-Rindal & Dougherty, 2000;
Gundersen-Rindal et al., 1999; Gundersen-Rindal & Lynn, 2003; Kim et al., 1996; McKelvey et al., 1996; Volkoff et al., 1999, 2001). Preliminary evidence has also been provided for in vivo integration of Cotesia congregata bracovirus (CcBV) DNA into Manduca sexta host DNA (Le et al., 2003). Hence, it has been suggested that PDVs could be used to transform pest insects, in addition to facilitating transformation of the parasitoid wasp itself.

In most known parasitoid systems, a number of PDV genes are transcribed in host cells and most of these transcripts persist throughout most of the embryonic and larval parasitoid development within the host caterpillar (Strand et al., 1992; Summers & Dib-Hajj, 1995; Theilmann & Summers, 1986). For example, expression of the early gene family (EP1, EP2 and EP3) from CcBV began 30 min post-parasitization (p.p.), with gene products comprising 10% of total haemolymph proteins at 24 h p.p. (Harwood et al., 1994). Among them, EP1 transcripts could be detected in larval development (Harwood & Beckage, 1994; after 6 days, which is more than half the time needed of total haemolymph proteins at 24 h p.p. (Harwood et al., 1994). Among them, EP1 transcripts could be detected in vitro after 6 days, which is more than half the time needed for larval development (Harwood & Beckage, 1994; Harwood et al., 1994). In contrast, the expression of the Cotesia rubecula bracovirus (CrBV) gene, CrV1, is unique in that its expression is highly transient, occurring 4–8 h p.p., after which transcript abundance dramatically declines (Asgari et al., 1996, 1997). Promoter studies of the CrV1 gene suggested that upregulation is achieved by the promoter and upstream regulatory elements, whereas downregulation has been proposed to be due to post-transcriptional effects in CrV1-producing cells (Asgari & Schmidt, 2001). As an intermediate example, Microplitis demolitor bracovirus (MdBV)-encoded epidermal growth factor (egf) genes produced high levels of transcripts in haemocytes at 12 h p.p., but levels were lower at 24 h p.p. and almost undetectable at 96 h p.p., in contrast with the constitutive expression pattern of most other MdBV genes (Strand & Pech, 1995). Although there is variation in temporal expression patterns, most PDV genes are expressed at the early phase of viral infection, which may be attributed to promoter activity, as demonstrated for CrV1 protein expression.

The solitary braconid endoparasitoid Cotesia plutellae is an important natural enemy of the diamondback moth, Plutella xylostella, the most problematic pest of brassica crops worldwide (Schuler et al., 2004). It also parasitizes the fall webworm, Hyphantria cunea, although parasitoid development takes longer in this host (Kim et al., 2004). C. plutellae wasps produce a PDV (C. plutellae bracovirus, CpBV) whose virions accumulated in the eviscerated lumen of females. The CpBV virion contains multiple nucleocapsids that are approximately 30 nm in diameter, of variable length (30–80 nm) and are surrounded by a single unit membrane envelope (Bae & Kim, 2004). Viral replication begins during pupal development (Kim et al., 2004). Parasitization of C. plutellae attenuates the immune capacity of P. xylostella and extends the host larval period (Bae & Kim, 2004; Lee & Kim, 2004). A proteomic analysis of parasitized P. xylostella revealed several parasitism-specific gene expression patterns, suggesting a functional regulation of CpBV genes (Lee et al., 2005).

To address CpBV gene function during parasitism, we have recently cloned 27 distinct CpBV genome segments, ranging in size from 0.1 to 25.5 kb, using a plasmid capture system (Choi et al., 2005). In the present study, we assessed the promoter activity of CpBV genes predicted from one of the largest genome segments, CpBV-S30, as a means of exploring their practical application.

METHODS

**Bacterial strains.** *Escherichia coli* strain JM109 (TaKaRa) was used in all experiments. All restriction endonucleases and modifying enzymes were from Roche.

**Insect cells and baculoviruses.** The *Spodoptera frugiperda* cell line SF9 and the *Bombyx mori* cell line Bm5 were maintained in TC-100 medium (JBI) supplemented with 10% heat-inactivated (56°C, 30 min) fetal bovine serum (JRH Biosciences). In addition, SF9 cells were maintained in SF-900 II SFM (Invitrogen). High-Five cells from *Trichoplusia ni* were maintained in Express Five SFM (Invitrogen) with l-glutamine. The LdFB cell line, developed from fat body cells of *Lymantria dispar*, was kindly provided by Dr Dwight E. Lynn (Agricultural Research Science, USDA, Beltsville, MD, USA) and was maintained in EX-CELL 420 serum-free medium (JRH Biosciences). All insect cell lines were incubated at 27°C and subcultured every 3–4 days. Wild-type and all recombinant *Autographa californica* nucleopolyhedrovirus (AcMNPV) used in this study were propagated in SF9 cells maintained in TC-100 medium.

