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Molecules and Cells

Improved Baculovirus Vectors Expressing Barnase Using Promoters from *Cotesia plutellae* Bracovirus

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The goal of this study was to create a novel baculovirus expression system that does not require recombinant virus purification steps. Transfection of insect cells with transfer vectors containing barnase under control of the *Cotesia plutellae* bracovirus (CpBV) promoters ORF3004 or ORF3005 reduced cell growth. Co-transfection with bApGOZA DNA yielded no recombinant viruses and non-recombinant backgrounds. To further investigate the detrimental effects of barnase on insect cells, two recombinant bacmids harboring the barnase gene under control of the CpBV promoters, namely bAcFast-3004ProBarnase and bAcFast-3005ProBarnase, were constructed. While no viral replication was observed when only the recombinant bacmids were transfected, recombinant viruses were generated when the bacmids were co-transfected with the transfer vector, pAcUWPolh, through substitution of the barnase gene with the native polyhedrin gene by homologous recombination. Moreover, no non-recombinant backgrounds were detected from unpurified recombinant stocks using PCR analysis. These results indicate that CpBV promoters can be used to improve baculovirus expression vectors by means of lethal gene expression under the control of these promoters.

INTRODUCTION

Baculoviruses, a family of large double-stranded circular DNA viruses, infect invertebrates such as insects and crustaceans (Hu, 2005). In the very-late phase of viral infection, polyhedrin, the major constituent of polyhedra, is synthesized in abundance (Choi et al., 2000). Because polyhedrin is under the control of a very-late strong transcriptional promoter and is not essential for viral replication, this promoter has been widely used for foreign gene expression (Choi et al., 1999).

Because the size of the baculovirus genome (81-160 kb) has made it difficult to engineer the genome through conventional restriction and ligation methods, homologous recombination in insect cells has generally been the preferred genetic engineering strategy. However, when this technique is used, the production efficiency of recombinant viruses is relatively low (0.1-2%), and repetitive screening needs to be performed to obtain the desired recombinant viruses. The use of linearized baculoviral genomic DNA increases the production efficiency of the desired recombinant virus by up to 90% (Kitts and Possee, 1993). Using this method, linear digested DNA segments, which are not subjected to recombination, are selectively excluded from the production of viral vectors. However, because it is not possible to achieve complete digestion with *Bsu36*I and because the digested DNA segments are fused with one another in insect cells by ligase, there is still the requirement for selection procedures. Peakman et al. (1992) developed another *in vitro* method that uses site-specific recombination mediated by a cre/loxP system. Although this method decreases the time required to produce recombinant viruses, the production efficiency of desired recombinant viruses is still low (0.2%-49%). Another approach that is used to generate recombinant baculovirus is to use a 'bacmid', which is a recombinant baculoviral genome carrying a miniF replicon, kanamycin resistance gene, and attTn7 recombination sequences (Luckow et al., 1993). Introduction of the transfer vector harboring the target gene between attTn7 termini into *Escherichia coli* cells carrying the bacmid, along with a helper plasmid that expresses an attTn7 transposase, results in transposition of the target gene into the bacmid. The resulting recombinant bacmid can then be isolated and transfected into insect cells to obtain recombinant virus particles. Recombinant baculoviruses can be obtained with relatively high efficiency using this site-specific transposition method. However, this method requires complex procedures including cloning of the target gene into Tn7 bacmid transfer vectors, transformation into *E. coli* DH10Bac for recombination between the bacmid and transfer vector, selection and isolation of bacteria harboring the recombinant bacmid, purification of the recombinant bacmid DNA from the isolated bacteria, and

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transfection of the DNA back into insect cells to produce recombinant virus. Furthermore, it is very difficult to select *E. coli* colonies harboring recombinant bacmid only, because there are several bacmid copies per *E. coli* cell. Thus, none of the above-mentioned methods are suitable as high-throughput systems for the preparation of recombinant viruses.