**Plasmids and transient expression.** To construct baculovirus transfer vectors expressing enhanced green fluorescent protein (EGFP) under the control of CpBV promoters, the *polyhedrin* gene promoter region of pDualBac was deleted by digestion with SacI and *BamHI*. The resulting fragment was blunt-ended with Klenow and self-ligated to obtain pDualBac-DelPro. The EGFP gene was amplified from the pEGFP vector (Clontech) using specific primers EGFP-KpnF and EGFP-EcoRR (all primer sequences are given in Supplementary Table S1, available in JGV Online). The PCR-amplified EGFP gene fragment was digested with KpnI and EcoRI and inserted into KpnI–EcoRI-digested pDualBac-DelPro to obtain pDualBac-8-DelProEGFP. In order to use the luciferase gene as an internal control for activity of promoters from CpBV, the luciferase gene was amplified from the pGLO-Control vector (Promega) using specific primers Luci-HindIII and Luci-SacII. The PCR-amplified luciferase gene fragment was digested with HindIII and SacII and introduced downstream of the ie-2 promoter from *Orgyia pseudotsugata* nucleopolyhedrovirus (OpMNPV) of the HindIII–SacII-digested pIB/V5-His vector (Invitrogen) to obtain pIB/V5-His-Luc. About 2.4 kb of the OpMNPV ie-2 promoter-luciferase-OpMNPV polyA cassette fragment amplified from the pIB/V5-His-Luc vector using primers OpIE2Pro-XhoF and OpIE2P-A-PstR was digested with XhoI and PstI and inserted into pDualBac-8-DelProEGFP digested with the same restriction endonucleases to obtain pDualBac-8-Luc-DelProEGFP. About 0.7 kb of the CpBV promoter regions of putative ORFs was amplified from the CpBV-S30 segment using specific sense and anti-sense primers and digested with XbaI and KpnI, respectively. In addition, the ie-1 and *polyhedrin* gene promoters from AcMNPV were amplified from the AcMNPV genome using the same strategy as that used for CpBV promoters. These PCR-amplified and restriction endonuclease-digested CpBV promoter regions were introduced upstream of the EGFP gene in pDualBac-8-DelProEGFP digested with XbaI and KpnI.
The 5'-region of the ORF3006 promoter was deleted by PCR. Sense primers and the antisense primer, GFP-CR, were used for PCR amplification. The PCR fragments were digested with PstI and KpnI and then inserted into pBac8-DelEVBa digested with the same restriction endonucleases.

For the transient expression assays, insect cells were seeded into 60 mm diameter tissue culture dishes at 3 \times 10^5 cells per dish and incubated at 27 °C for 30 min to allow cells to attach. Attached cells were washed with 3 ml incomplete TC-100 medium and transfected with 2 μg of the corresponding transfer vector. Transfection was performed using Cellfectin reagent (Invitrogen) according to the manufacturer's instructions. At 3 days post-transfection (p.t.), transfected cells were harvested by centrifugation at 500 g for 5 min, washed twice with PBS and stored at 4 °C.

Construction of recombinant baculoviruses. Recombinant AcMNPV's expressing luciferase and EGFP under the control of OpMNPV ie-2 and CpBV promoters, respectively, were constructed by co-transfection of corresponding transfer vectors with bApGOZA DNA (Ic et al., 2001) into Sf9 cells. Transfection was performed using Cellfectin reagent (Invitrogen) according to the manufacturer's instructions and the recombinant viruses were purified by plaque assay on Sf9 cells, as described previously (O’Reilly et al., 1992).

Infection of cells with baculoviruses. Insect cells were seeded into 60 mm diameter tissue culture dishes at 1 \times 10^5 cells per dish and incubated at 27 °C for 30 min to allow cells to attach. Attached cells were washed twice with 3 ml incomplete TC-100 medium and inoculated with 1 ml appropriately diluted viral stock. Sf9 and High-Five cells were infected with an m.o.i. of 10 p.f.u. per cell, and Bm5 and LdFB cells with an m.o.i. of 20 p.f.u. per cell. After incubation at 27 °C for 1 h with gentle rocking, the medium was replaced with 3 ml fresh medium and incubated at 27 °C. At specific time points, infected cells were harvested by centrifugation at 500 g for 5 min, washed twice with PBS and stored at 4 °C.

Inoculation of insect larvae was carried out using a previously reported method (Choudary et al., 1995). About 50 μl viral suspension (1 \times 10^7 p.f.u. ml^-1), containing 6 mg kanamycin ml^-1, was injected just underneath the dorsal cuticle of Spodoptera exigua 5th instar larvae using a 1 ml syringe fitted with a 30 gauge needle. After incubation at 25 °C for 3 days, haemolymph from infected larvae was collected by piercing the larval proleg using a 25 gauge needle plugged with Parafilm. To prevent melanization, haemolymph was collected at 4 °C and 1/10 volume of 100 mM DTT was added. The collected haemolymph was centrifuged at 8000 g for 1 min at 4 °C and the supernatant was stored at 4 °C.

Luciferase activity assay. Insect cells transfected with transfer vectors or infected with recombinant viruses were harvested and washed, as described above. The intracellular luciferase assay was performed using the Luciferase Assay System (Promega) according to the manufacturer’s protocols. Relative light units (RLUs) were measured with a chemiluminometer Autolumat LB953 (EG&G Berthold).

Quantification of EGFP. Insect cells transfected or infected with transfer vectors or recombinant viruses were harvested and washed, as described above. Cells were sonicated in PBS containing 1/100 volume of protease inhibitor cocktail (Sigma) and centrifuged at 22000 g for 15 min. Fluorescence of the resulting supernatant was measured using a SpectraMax Gemini XS microplate spectrofluorometer (Molecular Devices) with an excitation filter of 450 nm and an emission filter of 510 nm. EGFP levels were calculated from measured relative fluorescence units and compared to a standard curve (r^2=0.999), which was plotted as a log function of twofold serial dilutions of EGFP (BD Biosciences) and normalized to RLUs from the same cell.

SigmaStat Ver. 2.0 software (SPSS) was used for detecting statistical difference by Duncan's multiple range test.

RESULTS

Promoter activity of CpBV in transient expression

In order to investigate the promoter activity of putative ORFs on the CpBV-S30 segment, promoter regions of six ORFs were introduced into the pDualBac baculovirus transfer vector in place of the polyhedrin promoter along with the EGFP reporter gene. In addition, the luciferase reporter gene under the control of the ie-2 promoter from OpMNPV was introduced into the transfer vectors as an internal control. The nature of the constructed transfer vectors was confirmed by restriction endonuclease digestion and nucleotide sequence determination (data not shown).

To examine the activity of the CpBV promoters in transient expression assays, 3 \times 10^5 insect cells, including Sf9, High-Five, LdFB and Bm5 cells, were transfected with 2 μg of each transfer vector, and the intracellular quantity of EGFP was determined at 3 days p.t. (Fig. 1). In Sf9 cells, the promoter of ORF3004 and 3007 showed the highest activity among CpBV promoters, but the activity was 20% of that of the ie-1 promoter of AcMNPV. Interestingly, the ORF3007 promoter showed higher activity than that of the ie-1 promoter in High-Five cells. In addition, promoters of ORF3002, 3003 and 3004 showed relatively high activity in High-Five cells. In LdFB cells, the promoter of ORF3004 showed the highest activity among CpBV promoters and the level of activity was comparable to that of the ie-1 promoter. Promoters of ORF3002, 3006 and 3007 also showed relatively high levels of activity in LdFB cells. Although the promoters of ORF3004 and 3007 showed the highest activity among CpBV promoters, promoters from CpBV showed relatively low levels of activity in Bm5 cells.

Promoter activity of CpBV using recombinant baculoviruses

To analyse CpBV promoter activity in a self-replicating system, recombinant AcMNPVs, containing a luciferase reporter gene under the control of ie-2 promoter from OpMNPV and an EGFP reporter gene under the control of promoters from CpBV or ie-1 or polyhedrin gene promoters from AcMNPV, were generated using the bApGOZA system. The genomic structure of the polyhedrin gene loci of all recombinant AcMNPVs was confirmed by PCR analysis using specific primers (data not shown).

Expression of EGFP in Sf9 and High-Five cells, which are permissive to AcMNPV, at 3 days post-infection (p.i.) was investigated by infecting 1 \times 10^6 cells with recombinant virus at an m.o.i. of 10 p.f.u. per cell (Fig. 2a). In both cell types, the promoter of ORF3004 showed the highest activity, with a seven- and fivefold higher activity level than the ie-1 gene promoter in Sf9 and High-Five cells, respectively.
respectively. The ORF3006 promoter also showed a relatively high level of activity. In addition, promoters of other ORFs showed activity that is comparable or slightly superior to that of the ie-1 promoter.