Here, we describe the creation of a novel recombinant bacmid that can be used for the rapid generation of recombinant virus without the need to purify target recombinant viruses from a non-recombinant background. We introduced the barnase gene from *Bacillus amyloliquefaciens*, which is lethal to insect cells, into bacmids under the control of early promoters from *Cotesia plutellae* bracovirus (CpBV). The CpBV promoter-barnase cassette was replaced with the gene of interest by homologous recombination in insect cells. No selection steps were required, and only recombinant virus was obtained after co-transfection of recombinant bacmid with a transfer vector harboring the gene of interest, because replication of the recombinant bacmid in insect cells was blocked by host cell death at the early stage of viral replication.

MATERIALS AND METHODS

Cell lines and baculoviruses

The *Spodoptera frugiperda* insect cell line, Sf9, was maintained in TC-100 medium (JBI, Korea) supplemented with 10% heat-inactivated (56°C, 30 min) fetal bovine serum (JBI, Korea) and incubated at 27°C and sub-cultured every 3-4 days. Wild-type *Autographa californica* nucleopolyhedrovirus (AcMNPV) and all recombinant AcMNPVs used in this study were propagated in Sf9 cells maintained in TC-100 medium.

Construction of transfer vectors

To construct baculovirus transfer vectors expressing the gene for *B. amyloliquefaciens* extracellular RNase (barnase) under control of the CpBV promoters, ORF3003, ORF3004, or ORF3005, about 0.87 kb of the barnase-barstar cassette fragment was amplified from the pGR006 vector (kindly provided from Dr. Beom Seok Park, Rural Development Administration) using primers BaBa-*Xho*F and BaBa-*Eco*RR. The BaBa-*Xho*F (5′-CCCGCTCGAGAT-GGCACAGGTTATCAACACG-3′) and BaBa-*Eco*RR (5′-CCCGGAATTCTTAAGAAAGTATGATGGTGA-3′) primers contained *Xho*I and *Eco*RI sites at their 5′-ends (underlined). The PCR-amplified barnase-barstar cassette fragment was digested with the restriction endonucleases *Xho*I and *Eco*RI. This fragment was inserted downstream of CpBV promoters in the transfer vectors pBac8-3003ProEGFP, pBac8-3004ProEGFP, and pBac8-3005ProEGFP, from which the EGFP gene was deleted by digestion with the same restriction endonucleases. To construct the transfer vectors pFastBac-3004ProBarnase and pFastBac-3005ProBarnase, 1.58 kb of the ORF3004/ORF 3005 promoter-barnase-barstar cassette was amplified from pBac8-3004Pro-Barnase and pBac8-3005ProBarnase, respectively, using oligonucleotides 3004Pro-F/3005Pro-F and BaBa-KpnR (5′-GG-GCGGTACCTTAAGAAAGTATGATGGTGA-3′), which contains a *Kpn*I site at its 5′-end (underlined) and the PCR products were digested with *Kpn*I. The resulting fragment was inserted between the *Sna*BI and *Kpn*I sites of pFastBac1 (Invitrogen, USA) to obtain the transfer vectors pFastBac-3004Pro-Barnase and pFastBac-3005ProBarnase, respectively. To construct baculovirus transfer vectors expressing native polyhedrin, pAcUW31 (Clontech, USA) was digested with *Xba*I and *Sna*BI, and then ligated with 0.95 kb of the PCR-amplified and *Xba*I- and *Sna*BI-digested polyhedrin gene region to obtain pAcUW-Polh. The polyhedrin region was amplified using primers Polh-XbaF (5′-CCCGTCTAGACTATCAATATATAGTTGC-TGATAT-3′) and Polh-SnaBR (5′-AAAATACGTACAACAATT-GTCTGTAAA-3′) to enable inclusion of the polyhedrin promoter, CDS, and terminator as intact structures into the wild-type AcMNPV genome.