In order to study CpBV promoter activity by recombinant viruses in insect cells that are non-permissive to AcMNPV, \(1 \times 10^6\) LdFB or Bm5 cells were infected with recombinant virus at an m.o.i. of 20 p.f.u. per cell (Fig. 2b) and the intracellular quantity of EGFP was determined at 3 days p.i. As in AcMNPV-susceptible cells, the promoter of ORF3004 showed the highest level of activity in LdFB and Bm5 cells. Although other CpBV promoters showed some activity, overall quantities of expressed EGFP were much lower than those observed in Sf9 and High-Five cells.

**Time-course of CpBV promoter activity**

To study the activity of the CpBV promoter at different time points, \(1 \times 10^6\) Sf9 cells were infected with recombinant virus at an m.o.i. of 1 p.f.u. per cell. When the promoter activities were observed at 2 h intervals for 1 day, the EGFP gene was expressed as early as 2 h p.i. when driven by ORF3004, 3006, 3007 and AcMNPV ie-1 promoters (Fig. 3a). Among CpBV promoters, the ORF3002 promoter was the last to show activity, starting at 12 h p.i. Other CpBV promoters, including ORF3003 and 3005, showed activity after 6 h p.i.

The polyhedrin promoter showed the highest level of EGFP expression and reached a maximum level 4 days p.i. (Fig. 3b). While the ORF3003 promoter reached a maximum activity at 2 days p.i., other CpBV promoters
and the AcMNPV ie-1 promoter showed maximum activity at 3 days p.i. Among CpBV promoters, the ORF3004 promoter showed the highest level of activity, at approximately 24 % of that of the polyhedrin promoter. In general, the CpBV promoters showed earlier responses than the polyhedrin gene promoter of AcMNPV, which began showing activity after 14 h p.i. and reached a maximum at 4 days p.i.

**Promoter activity of CpBV in insect larvae**

In order to evaluate CpBV promoter activity in an in vivo system, 5th instar larvae of *S. exigua* were infected with recombinant virus by haemocoelic injection and the amount of EGFP in the haemolymph of infected larvae was assessed at 3 days p.i.

Expression of EGFP was observed from all promoters tested and the polyhedrin promoter showed the highest level of activity in AcMNPV-susceptible *S. exigua* larvae (Fig. 4). All CpBV promoters, except for the ORF3002 promoter, showed more activity than the AcMNPV ie-1 promoter. Among CpBV promoters, the ORF3006 promoter showed the highest activity, at about 30 % of that of the polyhedrin promoter.

**Deletion analysis of the ORF3006 promoter**

In order to investigate the core region necessary for promoter activity of ORF3006, various fragments of the 5′-flanking region up to bp 699 of the ORF3006 promoter upstream from the ATG translation initiation codon were cloned into baculovirus transfer vector pBacPAK8 in place of the polyhedrin promoter along with the EGFP reporter gene (Fig. 5a). The nature of the constructed transfer vectors was confirmed by restriction endonuclease digestion and nucleotide sequence determination (data not shown).

To examine the activities of CpBV promoters in transient expression assays, 3 × 10^5 LdFB cells were transfected with 2 μg of each transfer vector and the intracellular quantity of EGFP was determined at 3 days p.t. As a result, whereas the activity of pBac8-3006Pro582EGFP and pBac8-3006Pro482EGFP was similar to that of pBac8-3006ProEGFP, the activity of pBac8-3006Pro382EGFP, pBac8-3006Pro282EGFP, pBac8-3006Pro182EGFP, pBac8-3006Pro143EGFP and pBac8-3006Pro93EGFP was dramatically reduced (Fig. 5b). In addition, a significant decline in activity was seen with truncations downstream of position −402 (Fig. 5b).