Construction of recombinant bacmids

Construction of the barnase-expressing recombinant bacmids bAcFast-3004ProBarnase and bAcFast-3005ProBarnase was performed using a Bac-to-Bac baculovirus expression system (Invitrogen, USA). Five nanograms of each transfer vector were transformed into *E. coli* DH10Bac™ cells (Invitrogen, USA) according to the manufacturer's instruction. White colonies harboring recombinant bacmid DNA were screened twice on nutrient agar plates containing 50 μg/ml kanamycin, 7 μg/ml gentamicin, 10 μg/ml tetracycline, 100 μg/ml X-gal, and 40 μg/ml IPTG, and recombinant bacmid DNAs were then analyzed by PCR using specific primers.

Transfection

Approximately 1×10^6 Sf9 cells were seeded on a 60-mm diameter tissue culture dish and incubated at 27°C for 30 min to allow the cells attach. One microgram of transfer vector and 50, 250, or 500 μg of bacmid DNA were added to 100 μl of incomplete TC-100 medium in polystyrene tubes. In other polystyrene tubes, 20 μl of Cellfectin™ (Invitrogen, USA) was mixed with 100 μl of incomplete TC-100 medium. The two solutions were gently mixed and the matrix was incubated at room temperature for 45 min. The attached cells were washed once with 3 ml of incomplete TC-100 medium and refreshed with 2 ml of the same medium. The cellfectin-DNA complexes were added drop-wise to the medium covering the cells while the dish was gently swirled. After incubation at 27°C for 5 h, each dish was refreshed with 3 ml of TC-100 medium supplemented with 10% FBS, and the transfected cells were incubated at 27°C. At 5 days post-transfection, the transfection supernatant was harvested by centrifugation at 500 × *g* for 5 min and stored at 4°C. The recombinant viruses were purified by plaque assay on Sf9 cells according to the method of O'Reilly et al. (1992).

PCR and primers

To amplify the gp64 gene, oligonucleotides AcGP64-F (5′-AGCAAGATGGTAAGCGC-3′) and AcGP64-R (5′-GTACATT-TTCGAAGTGG-3′) were used. To amplify kanamycin resistance gene, oligonucleotides Kan-F (5′-AGCGAGTTTAAACG-ATATCG-3′) and Kan-R (5′-GTCGACACTAGTATTACCCTG-TTA-3′) were used. Furthermore, the polyhedrin gene region was amplified using oligonucleotides Bac3 (5′-GCCATTG-TAATGAGACG-3′) and Bac4 (5′-TCTGTAAATCAACAACGC-3′). The recombinant bacmid DNAs were subjected to 33 PCR cycles (1 min at 94°C, 30 s at 55°C, and 1 min at 72°C) followed by a 7 min final extension at 72°C using AccuPower™ PCR Premix (Bioneer, Korea) and a C1000™ Thermal Cycler (BIO-RAD, USA).

RESULTS

Cytopathic effect of barnase in insect cells

To examine the possibility of improved baculovirus expression system by expression of lethal gene under control of the CpBV promoters, transfer vectors pBac8-3003ProBarnase, pBac8-3004ProBarnase, and pBac8-3005ProBarnase expressing barnase under control of the promoters of ORF3003, 3004, and 3005, respectively, were constructed. The internal structures of these transfer vectors were confirmed by restriction endonuclease digestion and nucleotide sequencing.

Table 1. Growth of Sf9 cells transfected with transfer vectors expressing barnase under the control of CpBV promoters.

Transfected transfer vector	Number of cells ($\times 10^5$ cells/3 ml)	
	at 1 day post-transfection	at 2 days post-transfection
Mock-transfected	7.70 ± 0.59a*	17.24 ± 0.97a
pBac8-3004ProEGFP	4.69 ± 0.35b	13.46 ± 0.23b
pBac8-3003ProBarnase	4.66 ± 0.35b	9.83 ± 0.72c
pBac8-3004ProBarnase	5.19 ± 0.55b	10.44 ± 0.48c
pBac8-3005ProBarnase	4.65 ± 0.14b	13.31 ± 0.45b

*Different letters behind means indicate significant differences (n = 4, $p < 0.05$, Duncan's multiple range test).