**DISCUSSION**

In lepidopteran hosts, PDV genes are expressed as early as 2 h p.p. in the absence of viral replication (Fleming, 1992; Stoltz, 1993; Summers & Dib-Hajj, 1995; Yamanaka et al., 1996). The expression of these viral genes appears to be concomitant with physiological changes, such as immunosuppression and developmental disturbance observed in parasitized hosts (Beckage, 1993; Hayakawa, 1990, 1994, 1995; Hayakawa et al., 1994; Webb, 1998). Several PDV
proteins, such as the early proteins (EPs 1, 2 and 3) of CcBV, are abundantly expressed in parasitized Manduca sexta larvae, and together represent as much as 10–15% of total haemolymph protein at 24 h p.p. (Beckage, 1993; Beckage et al., 1987; Harwood & Beckage, 1994). This early and high expression profile of PDV genes reflects the activity of their promoters. Here, we assessed the activity of promoters predicted for the CpBV-S30 genome segment using the EGFP reporter gene and a baculovirus expression system. This segment encodes several types of putative genes, suggesting that its promoters are active during parasitism and that their activities may be compared using different expression systems.

In the transient expression assay, ORF3004 and 3007 promoters showed higher activities than the other CpBV promoters (Fig. 1). Also, the promoter activity of ORF3004 was superior to the other CpBV promoters in baculovirus expression assays (Fig. 2). Interestingly, compared with the activity measured during transient expression, the activity of CpBV promoters was upregulated when the promoters were linked to the self-replicating AcMNPV genome. These results suggest that some factors associated with the baculovirus may enhance the CpBV promoters. Transcriptional transactivators of baculovirus such as IE-0, IE-1 and IE-2 have been reported to activate and enhance viral gene expression in transient assays (Kovacs et al., 1991; Olson et al., 2002; Shippam-Brett et al., 2001). It is possible that these transactivators could enhance the transcription from CpBV promoters. In addition, amplification of the template through viral replication may also be an explanation for the increase in promoter activity.

The CrBV gene, CrV1, is expressed in a highly transient fashion. Whereas high levels of transcripts were observed in haemocytes of the host caterpillar Pieris rapae within 4 h p.p., no transcripts were detectable at 12 h p.p. (Asgari et al., 1996, 1997). When the promoter region of the CrV1 gene was cloned into the baculovirus expression system with the CAT reporter gene, the CrV1 promoter was active as early as 2 h p.i. and activity continued until the late phase of infection in Sf21 cells, suggesting that down-regulation of CrV1 expression in host haemocytes may be mediated by post-transcriptional mechanisms (Asgari & Schmidt, 2001). In the baculovirus genome, the CrV1 promoter behaved similarly to the AcMNPV ie-1 promoter (Asgari & Schmidt, 2001). In the present study, the promoters of ORF3004, 3006 and 3007 were activated as early as 2 h p.i., and the amount of expressed protein increased for at least 72 h p.i. Although the time-course response of these promoters was similar to an ie-1 promoter, activity levels were higher (Fig. 3).

Interestingly, the ORF3006 promoter showed the highest level of activity in S. exigua larvae (Fig. 4), while its activity was relatively high in the LdPB transient expression assay (Fig. 1) as well as in the baculovirus expression system (Fig. 2). The putative ORF3006 has significant similarity (about 87% aa identity) to the histone H4 family. Histone H4, one of the four histones, along with H2A, H2B and H3, forms the eukaryotic nucleosome core. Along with H3, it plays a central role in nucleosome formation (Turner, 1995). Since histones play a role in chromosome remodelling and control of gene activity, defects in the establishment of proper chromosome structure by histones may aberrantly activate or silence genes (Turner, 1995; Wyler-Duda et al., 1997). Moreover, transiently expressed CpBV H4 interrupted haemocyte spreading in non-parasitized P. xylostella (Gad & Kim, 2008). Taken together, these findings suggest that the CpBV protein histone H4 driven by this promoter may perform an essential role in host immune suppression and developmental arrest. Analysis of the ORF3006 promoter revealed that the region located between −382 and −422 from the translation start point are critical for activity of

Fig. 5. The structures of the transfer vectors containing ORF3006 promoter regions with 5' deletion (a) and expression of EGFP using these transfer vectors (b). Different letters next to error bars (indicating minimum and maximum values) indicate a significant difference by Duncan's multiple range test (P<0.05).
this promoter, though no conserved sequences or reported promoter motifs were identified from this region.

The CpBV promoters investigated in this study, which have early regulated and strong activity, could be exploited for a variety of purposes, such as insecticide development, gene expression and molecular therapy. Indeed, we have constructed recombinant baculoviruses expressing insecticidal neurotoxin, AaIT, under the control of early promoters from CpBV and these viruses showed improved insecticidal activity (LT₅₀ reduced by about 27% compared with AcMNPV) (Choi et al., 2008). Also, early CpBV promoters could be useful in improving baculovirus expression vectors by means of lethal gene expression under the control of these promoters, which would block the replication of non-recombinant background.

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REFERENCES