While Sf9 cells transfected with pBac8-3003ProBarnase and pBac8-3004ProBarnase showed reduced growth rates, Sf9 cells transfected with pBac8-3005ProBarnase showed no obvious differences in cell growth rate compared to those transfected with pBac8-3004ProEGFP, which was not lethal to transfected cells (Table 1). When the transfer vectors expressing barnase under the control of CpBV promoters were co-transfected with bApGOZA DNA (Je et al., 2001b) into Sf9 cells, no recombinant viruses or non-recombinant backgrounds were generated (Table 2). In contrast, recombinant viruses were generated when Sf9 cells were co-transfected with bApGOZA and pBac8-3004ProEGFP expressing EGFP instead of barnase with an efficiency of about 88%.

Construction of recombinant bacmids expressing barnase

To investigate the detrimental effects of barnase on insect cells and the associated viral replication, we intended to construct recombinant viruses expressing barnase under the control of CpBV promoters. Because these recombinant viruses cannot be generated in insect cells using traditional recombinant baculovirus techniques, we used a Bac-to-Bac system in which recombinant viral genomes are generated in *E. coli* cells as recombinant bacmids by site-specific recombination using the transposon Tn7. Transfer vectors pFastBac-3004ProBarnase and pFastBac-3005ProBarnase were constructed and confirmed by patterns of restriction digestion and DNA sequencing. The recombinant bacmids bAcFast-3004ProBarnase and bAcFast-3005ProBarnase that express barnase under the control of the ORF3004 and 3005 promoters, respectively, were generated by transforming those transfer vectors into *E. coli* DH10Bac™ cells containing bacmid DNA. The genomic structures of each recombinant bacmids in polyhedrin gene locus were schematically given in Fig. 1.

To investigate replication of these recombinant bacmids in insect cells, purified bAcFast-3004ProBarnase or bAcFast-3005ProBarnase were transfected into Sf9 cells to produce recombinant virus particles, and the resulting transfection medium was passaged twice in Sf9 cells. After a second passage in Sf9 cells, AcFast-3004ProBarnase and AcFast-3005ProBarnase viral DNA was extracted from infected Sf9 cells and PCR analysis was performed. However, the barnase-specific primers did not amplify any specific viral DNA fragments. Furthermore, the gp64 gene, which is essential for AcMNPV replication, was not detected by PCR amplification using a gp64 gene-specific primer set (Fig. 2).

Generation of recombinant baculovirus using recombinant bacmids

To assess the ability of the recombinant bacmids to serve as a

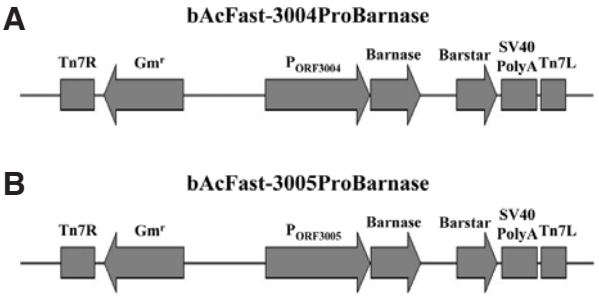

Fig. 1. Genomic structure of recombinant bacmids, bAcFast-3004ProBarnase (A) and bAcFast-3005ProBarnase (B). Schematic diagram of polyhedrin gene locus was represented and solid arrows indicate direction of transcription of each gene.

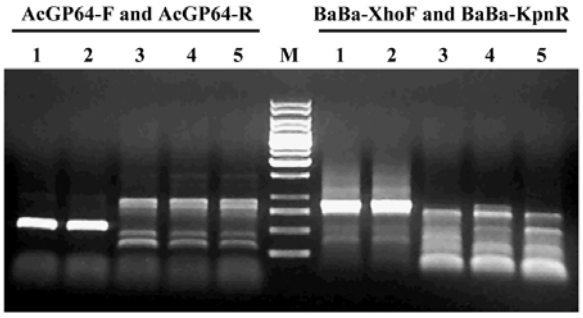

Fig. 2. Detection of viral DNA from Sf9 cells transfected with bAcFast-3004ProBarnase or bAcFast-3005ProBarnase. After the second passage of Sf9 cells transfected with bAcFast-3004ProBarnase or bAcFast-3005ProBarnase, viral DNA was extracted from transfected cells, and the presence of viral DNA was confirmed by PCR analysis targeting the AcMNPV gp64 gene (left) or Barnase gene (right), respectively. Lane 1, bAcFast-3004ProBarnase; lane 2, bAcFast-3005ProBarnase; lane 3, mock-infected Sf9 cells; lane 4, AcFast-3004ProBarnase; lane 5, AcFast-3005ProBarnase; lane M, 1 kb DNA Ladder.

parental viral genome for the generation of recombinant baculovirus, Sf9 cells were co-transfected with AcFast-3004Pro Barnase or AcFast-3005ProBarnase DNA purified from *E. coli* and the transfer vector, pAcUW-Polh, in which the polyhedrin gene region is intact, resulting in creation of the recombinant baculoviruses AcFast-3004ProPolh and AcFast-3005ProPolh, respectively. Polyhedra were produced by Sf9 cells infected with the AcFast-3004ProPolh or AcFast-3005ProPolh, as in Sf9 cells infected with wild-type AcMNPV or the recombinant virus AcPolh, which was constructed by co-transfection of Sf9 cells with pAcUW-Polh and bAcGOZA (Je et al., 2001a) (Fig. 3).

To verify that non-recombinant backgrounds were absent from the unpurified viral stocks, the transfection media of recombinant viruses, AcPolh, AcFast-3004ProPolh, and AcFast-3005ProPolh were passaged twice in Sf9 cells. After the second passage on Sf9 cells, AcPolh, AcFast-3004ProPolh, or AcFast-3005ProPolh viral DNA was extracted from infected Sf9 cells and the kanamycin resistance gene, which is replaced by the polyhedrin gene by homologous recombination, was targeted by PCR. Whereas the kanamycin resistance gene was amplified from the viral DNA of AcPolh, kanamycin resistance gene-specific primers did not amplify any specific fragments from the viral DNA of AcFast-3004ProPolh or AcFast-3005 ProPolh (Fig. 4).

Table 2. Production of recombinant baculoviruses expressing barnase under the control of CpBV promoters.

Viral DNA	Transfer vector	Number of recombinant plaques	Number of non-recombinant plaques	Recombination rate (%)
bApGOZA	pBac8-3004ProEGFP	15	2	88.2
bApGOZA	pBac8-3003ProBarnase		No plaques	
bApGOZA	pBac8-3004ProBarnase		No plaques	
bApGOZA	pBac8-3005ProBarnase		No plaques	

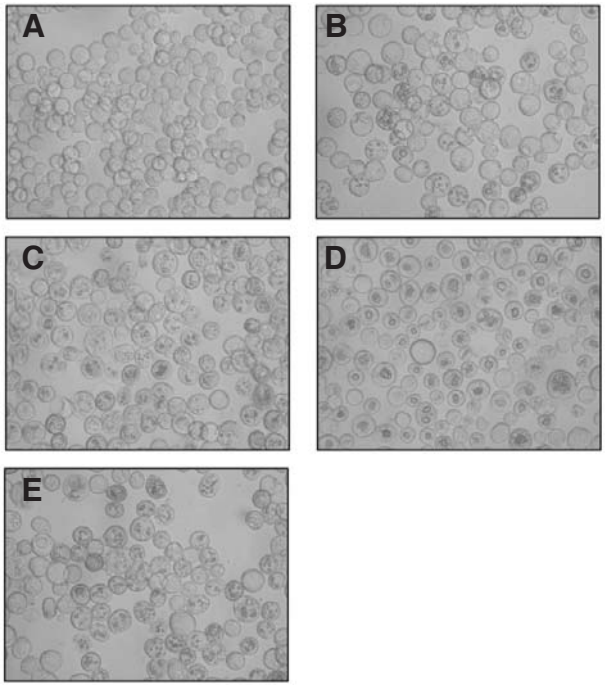

Fig. 3. Phase-contrast micrographs of Sf9 cells infected with recombinant AcMNPVs. (A) mock-infected; (B) wild-type AcMNPV-infected; (C) AcPolh-infected; (D) AcFast-3004ProPolh-infected; (E) AcFast-3005ProPolh-infected.

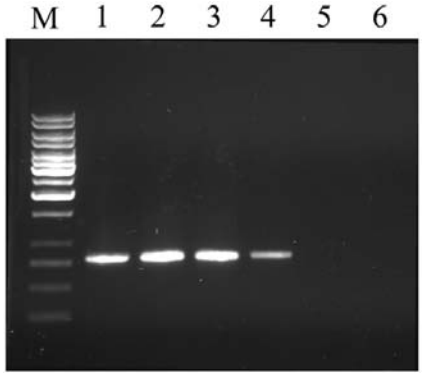

Fig. 4. Detection of non-recombinant backgrounds from Sf9 cells infected with unpurified viral stocks of AcPolh, AcFast-3004ProPolh, or AcFast-3005ProPolh, respectively. After the second passage of Sf9 cells, viral DNA was extracted from infected cells, and the presence of non-recombinant backgrounds was confirmed by PCR analysis using kanamycin resistance gene-specific primers. Lane M, 1 kb DNA ladder; lane 1, bAcGOZA; lane 2, bAcFast-3004Pro Barnase; lane 3, bAcFast-3005ProBarnase; lane 4, AcPolh; lane 5, AcFast-3004ProPolh; lane 6, AcFast-3005ProPolh.

To establish the optimal quantity of bacmid DNA to transfect to generate recombinant viruses, 50, 250, or 500 μg of recombinant bacmid DNA was transfected together with 1 μg of pAcUW-Polh into Sf9 cells. Viral DNA was extracted from the transfection media of second passage Sf9 cells and the introduced polyhedrin gene was targeted by PCR. This analysis revealed that target recombinant virus was only produced when cells were transfected with 50 μg of recombinant bacmid DNA (Fig. 5).

DISCUSSION

In the post-genomic and proteomics era, there is growing demand for high-throughput protein expression systems (Leder et al., 2007). A baculovirus expression system is a helper-independent system that has been used extensive to express heterologous genes. This technique's popularity stems from the combination of high-level expression and the ability to obtain authentic eukaryotic post-translational modifications (O'Reilly et al., 1992). AcMNPV is the prototype baculovirus strain and is the strain most commonly used in expression vector systems. A number of methods to conveniently and rapidly generate recombinant baculoviruses expressing gene of interest have been reported (Airenne et al., 2003; Je et al., 2001a; Kitts and Possee, 1993; Luckow et al., 1993; Peakman et al., 1992; Yao et al., 2007). Recently, semi-automated or automated systems that use recombinant baculoviruses for protein production have also been reported (Possee et al., 2008; Schlaeppi et al., 2006). However, the major drawback of these methods is the substantial effort required to purify recombinant virus from non-recombinant backgrounds.

To overcome this defect, we introduced the barnase gene into the AcMNPV genome under the control of CpBV promoters. Barnase is a major extra-cellular ribonuclease isolated from *B. amyloliquefaciens*, and is lethal to cells (Hartley, 1989). Barstar, a specific inhibitor of barnase, counteracts barnase by formation of a stable and nonequivalent one-to-one complex (Hartley and Smeaton, 1973). A previous study demonstrated that recombinant *Heliothis armigera* nucleopolyhedrovirus (HaNPV), rHa-Bar, carrying the barnase gene, could not replicate in cultured insect cells (Hz-AM1); this rHa-Bar could only replicate in AM1-NB cells in which the barstar gene was integrated into the cellular chromosome (Qin et al., 2005).

CpBV is a member of the polydnaviruses (PDVs), which have obligate symbiotic associations with parasitoid wasp species in the families Braconidae and Ichneumonidae (Drezen et al., 2003; Turnbull and Webb, 2002; Whitfield, 2002). Previously, we cloned, sequenced, and provided annotations for 27 discrete genome segments of CpBV (Choi et al., 2005). In lepidopteran hosts, PDV genes are expressed as early as 2 h post-parasitization (p. p.) in the apparent absence of viral replication

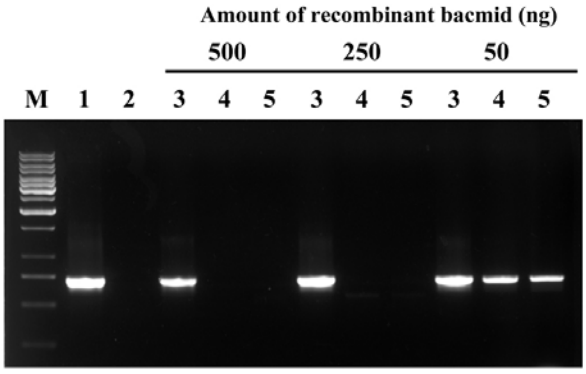

Fig. 5. Detection of viral DNAs from Sf9 cells co-transfected with pAcUW-Polh and various amounts of recombinant bacmids. After the second passage of Sf9 cells infected with AcFast-3004ProPolh or AcFast-3005ProPolh, viral DNA was extracted from infected cells, and the presence of viral DNA was confirmed by PCR analysis using polyhedrin gene-specific primers. Lane M, 1 kb DNA ladder; lane 1, pAcUW-Polh; lane 2, mock-infected Sf9 cells; lane 3, AcPolh; lane 4, AcFast-3004ProPolh; lane 5, AcFast-3005ProPolh.

(Fleming, 1992; Stoltz, 1993; Summers and Dib-Hajj, 1995; Yamanaka et al., 1996). Furthermore, several PDV proteins such as early proteins 1, 2, and 3 (EPs) of *Cotesia congregata* bracovirus (CcBV) are expressed at high levels in parasitized *Manduca sexta* larvae; these three proteins together can comprise up to 10 to 15% of total hemolymph proteins 24 h p. p. (Beckage, 1993; Beckage et al., 1987; Harwood and Beckage, 1994). This early and abundant expression of PDV genes is due to their promoter activities. Recently, the activities of promoters associated with six predicted ORFs from one of the largest CpBV genomic segment, CpBV-S30, were investigated using both transient and baculovirus expression assays using an EGFP reporter gene (Choi et al., 2009). CpBV promoters showed activity earlier than the *polyhedrin* promoter, and the activity of some of these promoters was superior to that of the AcMNPV *ie-1* promoter in baculovirus expression assays. Therefore, it was hypothesized that by using these early promoters, we could generate improved baculovirus expression vectors.

Insect cells transfected with the transfer vectors containing barnase under the control of the CpBV promoters ORF3003 and ORF3004 showed reduced cell growth (Table 1); furthermore, co-transfection of these transfer vectors with bApGOZA DNA did not yield any recombinant viruses or non-recombinant backgrounds (Table 2). In addition, no replication of recombinant viral genomes harboring the barnase gene under the control of CpBV promoters was observed in insect cells transfected with these recombinant viral genomes (Fig. 2). These results suggest that barnase expressed under the control of CpBV promoters on the transfer vector or viral genome is lethal to transfected insect cells. In contrast, transient expression of barnase under the control of the ORF3005 promoter did not trigger any cytopathic effect (Table 1), because this promoter has been reported to be active only when present in the viral genome (Choi et al., 2009). Therefore, the ORF3005 promoter is a suitable candidate for expression of lethal genes using a baculovirus expression system.

As expected, no non-recombinant backgrounds were present in unpurified stocks of recombinant viruses generated using recombinant bacmids harboring barnase (Fig. 4, lanes 5 and 6). Because the non-recombinant backgrounds express barnase, which is lethal to insect cells, increasing amount of bacmids prevent the replication of co-existing recombinant viruses by means of host cell death. This was demonstrated by the relatively small amount of bacmids (as small as 50 μg) required to generate recombinant viruses (Fig. 5, lanes 4 and 5). These results confirm that baculovirus expression vectors can be improved by means of lethal gene expression under control of early CpBV promoters.

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